THE OXIDATION OF CHALCONE CATALYSED BY PEROXIDASE

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SUMMARY

The studies recorded in this thesis began with the aim of elucidating the enzymology of the recently reported oxidation of 2',4,4'-trihydroxychalcone (isoliquiritigenin) to a hydrated aurone product, isolated as the diastereoisomers Y_1 and Y_2. On partial purification of this activity from cell-free extracts of seedlings of garbanzo bean (Cicer arietinum) it emerged that a peroxidase in the extract was responsible for the catalysis initially observed. Of even greater eventual significance, however, was the concurrent finding that the peroxidase-catalysed oxidation of isoliquiritigenin (chalcone) led mainly to products other than Y_1 and Y_2. Much of the work recorded here is consequently concerned with the elucidation of these major products of the reaction, which are new compounds, and with studies of the biochemistry of their formation. From the results of these chemical and biochemical studies a scheme which rationalises the enzymic reaction is presented.

Preliminary studies on enzyme purification are described which led to the finding that a peroxidase in cell-free extracts of garbanzo was responsible for Y_1 and Y_2 formation from added chalcone in the crude extract. A further finding was that when certain soluble substances were removed from the enzyme preparation, the reaction of chalcone became much more extensive and the major product was then a hitherto unknown compound which was isolated chromatographically and referred to as OC. Under these

conditions, Y_1 and Y_2 production decreased considerably. The soluble substances were thus seen to exert the dual effect of inhibiting OC formation and promoting Y_1 and Y_2 formation. Dialysis of the crude enzyme or precipitation of the protein (enzyme) therein by ammonium sulphate or acetone treatment effectively removed the soluble substances and wrought the changes noted in the extent of reaction and product pattern. The reaction was found to be accelerated by the addition of hydrogen peroxide.

Following the identification of peroxidase as the enzyme responsible for the activities of interest, a procedure for the partial purification of this enzyme from the cell-free extract was developed as a prelude to more detailed biochemical studies.

With the availability of purified enzyme, a system was developed for the successful production and isolation of preparative amounts of the major product, OC, hitherto isolated solely by chromatography. In handling this OC, evidence of its unstable nature was obtained. This led to spectrophotometric study of the reaction in an attempt to obtain quantitative estimates of product formation by eliminating as far as possible any product breakdown.

Spectrophotometric monitoring of the reaction product formed in a rapid complete consumption of the added chalcone revealed surprisingly a spectrum other than that of OC in the reaction buffer. Thus the first evidence was obtained that the initial reaction product consisted mainly of a precursor of OC. This initial product rapidly gave

rise to mainly OC on careful treatment with dilute alkali. Further, it could be recovered unchanged by solvent extraction from weakly acidic media. Chromatography then revealed that it consisted largely of two compounds each giving rise to OC on treatment with dilute alkali. These compounds which had chromatographic properties that overlapped with those of Y_1 and Y_2, are referred to as $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. These studies also revealed that the initial product contained small amounts of Y_1 and Y_2 (converted to the aurone, hispidol, on alkali treatment). Further, they showed that small amounts of the known flavonoid, 4',7-dihydroxyflavonol (flavonol) were also formed on treatment of the initial product or of chromatographically isolated $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ preparations with dilute alkali. Thus the initial product was identified as consisting of precursors of three distinct compounds; the new compound OC and the recognised flavonoids 4',7-dihydroxyflavonol and hispidol.

Additional studies showed how the chromatographic product pattern changed according to the treatment the initial product received at various stages prior to or during 2-D chromatography. The highly unstable nature of the $\not{\alpha}Y$ precursors of OC was clearly evident. This work uncovered another precursor of OC, referred to as $\not{\alpha}OC$, which arose apparently from the $\not{\alpha}Y$ compounds under acidic conditions. This extensive array of products arising from the enzymic reaction was simplified somewhat when it was shown that the $\not{\alpha}Y$ compounds were common precursors of OC and flavonol.

In the biochemical studies the peroxidase preparation from garbanzo was compared with the action of commercial

horseradish peroxidase (HRP). Very similar results were obtained throughout with these two enzymes.

Both enzymes showed a pH optimum for catalysis of chalcone oxidation at slightly above neutrality (garbanzo, pH 7.5 and horseradish, pH 7.9). The rate of reaction was found to be dependent on the concentration of added hydrogen peroxide in a manner indicative of a substrate requirement for this compound. Under the conditions employed, the concentration of hydrogen peroxide giving maximum rate with garbanzo enzyme (0.15 mM) was about half that required with HRP (0.30 mM). Typical substrate concentration curves were obtained on varying the concentration of chalcone, both enzymes being apparently saturated at concentrations above about 45 μM. An oxygen requirement for the reaction was demonstrated manometrically with a chalcone:oxygen stoicheiometry of approximately 1:1. This result was independent of the concentration of hydrogen peroxide tested and indicated that this compound did not substitute for oxygen. This was demonstrated by inhibition of the reaction in the absence of oxygen but in the presence of substrate amounts of hydrogen peroxide. Direct measurement showed only a very small (catalytic) net consumption of hydrogen peroxide in the course of the aerobic reaction, in keeping with the stoicheiometric oxygen requirement. The reaction was found to be strongly inhibited by manganous ion, mercaptoethanol and diethyldithiocarbamate and by the redogenic donors, hydroquinone, catechol, pyrogallol and p-phenylenediamine. The oxidogenic donors tested were not found to be stimulatory. Qualitative studies indicated the

effect of substitution pattern on the enzymic reaction of a range of chalcones; for both enzymes a free phenolic group in the B ring, preferably in the 4-position, was required.

While the enzymic reaction was treated, for the purposes of the quantitative biochemical studies, as a $\not{a}Y_1$ and $\not{a}Y_2$ (CC)-forming reaction, additional chromatographic studies of Y_1, Y_2 and flavonol formation were made. Thus Y_1 and Y_2 were found to be ubiquitous minor products of the aerobic reaction; the combined yield was measured (as aurone) at approximately 1% of the chalcone consumed under typical reaction conditions with purified systems. As expected, Y_1 and Y_2 formation was inhibited by redogenic donors yet with diethyldithiocarbamate, an inhibitor of the main ($\not{a}Y$-forming) reaction, a relative increase was noted. The promotor(s) of Y_1 and Y_2 formation in the cell-free extract were found to be substances with hydrophilic sugar-like properties. They were considered to have the added capacity to simultaneously inhibit the formation of $\not{a}Y$ compounds (observed as OC).

The source of flavonol noted in small amounts on all chromatograms of reaction products was found to be the $\not{a}Y$ compounds. When treated with alkali these compounds gave rise to both OC and flavonol. A competitive formation of OC and flavonol occurred and at higher concentrations of alkali (0.1-0.5 N) the flavonol product predominated. A more facile reaction was observed with $\not{a}Y_1$ than with $\not{a}Y_2$. An apparently specific inhibition of flavonol formation in the enzymic reaction by cupric ion was initially observed chromatographically and on the identification of the

precursors of flavonol, it was shown that cupric ion caused extensive degradation of ØY compounds and could effectively inhibit flavonol formation at low alkali concentrations.

The finding that the ØY compounds were the precursors of flavonol in the peroxidase-catalysed system was in contrast to the accepted role of the dihydroflavonol as precursor of flavonol in flavonoid biosynthesis. Consequently the place of the specific dihydroflavonol (garbanzol) in the peroxidase reaction was investigated. Garbanzol was found not to be oxidised to flavonol in the enzymic system. Further, with the aid of labelled chalcone, evidence was obtained for the production of traces of garbanzol in the products of the enzymic reaction.

The new compounds which were isolated from the enzymic reaction product, namely $ØY_1$, $ØY_2$, OC and ØOC, were obtained in preparative amounts and characterised using u.v., i.r., NMR and mass spectral techniques. From these data the structures of these closely related compounds were formulated as the benzoxepin derivatives given in Figure 61. Thus $ØY_1$ and $ØY_2$ are diastereoisomeric diols and OC is the enolic form of the α-diketone (ØOC) derived from the diols. Schemes are presented to rationalise these transformations and also the formation of flavonol in competition with OC from the ØY compounds. Base-catalysed elimination of the elements of water occurs in the conversion of ØY compounds to OC and to flavonol; in the latter case skeletal rearrangement also occurs. A pinacolic rearrangement accounts for the generation of ØOC from the ØY compounds under acidic conditions and enolization for the conversion of ØOC to the more stable enol, OC. A very significant facet of the

characterisation studies was the resolution of $\not{\alpha}Y_2$ into enantiomers by paper chromatography as confirmed by optical rotation and spectral data. No similar resolution of the virtually optically inactive $\not{\alpha}Y_1$ was observed.

The chemical oxidation of chalcone with hydrogen peroxide was also studied. At room temperature and slightly alkaline pH (7-11) it was found that the products isolated were chiefly OC and flavonol. Direct evidence that the precursor relationships of the enzymic reaction also applied to the chemical reaction was obtained with the isolation of $\not{\alpha}Y$ compounds as the only significant product under carefully controlled conditions. The product of the chemical reaction under Algar-Flynn-Oyamada (A.F.O.) conditions (high pH, low temperature - 0^{o}) was found, however, to be flavonol with no trace of OC. It is nevertheless proposed that under A.F.O. conditions, the intermediates ($\not{\alpha}Y$ compounds) of both the enzymic and the room temperature, lower pH, chemical reaction are still produced and that they rearrange spontaneously at the high pH and low temperature to flavonol. This conclusion is in contrast to the role normally ascribed to dihydroflavonol as the precursor of flavonol in the A.F.O. reaction.

Schemes are presented and discussed to explain the enzymic and chemical reaction of chalcone to form $\not{\alpha}Y$ compounds. A central feature of these schemes is the postulated formation of an enantiomeric epoxide as the true initial reaction product. Hydrolytic opening of the epoxide ring of this structure would give rise to the $\not{\alpha}Y$ (diol) structure. Since $\not{\alpha}Y_2$ occurs in enantiomeric form, it is also proposed that both isomers of $\not{\alpha}Y_1$ similarly occur and therefore that

all four isomeric diols occur in the product. The formation of these four diols from the epoxide enantiomers is discussed. An attractive feature of the epoxide as true initial product is that it permits a scheme for the enzymic reaction to be advanced based on a chain reaction which utilises only known redox states of peroxidase and which accommodates all features of the biochemical data.

The enzymic reaction of chalcone is thus a four-equivalent oxidation (dehydrogenation plus oxygenation), the postulated epoxide precursor, the ØY structure, OC and ØOC all being at the one oxidation level (4 equivalents higher than chalcone). The proposed oxygenating species of the enzyme (formally Compound III) is discussed in relation to other examples of this activity of peroxidase and also with respect to selected examples of oxygenation by mixed function oxidases.

This new reaction of chalcone is held to provide a particularly clear-cut example of the incompletely understood oxygenating activity of peroxidase which occurs in this case without detectable requirement for any organic cofactors or of metal ions. In relation to the original starting point of flavonoid biosynthesis, the reaction accounts for the formation of Y_1 and Y_2 initially observed in the crude system and may account for the biosynthesis of garbanzol also previously recorded in that system. Significantly, the enzymic reaction provides the first example of an *in vitro* system leading to flavonol formation through the hitherto unknown ØY structure rather than by way of the anticipated dihydroflavonol. The question of the *in vivo* significance of this complex activity of peroxidase remains open.

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Chapter 1

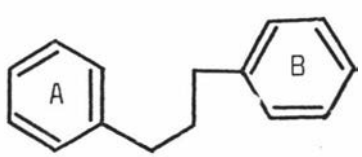
INTRODUCTION

This thesis deals with studies which have as their origin the intention to investigate select aspects of the enzymology of flavonoid biosynthesis and which lead to the finding that a peroxidase-catalysed reaction is of central importance in the system under scrutiny. In the course of this Introduction, therefore, a brief background is developed in relation to not only flavonoids but also peroxidase-catalysed reactions.

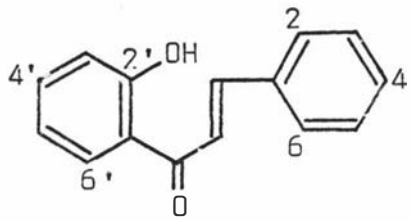
1A CHEMISTRY AND BIOCHEMISTRY OF FLAVONOIDS

1A-1 Structure and classes of flavonoids

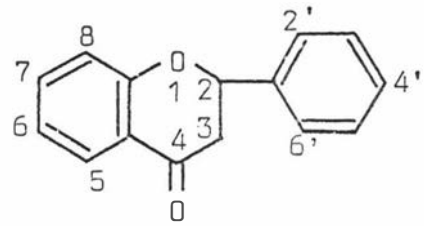
Flavonoids are natural phenolic compounds having in common the 1,3-diphenylpropane skeleton of 15 carbon atoms shown below.



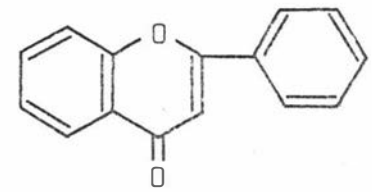
The A ring characteristically bears an ortho-hydroxyl substituent which is usually involved in heterocyclic ring formation with the 3-carbon link between the A and B rings. Oxidation state and substitution in the central 3-carbon fragment define the different classes of flavonoids (1), for which skeletal structures are presented in Figure 1. (NB The numbering systems employed for chalcone and aurone differ



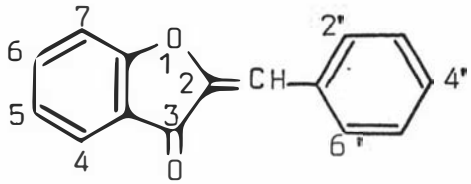
Chalcone



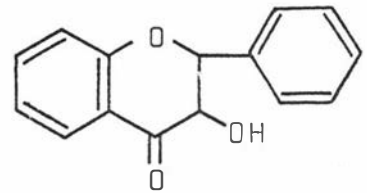
Flavanone



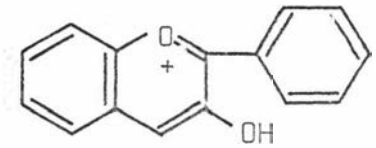
Flavone



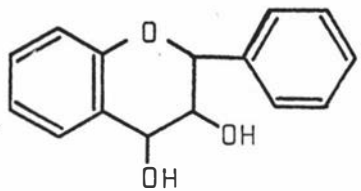
Aurone



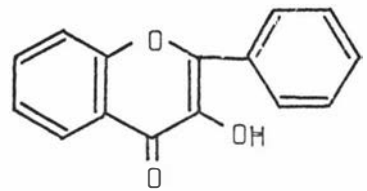
Dihydroflavonol



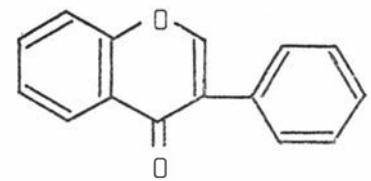
Anthocyanidin



Flavan-3,4-diol



Flavonol



Isoflavone

Figure 1. Skeletal structure of various flavonoid classes

from those of the other classes, which follow the flavanone pattern).

Individual compounds within classes arise by varied substitution of the A and B rings with hydroxyl groups which may be further modified by ether or glycoside formation (1).

1A-2 Occurrence of flavonoids

Virtually all higher plants synthesize flavonoids as secondary metabolites (2,3). Many flavonoids are known in angiosperms, gymnosperms and ferns and a few in mosses, but these compounds are apparently absent from bacteria, fungi and algae (2,3).

Those flavonoid compounds most commonly found and widely distributed in plants belong to the flavonol, flavone and anthocyanin classes (4,5). Many representatives of a class may exist in one plant; for example, complex mixtures of anthocyanin pigments in flowers (4). The number of compounds now known is impressive. The flavones and flavonols for example number between 200 and 300 (5).

The other classes of flavonoids are currently represented by fewer known compounds for a variety of reasons (6). For some classes, such as chalcone and dihydroflavonol, compounds have been found in quantity in only a few species of plants yet representatives of these classes probably occur in trace amounts in most plants in view of their probable biosynthetic roles. Flavanones may have been overlooked frequently in surveys because they are difficult to detect. Yet other classes are of limited taxonomic distribution within the plant kingdom; isoflavones are restricted largely to

the legumes, while aurones are thus far found frequently only in the flowers of the Compositae.

Flavonoids normally exist in plants in a combined form, usually as glycosides where the linkage to the sugar is through one or more of the hydroxyl groups (1). A simple phenol (aglycone) may occur in several different glycosidic forms in the plant (4,5).

1A-3 Biosynthesis of flavonoids

To date, the biosynthesis of flavonoid compounds has been investigated largely by means of *in vivo* tracer experiments. Progress has been made to the point where the origin of the flavonoid skeleton from primary metabolites in the plant has been defined and probable interrelationships between the classes of flavonoids deduced with some certainty (7,8,9,10).

Biogenesis of the C15 flavonoid skeleton from a combination of two different pathways to aromatic compounds has been firmly established (7). The A ring of flavonoids is acetate-derived, while the C6C3 unit forming the B ring and the central 3-carbon atom fragment is of phenylpropanoid origin. The phenylpropanoid precursor, probably a cinnamic acid, arises by way of the shikimic acid pathway (11) leading to the aromatic amino acids phenylalanine and tyrosine. Specific enzymic deaminations of phenylalanine and tyrosine lead to the irreversible formation of trans-cinnamic and p-hydroxycinnamic acids respectively (12).

Grisebach and others have postulated (7,8) that the flavonoid skeleton is formed as outlined in Figure 2.

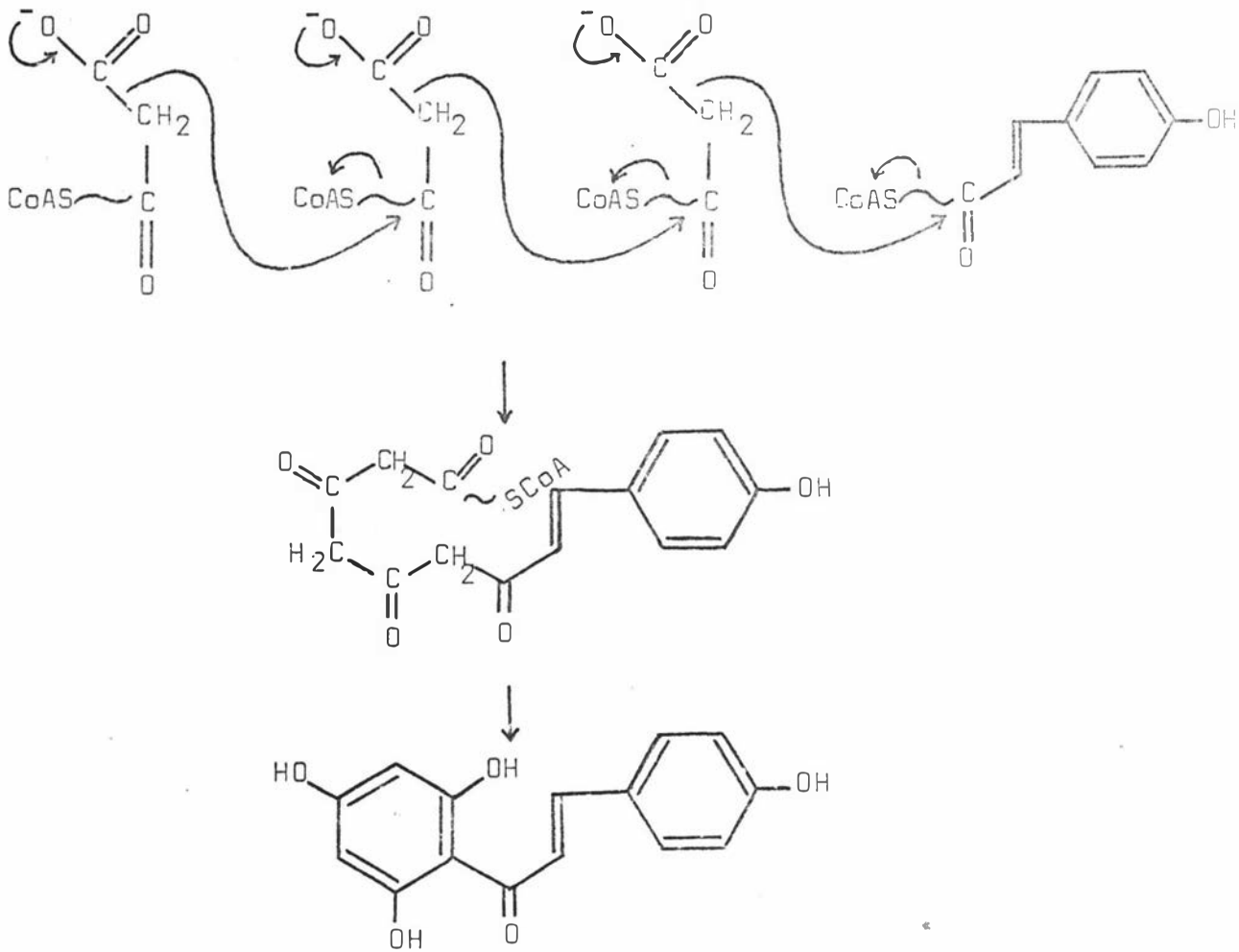
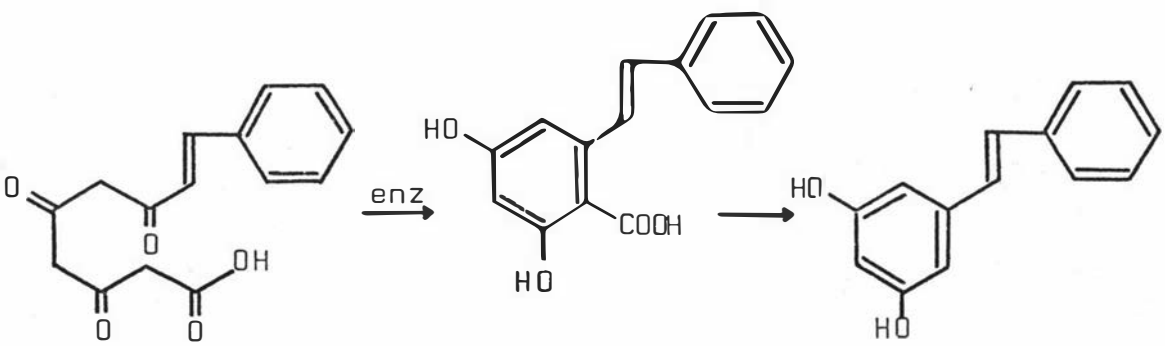


Figure 2. Postulated pathway of chalcone biosynthesis

Three molecules of malonyl-CoA (activated form of acetate as in fatty acid biosynthesis) condense with a molecule of cinnamic acid, activated probably as the CoA ester. Thus the β-polyketide progenitor of the aromatic A ring is formed. On intramolecular ring closure of this polyketide, a chalcone is formed as the first stable product and flavonoid.

While no enzyme system catalysing any part of this chalcone-forming reaction has yet been found, it is of interest that recently an enzyme catalysing a closely related cyclization of the polyketide compound, cinnamoyl triacetate, to the stilbene, pinosylvin, has been reported (13).



Cinnamoyl triacetate Pinosylvin

The common phloroglucinol-type A ring oxygenation pattern in flavonoids can now be seen to reflect the biosynthetic origin of the A ring from acetate. In those compounds which have resorcinol-type A ring substitution (5-deoxy compounds), the oxygen function normally in the 5-position (flavanone numbering) is probably lost by reduction at the polyketide stage (7). p-Hydroxylation in the B ring may occur at the cinnamic acid precursor level but further hydroxylation probably takes place subsequent to the chalcone formation. These finer details are however

uncertain and differences may exist between plants (8). Methylation and glycosylation of hydroxyl groups are very likely some of the final steps in the elaboration of the flavonoid molecule (9).

Results from many tracer experiments over a number of years have enabled certain biosynthetic relationships between the classes of flavonoids to be deduced (7,8,10,14). These interclass relationships are currently held to be as shown in Figure3(8,10).

The relative roles of chalcones and flavanones as precursors of other classes of flavonoids have recently been critically evaluated by Wong (10,14) in tracer studies and the results have implicated chalcones as the more direct precursors of the other classes investigated. This interpretation of interclass relationships shifts flavanones from their former, central role in flavonoid biosynthesis (8) to a side-branch position and is of major significance in relation to probable mechanisms for the biosynthesis of the other flavonoid classes (10).

Little is known of the enzymology of flavonoid biosynthesis or of the chemical details of the presumably multi-step transformations required in the interclass conversions spanned by single arrows in Figure 3. Enzymic studies, when possible, will obviously play a key role in the further elucidation of flavonoid biosynthesis.

A first step taken in the study of enzymes in flavonoid biosynthesis was the partial purification and study of the properties of the isomerase enzyme from soybean (Soja hispida) which catalyses the reversible inter-conversion of chalcones and flavanones (15). More detailed studies

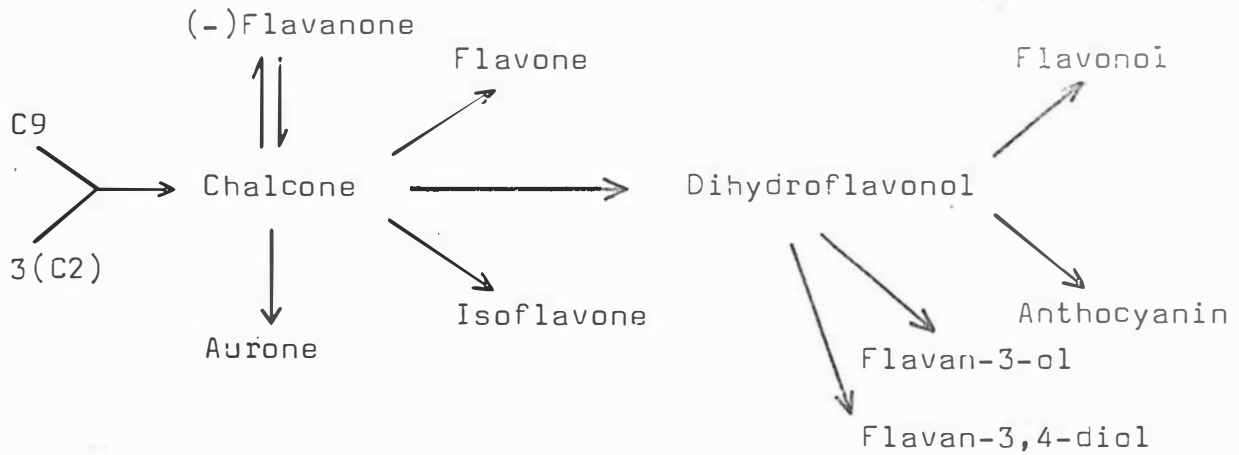


Figure 3. Probable interrelationships of flavonoid classes

on isomerase isoenzymes from a variety of plants have since been made (16). With the recent displacement of flavanones from the mainstream of flavonoid biosynthesis (10), the role of the isomerase would not now be of central importance to flavonoid biosynthesis.

Wong has also opened up the possibility of studying the enzymology of biosynthesis not only of aurone but also of dihydroflavonol. Cell-free extracts of soybean seedlings catalyse the enzymic oxidation of 2',4,4'-trihydroxychalcone (isoliquiritigenin) to the corresponding 4',6-dihydroxyaurone (hispidol) (17), probably by way of the intermediate hydrated aurone*, 4',6-dihydroxy-2-(α-hydroxybenzyl) coumaranone, which exists in diastereoisomeric forms, Y_1 and Y_2 (18). Similar cell-free enzyme preparations of Cicer arietinum seedlings catalyse the oxidation of the same chalcone to 4',7-dihydroxydihydroflavonol (garbanzol), besides Y_1 and Y_2 and hispidol (19). These enzyme products of chalcone oxidation are set out

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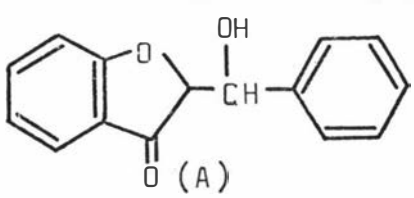
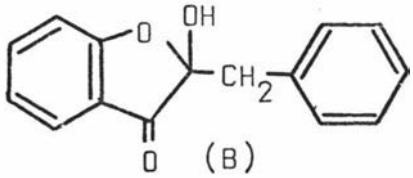
* The "hydrated aurone" structure referred to in this thesis is the β-hydroxyketone (A) rather than the alternative α-hydroxyketone (B) isomer, both of which may be formally considered as a hydrated aurone.



(A)



(B)

Isomer (A) should dehydrate readily to aurone, while isomer (B) is likely to be resistant to dehydration (see F.M. Dean, "Naturally Occurring Oxygen Ring Compounds", 1963, p 614, London, Butterworth and Co.)

in Figure 4.

Studies recorded in this thesis have developed from a further investigation of the enzymic conversion in cell-free systems of isoliquiritigenin into Y_1 and Y_2 and hispidol. Following a series of enzyme purification experiments it became apparent that Y_1 and Y_2 formation constituted but part of a complex system. In this system the chalcone was undergoing a peroxidase-catalysed oxidation to yield, in addition to Y_1 and Y_2, several other products, some of which turned out to be new variants of flavonoid compounds.

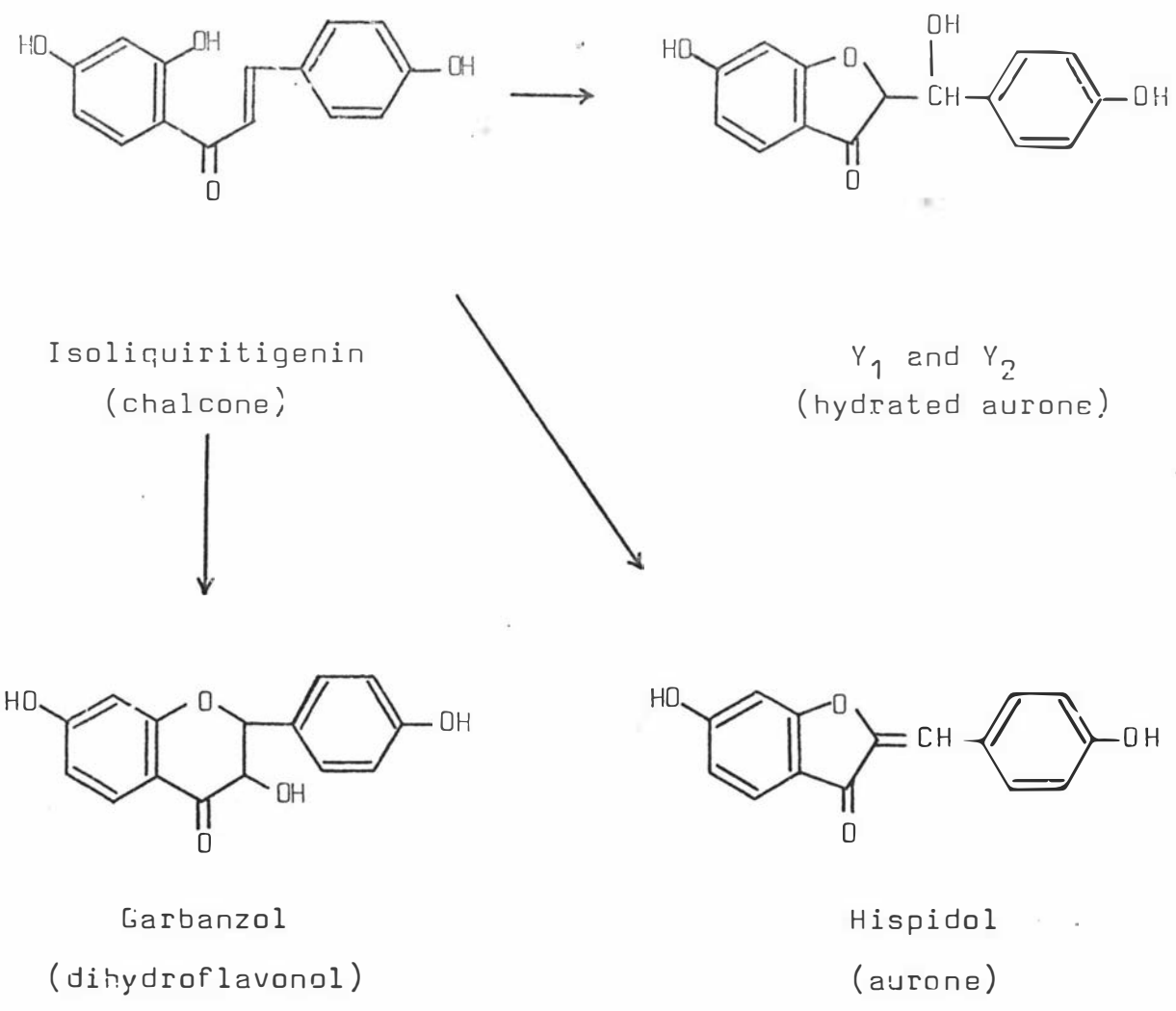
An introduction to the features of peroxidase-catalysed reactions which will be relevant to an appreciation of the chalcone-oxidising reaction will now be presented.

1B PEROXIDASE ENZYME AND ITS CATALYTIC FUNCTIONS

Plant peroxidases are in general haemoproteins of molecular weight about 40,000, with one haem group and a carbohydrate component in the molecule (20). One of the richest, and a traditional source of peroxidase is horseradish root (*Armoracia rusticana*), the first source of the crystalline enzyme (21). Apart from its enzymic function, horseradish peroxidase, as a haemoprotein, has many similarities to haemoglobin and myoglobin (22). Peroxidases are widespread in higher plants (23,24) and have been extensively purified from a number of sources (21). Animal sources of peroxidase enzymes are also well known (24).

In the discussion which follows, peroxidase will

Figure 4. Products of chalcone oxidation with cell-free enzyme preparations from soybean and cicer



refer chiefly to the preparation from horseradish, which is typical of most plant peroxidases in properties and mode of action (24).

1B-1 Enzyme activity

Peroxidase is a catalyst in several types of oxidative reactions in which a great variety of molecules are oxidised to yield an even greater variety of products (21,24).

Firstly, in its classical peroxidatic activity, the enzyme catalyses the oxidation of electron (hydrogen) donors by hydrogen peroxide, the electron acceptor.

$$H_2A + H_2O_2 \xrightarrow{\text{enzyme}} A + 2H_2O$$

This constitutes the common activity on which the classification of the enzyme rests, (donor: H_2O_2 oxidoreductase, EC 1.11.1.7).

A second, more complex, reaction catalysed is the oxidatic one, (peroxidase-oxidase reaction). In this case, molecular oxygen replaces hydrogen peroxide as the electron acceptor in the oxidation of a restricted number of electron donors (25).

$$H_2Y + O_2 \xrightarrow{\text{enzyme}} Y + H_2O_2$$

Finally, a direct oxygenation reaction, in the form of non-specific and non-stoicheiometric aerobic aromatic hydroxylation, is supported in a system based on the enzyme-catalysed oxidatic reaction (26).

$$H_2Y + O_2 \xrightarrow{\text{enzyme}} Y + H_2O + (O)$$

$$ArH + (O) \longrightarrow ArOH$$

1B-2 Enzyme-peroxide compounds

Peroxidase contains the iron porphyrin, protohaematin IX, as a prosthetic group which functions in the enzyme action (24). Of the six co-ordination positions of the haematin iron, four are occupied by the porphyrin nitrogens, a fifth by the linkage to a protein group and the sixth binds a molecule of water. Displacement of this group in the sixth position by a molecule of hydrogen peroxide (substrate) leads to the formation of distinct enzyme-substrate compounds through which the enzyme mechanism operates (21,24,26). These redox compounds of the enzyme are conveniently monitored by their distinctive absorption spectra (27,28).

Three redox compounds, known as Compounds I,II and III, are formed on treatment of the enzyme with hydrogen peroxide under certain conditions. Compound I, the green primary compound discovered by Theorell, is formed on reaction of the normal ferric form of the free enzyme with an equimolar amount of hydrogen peroxide (24,27). Given an oxidation state of +3 for the free ferric enzyme, Compound I has formally an oxidation state of +5. Compound II, which has a formal oxidation state of +4, may be formed either by a one-electron reduction of Compound I by a suitable electron donor or by treatment of the free enzyme with a slight excess of hydrogen peroxide (21,24,27). Kinetic evidence has been presented by Chance that in the latter case Compound II is still formed only through Compound I (21). Highly pure enzyme preparations are required to observe Compound I formation since any endogenous electron donor present will convert Compound I to Compound II.

A one-electron reduction of Compound II releases free enzyme. Redox Compounds I and II function as normal intermediates in the peroxidatic reaction (see next section) (27). With higher concentrations of hydrogen peroxide the enzyme forms the red-coloured Compound III (21,24,27) which is catalytically inactive in the peroxidatic reaction but which may be active in the oxidatic reaction (28). Compound III, as shown by George, is formed in the reaction of a molecule of hydrogen peroxide with a molecule of Compound II (27) and has formally an oxidation state of +6 (28). Electron donors invariably react faster with Compounds I and II than with Compound III (28).

Ferroperoxidase, obtained on reduction of the ferric enzyme either chemically or by the free radicals of semi-oxidised biological electron donors, reacts rapidly with oxygen to form a compound, oxyperoxidase, which is probably identical with Compound III (22,28). Dissociation of oxyperoxidase or of Compound III into ferrous enzyme and oxygen could not be detected (22,28). Recently Noble and Gibson (29) have shown that ferroperoxidase is converted to oxyperoxidase by hydrogen peroxide in two steps, each of which is a two-electron oxidation, Compound II being an intermediate in this conversion. A further mode of Compound III formation suggested (28) is the reaction of perhydroxyl radical ($HO_2^{\cdot}$), or superoxide anion ($O_2^{-\cdot}$), the ionic equivalent, with free enzyme. This radical is a postulated intermediate in the oxidatic reaction (25).

Interrelationships between the five oxidation states

of peroxidase are summarised in Figure 5 (28). Oxyperoxidase and Compound III have been equated in the Figure (22,29). The number in parenthesis gives the formal oxidation level of each state.

18-3 Peroxidatic reaction

The mechanism of this classical reaction catalysed by peroxidase embodies the cycle of reactions linking the free enzyme, Compound I and Compound II in Figure 5 and is set out in the equations below (21,27,28).

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + H_2A \longrightarrow \text{Compound II} + HA^{\cdot} + H_2O$$

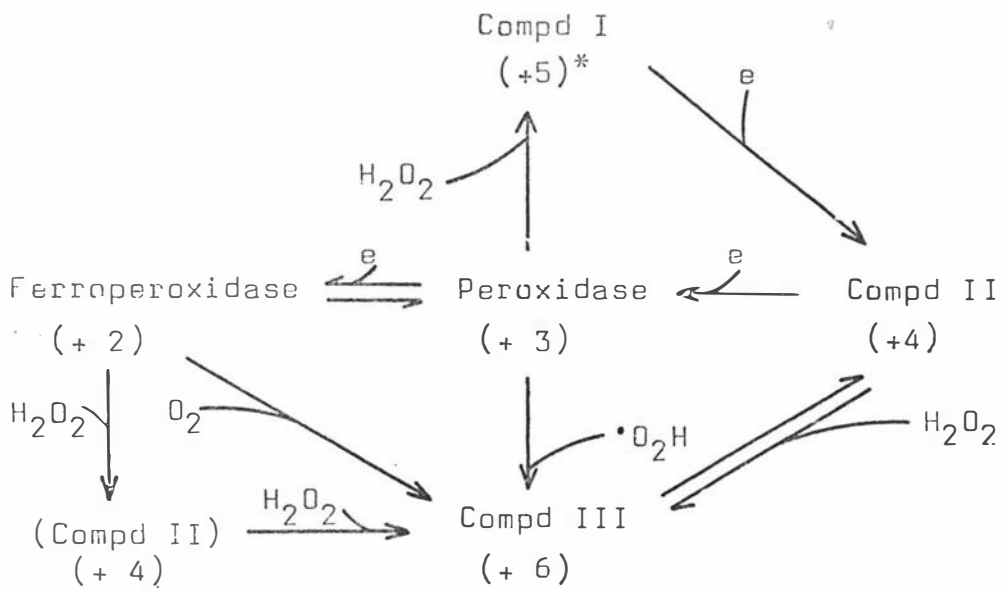
$$\text{Compound II} + H_2A \longrightarrow \text{Peroxidase} + HA^{\cdot} + H_2O$$

$$2HA^{\cdot} \longrightarrow A + H_2A$$

$$\text{Overall:} \quad H_2A + H_2O_2 \longrightarrow A + 2\,H_2O$$

The donor molecule (H_2A) being oxidised by hydrogen peroxide supplies the electrons to reduce Compound I to free enzyme in two one-electron steps through Compound II. Free radicals of the donor which are consequently formed are thought to remain free in solution and to undergo a dismutation reaction, being apparently poor electron donors for Compounds I and II (28,30). The above peroxidatic mechanism is founded on the studies of Chance and George who showed that the transitions, Compound I to Compound II to free enzyme, were both one-electron reductions (21,27,28). In the course of the reaction, free radicals of donors have been detected by titration with suitable electron acceptors and more recently by electron paramagnetic resonance (EPR) spectroscopy (28,30). Typical donors in the

Figure 5. Interrelationships of the five redox states of peroxidase



* The number in parenthesis gives the oxidation level of that redox state.

peroxidatic reaction include guaiacol, ascorbate, p-phenylenediamine, pyrogallol and hydroquinone.

1B-4 Oxidatic reaction

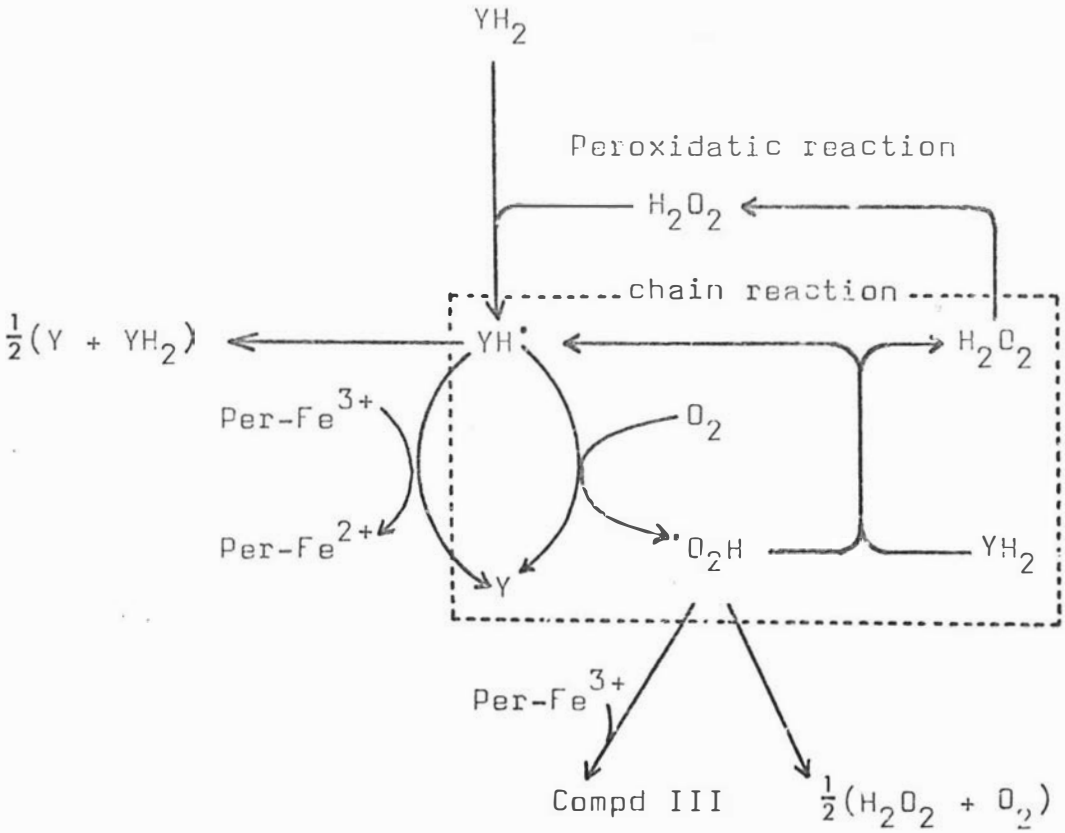
While the mechanism of the peroxidatic reaction is well established that of the oxidatic reaction is still somewhat indefinite (28). The first example of the oxidatic reaction was the aerobic oxidation of dihydroxy-fumaric acid, reported by Swedin and Theorell (31). Since then, the reactions of several additional electron donors have been investigated (see ref. 25). Basically, the oxidatic reaction occurs by virtue of molecular oxygen accepting electrons from the donor molecule in the presence of enzyme, to yield most simply hydrogen peroxide and oxidised donor as products. This multi-step sequence may be summarised in essence in equation 1.

$$YH_2 + O_2 \xrightarrow{\text{enzyme}} Y + H_2O_2 \quad \text{Eqn. 1}$$

Since the donors are not appreciably autoxidisable under the conditions of the reaction, it is the function of the enzyme to activate donor or oxygen or both (28).

Proposed mechanisms (25) for the oxidatic reaction have been reviewed by Yamazaki and co-workers (28,32). In Figure 6, the free radical mechanism postulated by this group to explain the essential features of the oxidatic reaction with a number of donors (YH_2) is reproduced. Donors specifically included are dihydroxyfumaric acid, indole acetic acid, triose reductone and reduced nicotinamide adenine dinucleotide (NADH). An added complication

Figure 6. Postulated mechanism for the oxidatic reaction catalysed by peroxidase.



Per-Fe^{3+} = ferriperoxidase

is that the properties of the overall reaction differ somewhat for each donor (25,33). Relative velocities for the component steps of the overall reaction with each of the four donors mentioned above have been summarised (33) and illustrate the significance of this donor effect.

To initiate the oxidatic reaction, the donor free radical (semiquinone) is thought to be formed in a classical peroxidatic reaction using hydrogen peroxide. Such free radicals have not yet been found, however, in measurable concentrations (ESR) under aerobic conditions, but indirect evidence for their existence has been obtained (28,34). Semiquinones of donors in the oxidatic reaction are considered able to reduce molecular oxygen to superoxide anion (perhydroxyl radical), a postulated key intermediate. This reaction has been indicated experimentally for the semiquinones of dihydroxyfumaric acid, triose reductone (25) and vitamin K_3 (28), using ESR techniques. In the next step, superoxide may then oxidise another donor molecule to the semiquinone, being itself reduced to hydrogen peroxide (28). A non-enzymic chain reaction, expressed in the simple stoicheiometry of equation 1 above, is thereby established. Recently, it has been possible to demonstrate, by ESR, suspected superoxide radical formation during the oxidation of dihydroxyfumaric acid catalysed by horseradish peroxidase (35). The correctness of the identification of this radical has since been confirmed (36). Further, superoxide has also been shown to react with dihydroxyfumaric acid (37). These recent findings, therefore, greatly strengthen the argument for the mechanism presented in Figure 6.

While a small amount of hydrogen peroxide, possibly already present in solutions of the donor, is necessary to start the oxidatic reaction, the system is autocatalytic, given an efficient chain reaction (34). Efficiency of the chain reaction may be increased by manganous ion, possibly functioning as shown in Figure 7 to increase the effectiveness of the chain-maintenance reaction over the potentially rapid chain-breaking dismutation of superoxide to peroxide and oxygen (25,28). At higher pH, manganous ion is especially effective to bridge between the mutually repulsive superoxide and ionized donor anions. Activation by manganous ion is strong in dihydroxyfumaric acid, indole acetic acid and NADH oxidations, but is weak in the triose reductone system (25).

A further chain-breaking reaction, dismutation of the semiquinone as in the peroxidatic sequence, depends on the nature of the donor, being prominent in triose reductone oxidation (25).

Reduction of the free ferric enzyme to the ferrous state by the semiquinone is shown simply as an apparent chain-breaking reaction in Figure 6. This reaction is of much further interest, however, for it is the starting point of an alternative pathway proposed for oxygen reduction in the oxidatic reaction. Unlike the previously considered reaction sequence, this additional chain reaction is enzyme mediated. Thus two pathways of electron flow from the donor molecule to molecular oxygen acceptor, one basically non-enzymic and the other enzymic, may operate in the oxidatic reaction as depicted in Figure 8 (28,32,33). The distribution of the total electron flux

Figure 7. Suggested mechanism of activation of the oxidatic reaction by manganous ion.

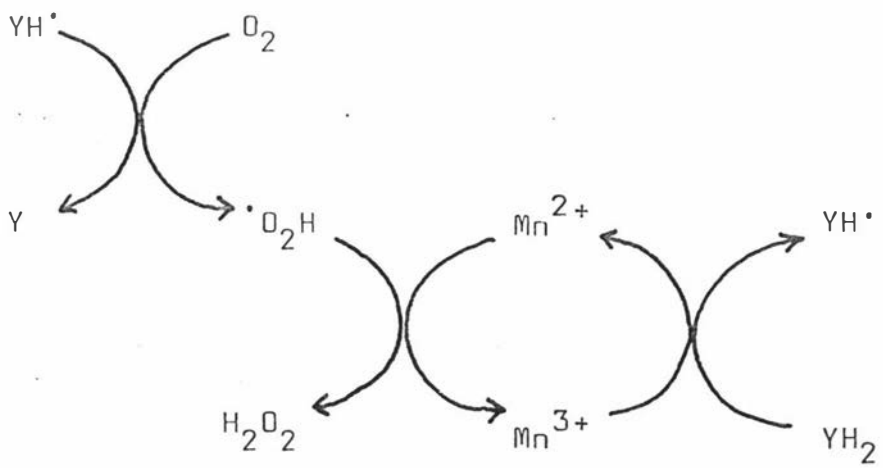
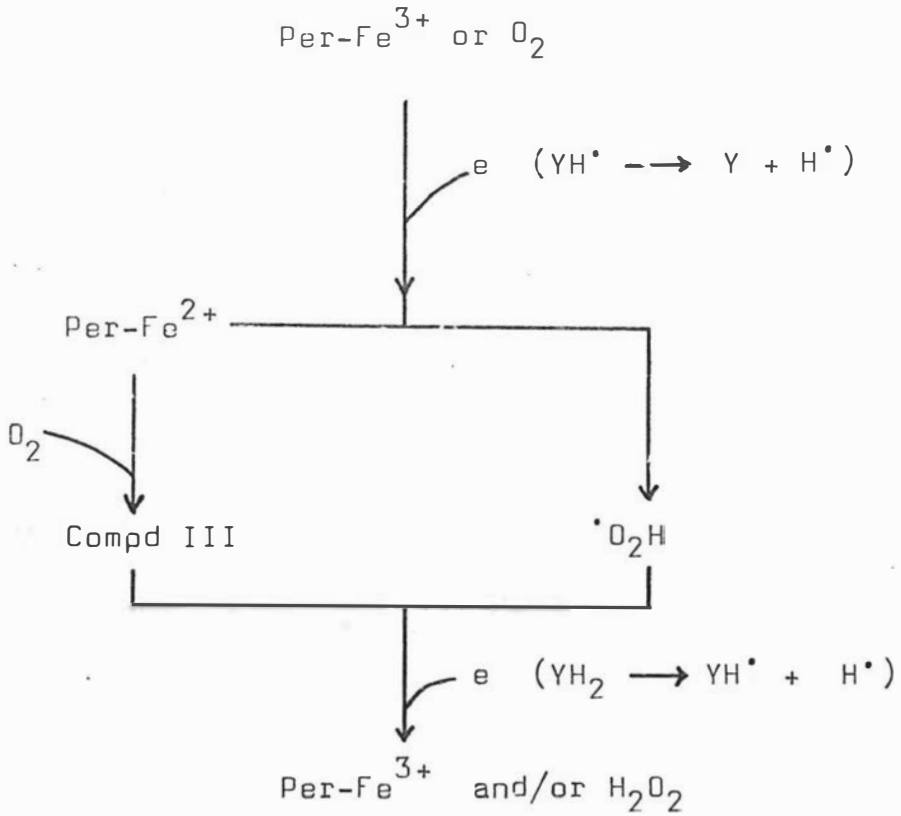


Figure 8. Competing pathways of electron flow in the oxidatic reaction.



between these competing pathways is donor dependent (32,33); the enzymic pathway being particularly competitive with indole acetic acid as donor (28,33). The envisaged mode of operation of the enzymic pathway depends on the semiquinone of the donor reducing free enzyme rather than molecular oxygen directly (Figure 8). Molecular oxygen then reacts with the ferroperoxidase to form oxyperoxidase, or Compound III, in which oxygen is considered activated possibly by a partial movement of an electron from iron to oxygen (25,28). To complete the chain reaction in Figure 8, Compound III is considered to react with another molecule of hydrogen donor to yield free enzyme and semiquinone of the donor. Thus the chain reaction again accounts for the oxidatic reaction as earlier summarised in equation 1.

The degree of experimental support for this enzyme-mediated chain reaction varies depending on the hydrogen donor. Thus while reaction of certain hydrogen donors with Compound III is rapid, as in the case of indole acetic acid (28), it remains that with dihydroxyfumaric acid (25,32) and NADH (15) donors, Compound III formed during the oxidatic reaction is evidently not involved in the chain reaction sequence. This latter situation may reflect essentially the exclusive operation of the non-enzymic chain reaction sequence in dihydroxyfumaric acid oxidation. Formation of Compound III may still occur, not by oxygenation of the ferrous enzyme but by reaction of the ferric enzyme with perhydroxyl radical (see Figure 5). In Figure 8, an essential role of the ferrous enzyme in the chain reaction is envisaged, yet

the role of the ferrous enzyme in the oxidatic reaction has been much contested in the past, no doubt partly as a result of the variable effects of carbon monoxide on the system (25,28).

18-5 Classification of hydrogen donors

While a great variety of compounds are electron donors in peroxidase-catalysed reactions, they can be simply classified into two types on the basis of the redox properties of their free radicals. This classification is of interest in the study of peroxidase-catalysed reactions for it indicates those donors which may operate either to promote or inhibit the reaction of another donor.

Yamazaki has classified hydrogen donors in the peroxidatic reaction as redogenic or oxidogenic, according to whether their free radicals exhibit reducing or oxidising activity respectively (38,27,25).

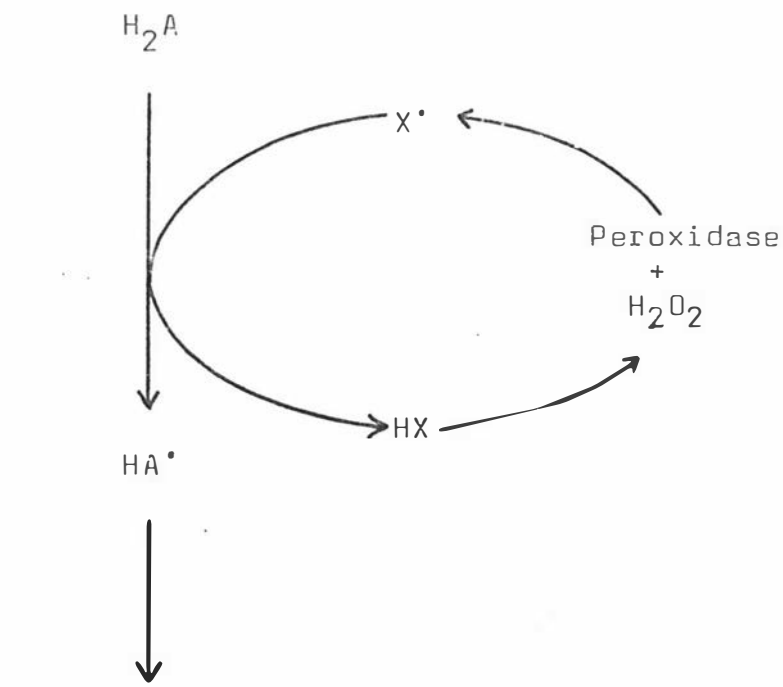
$H_2A \rightleftharpoons HA^{\cdot} \rightleftharpoons A$ Redogenic donor

$HX \rightleftharpoons X^{\cdot} \longrightarrow$ Dimers etc. Oxidogenic donor

Substrates for the oxidatic reaction are always redogenic donors, but both types of donor may undergo peroxidatic reaction.

Oxidogenic donors can accelerate both peroxidatic and oxidatic reactions by virtue of the oxidising power of the enzymically generated free radical, as shown in Figure 9 (27,39). On the other hand, redogenic donors are normally inhibitory, being competitive with the other donor in the reaction (27).

Figure 9. Mechanism of accelerating action of oxidogenic donors.



1B-6 Aerobic aromatic hydroxylation

In the aerobic dihydroxyfumarate-peroxidase system, Mason and co-workers have reported non-specific aromatic hydroxylation in which the oxygen incorporated originated from molecular oxygen (26). The oxidatic reaction of dihydroxyfumarate which occurs serves not simply as a source of hydrogen peroxide, but of some oxygen-derived radical species which functions as the hydroxylating agent. Superoxide (perhydroxyl radical) has been suggested as the hydroxylating agent (25,26,28), but as this radical appears incapable of hydroxylating benzene or naphthalene, Daly and Jerina have recently suggested that a more reactive, possibly enzyme-bound, radical is involved (40).

Addition of manganous ion to the system inhibits hydroxylation completely despite the increased rate of dihydroxyfumarate disappearance. This effect on hydroxylation has been explained as a depletion of the oxygen-derived hydroxylating radical, active in the coupled hydroxylation, as a result of catalysis by manganous ion of the chain reaction which reduces superoxide to hydrogen peroxide (26) (see Figure 7). This explanation, of course, equates the hydroxylating species with superoxide.

Absence of the NIH shift, both in this peroxidase system and in model systems involving radical species, supports the view that the hydroxylating agent is radical in nature (40). The peroxidase hydroxylating system is unique in the absence of the NIH shift, for the shift is always observed in similar hydroxylations of aromatic substrates catalysed by mixed-function oxidases (40).

1B-7 The present study

The study reported in this thesis has uncovered a new example of peroxidase acting as a catalyst in a reaction which combines in the same substrate, features of both the oxidatic and hydroxylation reactions discussed above. Products of chalcone oxidation in this new reaction include not only recognised flavonoids but also new variants of flavonoid compounds.

Chapter 2

RESULTS

2A NATURE AND PRODUCTS OF THE ENZYMIC REACTION

Wong has previously reported that cell-free enzyme extracts of garbanzo seedlings catalyse the conversion of 2',4,4'-trihydroxychalcone (isoliquiritigenin) into 4',6-dihydroxy-2-(α -hydroxybenzyl)coumaranone isomers (Y_1 and Y_2) and 4',7-dihydroxydihydroflavonol (garbanzol) - see Introduction, Figure 4 (14,18,19). The yields of Y_1, Y_2 and particularly garbanzol are low; most of the chalcone consumed is isomerised to flavanone by the isomerase enzyme (15,16) which is also active in these cell-free extracts.

The present work was commenced as an investigation of the enzymology of Y_1 and Y_2 formation in this garbanzo system. However, it expanded to include the study of a hitherto unknown product which first became prominent following certain fractionations of the protein of the garbanzo system. Subsequently, it was recognized that Y_1, Y_2, garbanzol, this new compound and 4',7-dihydroxyflavonol were all co-products of a complex reaction system. The enzyme responsible for their formation was recognised to be a peroxidase.

2A-1 Effect of enzyme purity on products* observed

At the outset, cell-free extracts of garbanzo seedlings were subjected to a variety of protein fractionation procedures and the resulting preparations were assayed for enzyme activity by incubation with chalcone in 0.05 M tris buffer, pH 7.5, at 37° for 1.5 hours. Products and unchanged chalcone, recovered after incubation by ether extraction, were separated by 2-D paper chromatography. Results were assessed visually by inspection of the chromatograms either in u.v. light or after spraying with diazotised sulphanilic acid.

With cell-free extracts, the expected low yields of Y_1 and Y_2 were obtained. Heat treatment of the cell-free extract (50° for 60 min) was without apparent effect on the Y_1 and Y_2-forming system, yet much protein was denatured and removed by centrifugation and the isomerase enzyme inactivated. Consequently, this heat treatment was applied routinely in subsequent fractionation experiments since the absence of isomerase activity in the supernatant meant economy in the chalcone substrate required.

Fractionation of the heat-treated supernatant with solid ammonium sulphate in sequential 10% saturation increments from 30 - 100%, yielded protein fractions which all showed unexpected, though similar, catalytic activity

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* Unless specifically stated otherwise, in the rest of this thesis 'chalcone' refers to isoliquiritigenin while 'flavonol' and 'aurone' refer to the corresponding 4',7-dihydroxy-compounds, 4',7-dihydroxyflavonol and hispidol respectively.

towards chalcone. Firstly Y_1 and Y_2-forming activity was very low in all fractions and a considerable overall loss of this activity was evident. Secondly, an unknown, extra product was formed in all cases in amounts many times greater than Y_1 and Y_2. This new compound, designated OC (oxidised chalcone), was readily detected on chromatograms under u.v. light as a dull purple spot which turned bright blue on fuming with ammonia. The occurrence of OC was accompanied by greatly increased, and sometimes complete, utilisation of chalcone substrate during incubation. In contrast, much chalcone invariably remained after incubations with heat-treated preparations in which only Y_1 and Y_2 formation was evident.

In addition, protein which was first precipitated from heat-treated supernatant with ammonium sulphate added to 70% saturation and then fractionated by five successive increments in acetone concentration, provided enzyme preparations which displayed catalytic activity similar to the ammonium sulphate-derived fractions above.

Some concentration of OC-forming activity was evident particularly in the preparations precipitated at lower acetone concentrations, but no clear separation of Y_1 and Y_2- from the OC-forming activity could be detected in any preparation.

Following successful repetition of these experiments in protein fractionation with ammonium sulphate and acetone, it was clear that where the enzyme preparation had been through a precipitation step before assay, two effects would invariably show up in the assay results; firstly, very substantial apparent loss of Y_1 and Y_2-forming activity

and secondly, appearance of the OC product.

2A-2 Evidence for the presence of promotors and inhibitors

In an investigation of the cause of low recoveries of Y_1 and Y_2-forming activity in ammonium sulphate-precipitated fractions, it was first checked that the enzyme responsible had not remained largely in solution in the presence of ammonium sulphate and had thereby escaped detection. Thus, dialysis of the fraction of heat-treated supernatant which was soluble at 70% saturation with ammonium sulphate, against 0.05 M tris pH 7.5 to remove the ammonium salt, gave an enzyme preparation with activity characteristic of an ammonium sulphate-precipitated fraction.

Activity in this dialysed preparation typical of a precipitated preparation, rather than of the original heat-treated one, suggested the absence of dialysable factor(s) could be responsible for the changes asscciated with precipitated enzyme. As a test of this hypothesis, boiled supernatant, prepared from cell-free extract inactivated by heating at 100^{o} for 15 minutes, was added in varying amount to an ammonium sulphate-precipitated enzyme preparation (heat-treated supernatant saturated to 70%) and the mixture incubated with chalcone. Chromatograms of the products showed clearly that the supernatant preparation had the dual effect of restoring Y_1 and Y_2 yields to normal and eliminating evidence of the OC product. Appropriate controls demonstrated the expected product distribution with the ammonium sulphate-precipitated enzyme and the absence of enzyme activity in boiled supernatant alone.

The concentration of boiled supernatant in the

incubation mixture had an important effect on the chromatographic pattern of products; Y_1 and Y_2 levels increased and OC levels decreased progressively as the concentration of this supernatant increased in the incubation volume. Levels of Y_1 and Y_2 increased detectably at supernatant concentrations well above those at which OC first disappeared. On the basis of quantities added in these runs, cell-free extracts as routinely prepared were estimated to contain normally a concentration of inhibitory factor(s) slightly greater than required for complete suppression of OC. The concentration dependence of OC inhibition was also clearly shown when cell-free extract, normally assayed with minimum dilution, was diluted serially up to 8-fold and assayed. Production of OC appeared with the first 2-fold dilution and increased with dilution thereafter. Chalcone remaining decreased as OC increased, till finally all chalcone added was consumed, indicating that throughout this series of incubations the level of inhibitor rather than the level of enzyme for OC formation was limiting the reaction. Within the dilutions employed, little effect on Y_1 and Y_2 level was evident.

2A-3 Treatment effects on Y_1 and Y_2- and OC-forming activities

Further experiments were undertaken to obtain evidence on the possible relationships of the Y_1 and Y_2 and OC-forming activities. A check was made for both activities in enzyme preparations which had been subjected to a variety of treatments with the view to finding any major differential effects of the treatments on the two activities. Assays of OC-forming activity were made after the protein had been

put through a precipitation step, normally with ammonium sulphate (to 70% saturation) while boiled supernatant was added to precipitated enzyme to check Y_1 and Y_2-forming activity. Since major effects were being looked for, activities were assessed visually on 2-D chromatograms of the products.

Both activities persisted after heat treatment of a cell-free extract at 50^{o} for 2 hours. Likewise, the effect of lower pH treatments down to pH 3.5, for a time of about 10 minutes at 0^{o} during which the precipitated protein was removed by centrifugation and the acid supernatant then titrated back to pH 7.5, showed no differential effect on the activities assayed. Fractional precipitation of protein with either ammonium sulphate or acetone, as mentioned earlier, failed to separate the activities. Enzyme precipitated from solution 70% saturated with ammonium sulphate was found to store at 0 - 4^{o} in solution in 0.05 M tris buffer pH 7.5 for up to 6 months with considerable retention of both activities. Boiled supernatant was also fully active after several months in similar storage.

Collectively, these studies indicated that both activities were very stable and were retained in a single preparation after a wide variety of treatments and a considerable time in storage. On the basis of this parallel behaviour it was believed possible that the same enzyme was responsible for both activities.

2A-4 Detection and identification of OC-forming enzyme

Polyacrylamide gel electrophoresis of an enzyme

preparation precipitated from 70% saturated ammonium sulphate solution was undertaken using a tris-glycine buffer system, to effect rapid and efficient separation of enzymes present in a medium in which any zones of Y_1 and Y_2 and OC formation could be detected by decolorisation of a yellow chalcone stain.

After the gel had been run with normal polarity settings so that only net-negatively charged molecules entered the vertical gel slab from wells formed in its upper edge, a chalcone stain applied showed gradual bleaching to occur over the next 30 minutes in one zone a small distance within the gel from the sample position. Viewed under u.v. light, this bleaching appeared to be due to OC formation. In further similar runs this active zone was shown to coincide with peroxidase activity as detected by a benzidine staining reaction in the presence of hydrogen peroxide. Addition of a little hydrogen peroxide to a further chalcone-stained gel in tris buffer at pH 7.5 produced very rapid chalcone decolorisation in the zone where slow formation of OC had previously been suspected. Using a stained guide strip, the appropriate zone was excised from the rest of another gel and the enzyme eluted by crushing the gel segments in buffer. Incubation of filtered aliquots of this eluted enzyme with chalcone showed, on chromatography, that a low production of OC occurred, which was greatly stimulated on the addition of a little hydrogen peroxide. Addition of boiled supernatant to the enzyme appeared to suppress all reaction; no Y_1 and Y_2 were evident as products of any of these incubations.

Unsuccessful attempts to locate a zone of Y_1 and Y_2 formation in the gel were made by incubation for up to several hours of chalcone-stained gel strips in boiled supernatant, which was known to promote the Y_1 and Y_2-forming reaction. No evidence of chalcone decolorisation in visible or u.v. light could be detected; OC formation in the previously detected zone was inhibited by the boiled supernatant. Since the Y_1 and Y_2-forming reaction was not expected, on previous chromatographic evidence, to cause complete removal of the chalcone, a further check for low levels of Y_1 and Y_2 was made under u.v. light by cautious treatment of the incubated gel strips with dilute (0.1 N) alkali for a few minutes. No evidence of the formation of the characteristically fluorescent aurone product was noted on acidification of the alkali-treated gel strips.

Gels were then run as before, except with reverse polarity in order that net-positively charged molecules would enter the gel. Application of the procedures used with normal polarity gels showed that two distinct zones of OC formation coincided with similar zones of peroxidase activity, a short distance within the gel from the sample position. Samples of the less mobile and much stronger peroxidase band were eluted and the enzyme assayed with chalcone to confirm, upon chromatography, OC formation. Again, no evidence for Y_1 and Y_2-forming activity could be detected in these gels.

Thus, from this work with polyacrylamide gels, OC formation appeared to be catalysed by two and probably three separate peroxidase isoenzymes present in the garbanzo seedling enzyme preparation. Further, added hydrogen

peroxide greatly accelerated the reaction as judged by its effect on the rate of decclorisation of the chalcone stain in the active zones in the gel.

To extend these results, the ammonium sulphate-precipitated enzyme used in gel electrophoresis was incubated with chalcone (0.75 µmole) in 0.05 M tris buffer pH 7.5 (4 ml) at room temperature and the normally slow reaction was found to proceed by rapid decolorisation on addition of hydrogen peroxide (1 µmole). Incubation time was therefore reduced to 2 minutes in experiments of this nature. Chromatograms of the ether extracted products showed much increased OC and decreased residual chalcone where hydrogen peroxide had been added.

In a further otherwise identical experiment, the garbanzo enzyme in the above reaction mixture was replaced by horseradish peroxidase (HRP) enzyme of equivalent peroxidatic activity in the guaiacol assay. Product chromatograms demonstrated that the HRP enzyme gave results which were very similar to those for the garbanzo preparation. At this point it was accepted that typical peroxidase enzyme in the garbanzo preparations catalysed OC formation.

2A-5 Reaction products in addition to OC

The chromatograms of products of rapid incubations carried out in the presence of added hydrogen peroxide (not only those of the experiments mentioned above but also numerous other similar ones) revealed a pattern of spots much more complex than was expected from the earlier pattern established from incubations of precipitated garbanzo enzyme for 1.5 hours at 37^{o} without added hydrogen peroxide.

A typical chromatographic pattern for the latter type of incubation is shown in Figure 10. Identification and colours of the spots are given in Table 1. The main product was OC; Y_1 and Y_2 were detectable spots either after the chromatogram was sprayed with diazotised sulphanilic acid or kept for a time to permit spontaneous formation of aurone artifact. An aurone spot (A), clearly visible but quantitatively very minor, was also present, presumably as a result of dehydration of Y_1 and Y_2 prior to chromatography. Residual chalcone (C) and a trace of flavanone (F) could also be present. Some weak background spots representing native phenolic compounds present in the garbanzo enzyme preparation, despite precipitation treatments, were also present but are omitted from Figure 10 in the interest of clarity.

The pattern typical of short-time incubation in the presence of added hydrogen peroxide is shown in Figure 11. (See also Table 1 for colour reactions). The major spot was again OC. Most significant and variable were the occurrences of the three quantitatively minor new spots, Y_1', Y_2' and YOL. The Y' spots occupied approximately the positions of the true Y spots (hydrated aurone isomers), while the YOL and aurone spots partially overlapped. Normally the aurone spot was very weak, being frequently barely discernible, in contrast to the situation in Figure 10. A number of quantitatively minor background spots, not shown in Figure 11, were invariable present, in addition to the traces of native phenolics when the garbanzo enzyme preparations were used.

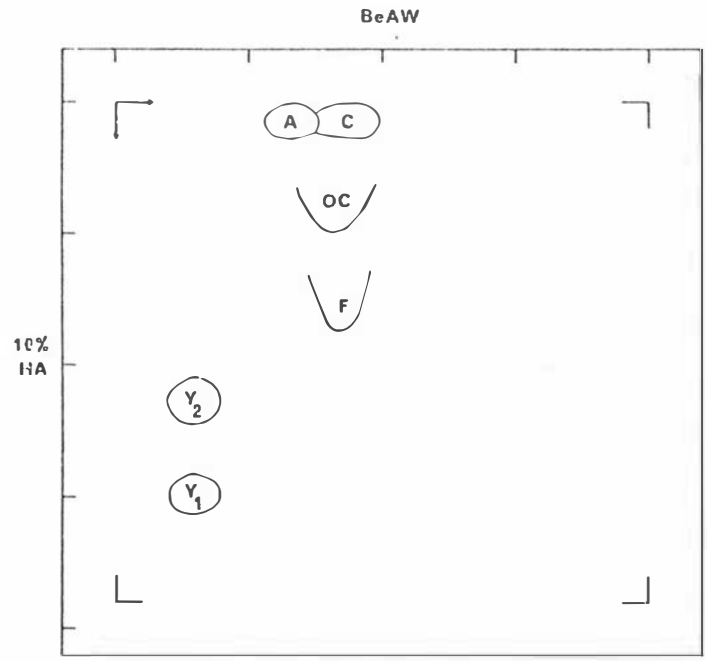
Identification of YOL as the known compound

Figure 10

Pattern, on 2-D chromatogram, of products of incubation of chalcone with precipitated garbanzo enzyme for $1^1/2$ hours at 37^o, hydrogen peroxide not added. See Table 1 and text for identification of spots. BeAW, benzene-acetic acid-water, 125: 72:3. HA, acetic acid in water.

Figure 11

Pattern, on 2-D chromatogram, of products of incubation of chalcone in the presence of added hydrogen peroxide for two minutes with either precipitated garbanzo or HRP enzyme. See Table 1 and text for identification of spots.


BeAW
A
C
OC
F
10%
HA
Y2
Y1

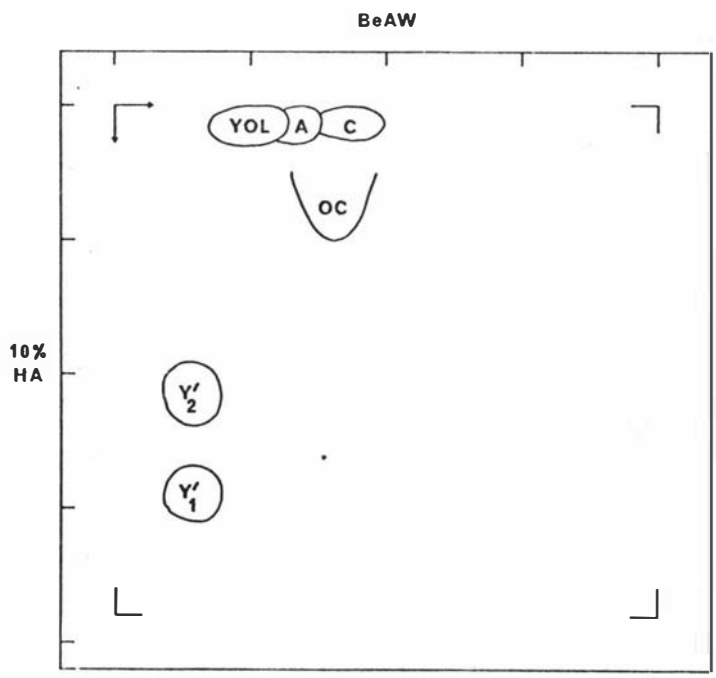

BeAW
YOL
A
C
OC
10%
HA
Y'2
Y'1

Table 1

Colours of spots detected on paper chromatograms..

Spot	Identification	colour[x] in			
		Visible	u.v.	u.v.+ NH_3	u.v. + visible
C	Chalcone	Gold	dkBr	bO-Y	-
OC	-	-	dkPu	bB	-
Y_1 or Y_2*	Hydrated aurone	-	-	-	-
A	Aurone	Y	bGr-Y	Gold	flGr-B
YOL	Flavonol	fY-Gr	bY	bY	flY
Y_1' or Y_2'ø	Mixture /	f dull Y	B-Gr-Y	dull Y	dull flY
F	Flavanone	-	V	V	-

.. Refer to Figures 10 and 11

x dk = dark, f = faint, l = light, b = bright, fl = fluorescent, Y = yellow, Gr = green, B = blue, O = orange, Br = brown, Pu = purple

* Detected as aurone artifact or with diazotised sulphanilic acid

ø Colours represent artifacts which form rapidly

/ Consists of precursors of OC and flavonol, besides hydrated aurone

4',7-dihydroxyflavonol (flavonol) was readily made by comparison with an authentic synthetic sample.

Chiefly on the basis of colour reactions in u.v. light, the Y'spots were first distinghished from the true Y spots. Elution and rechromatography of the Y' spots, separately, indicated each gave rise to a number of other spots, those recognised being Y_1 or Y_2, A, OC and YOL. This complex result will be rationalised in the course of this thesis. However, the immediately significant findings were as follows. Firstly, the existence of Y_1 and Y_2 in the respective Y' spots, from HRP-catalysed reactions in particular, strongly suggested that these isomers were also produced in the peroxidase-catalysed reaction which formed OC. Rechromatography of the Y' spots showed complete transformation of the original components had occurred except for Y_1 and Y_2, the residual amounts of which then became clearly visible. At the same time, some dehydration of Y_1 and Y_2 had also occurred as shown by the appearance of aurone on rechromatography. Secondly, the occurrence of a new product, flavonol, was clearly shown; its presence in the HRP reaction products again implicated peroxidase enzyme catalysis. Thirdly, precursors of both OC and flavonol existed in each Y' spot, together with the recognised aurone precursor, Y_1 or Y_2.

Thus, in the light of these results, the peroxidase-catalysed reaction of chalcone potentially assumed new dimensions in relation to not only variety of products but also precursor relationships.

The interpretation that a peroxidase enzyme was responsible for catalysis which resulted in the formation

of all compounds shown in Figure 11 was tested with cell-free garbanzo enzyme and found to be supported by the results. In this test, redogenic donors, catechol and hydroquinone, were used in separate experiments to specifically inhibit peroxidase-catalysed reactions of chalcone. Preliminary investigation showed an acid-treated cell-free enzyme preparation (see section 2A-6 Figure 12) catalysed chalcone oxidation very rapidly on addition of hydrogen peroxide to yield a chromatographic pattern of products as in Figure 11. Therefore the inhibition of OC formation of this acid-treated preparation, assayed in the absence of added hydrogen peroxide, could be overcome satisfactorily by the addition of that compound. When the same reaction mixture of chalcone (0.75 μmole) and acid-treated cell-free enzyme (4 ml in pH 7.5 tris) was supplied separately with catechol and hydroquinone (1 mM final concentration) and hydrogen peroxide (1 μmole) finally added, only a very slight reaction of chalcone was noted from product chromatograms, even for an incubation time of 30 minutes in the case of hydroquinone. A very faint OC spot was present, together with possibly a trace of Y_1 and Y_2, but no flavonol. Both redogenic donors were therefore found to almost completely inhibit chalcone conversion to OC, Y_1 and Y_2 and flavonol, which supported each of these compounds being products of peroxidase-catalysed conversions.

Purification of the peroxidase enzyme from garbanzo seedlings was therefore undertaken in reasonable certainty that the desired enzyme activities would be isolated in this one preparation.

2A-6 Partial purification of peroxidase enzyme from garbanzo seedlings

A procedure was developed to purify the soluble peroxidase present in cell-free buffer extracts of garbanzo seedlings in preparation for studies on the enzyme-catalysed transformations of chalcone.

Enzyme activity was assayed spectrophotometrically throughout the purification procedures using guaiacol as the peroxidatic hydrogen donor. Periodic tests of activity toward chalcone confirmed that the desired activity was being monitored in this manner.

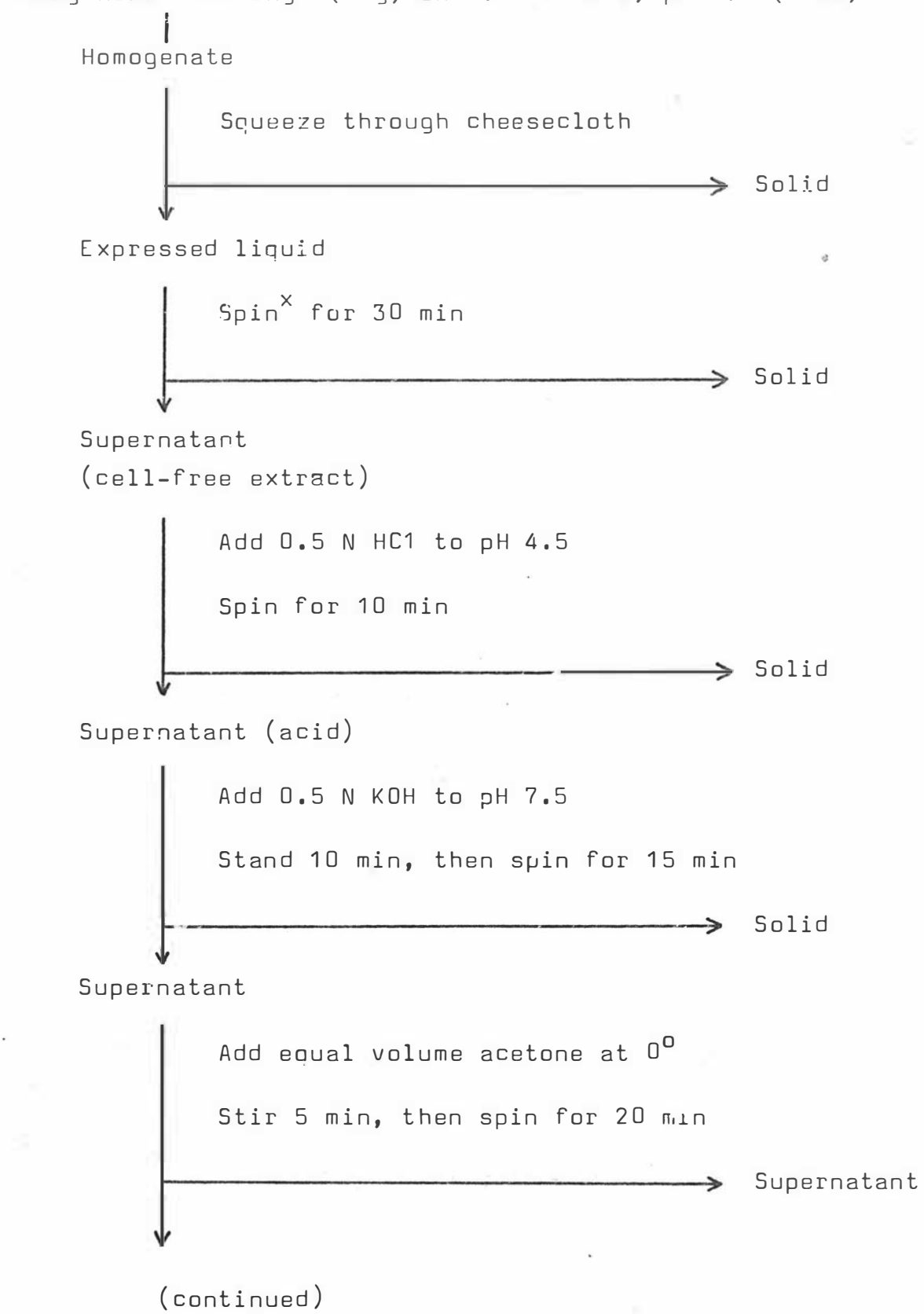
A flow sheet of the standard purification procedure (see Experimental) is presented in Figure 12. All manipulations were carried out at 0 - 4° in tris buffers, pH 7.5. Purified enzyme could be obtained 30-36 hours after the purification began.

After the acetone precipitation step of the purification (Figure 12) the redissolved enzyme was filtered through Sephadex G-75, (column 35 x 3 cm) overnight. Enzyme activity and protein content in each approximately 8.5 ml fraction collected from this column at 30 minute interval are recorded in Figure 13. Fractions 15-18 inclusive, which accounted for 86% of the enzyme activity in the sample applied to the column, were bulked for further purification by ion-exchange chromatography on DEAE-A50 Sephadex, either immediately or after storage in freeze-dried form at -10°.

A 5ml aliquot of the enzyme, fractions 15-18, from Sephadex filtration was applied to the ion-exchange column (10 x 1 cm) which was eluted with further 0.05 M tris pH

Figure 12

Scheme for partial purification of peroxidase from garbanzo seedlings



(continued)

Figure 12 continued

Precipitate

Suspend in 4 ml 0.05 M tris pH 7.5

Spin for 20 min

→ Solid

Supernatant

Filter through Sephadex G-75, 0.05 M tris pH 7.5

Collect fraction each 30 min

Pool 4 most active fractions (see Fig. 13)

→ Other fractions

Pooled fractions

Chromatograph on DEAE A-50 Sephadex

Apply 5ml sample, elute 0.05 M tris pH 7.5

Collect fraction each 15 min

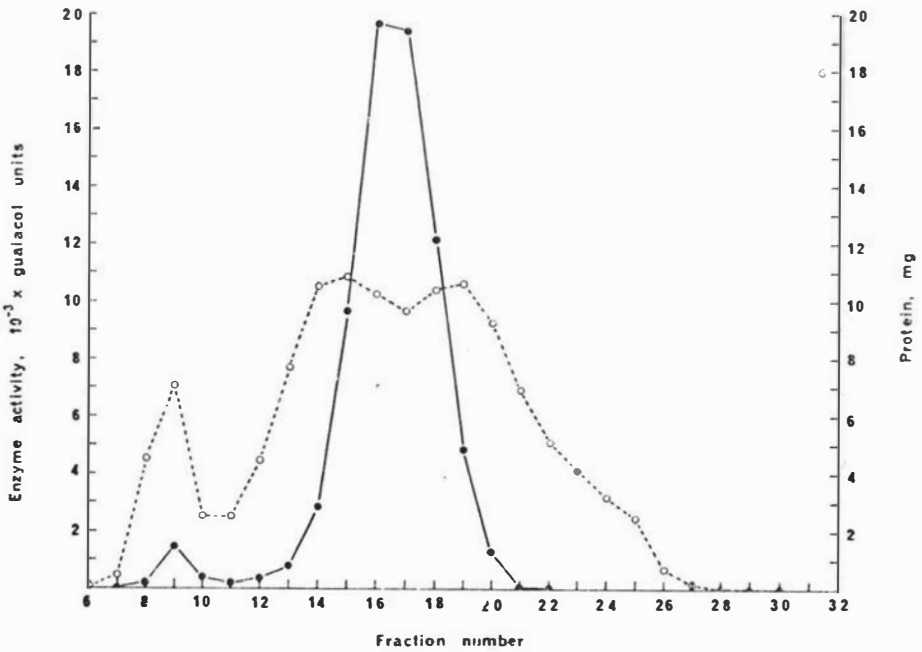
→ Other fractions

Use active fractions (see Fig. 14)

× Centrifuge at 5,000g in all instances

Figure 13

Sephadex G-75 filtration of acetone precipitated peroxidase enzyme preparation from garbanzo seedlings (See Figure 12).



●—● Peroxidase activity per 8.5ml fraction, 10^{-3} x guaiacol units.

o---o Protein per fraction, mg.

7.5 buffer. The enzyme was recovered largely in the early fractions (about 2.5 ml each) slightly ahead of a very small protein peak (Figure 14). Most of the protein in the applied sample remained firmly bound at the top of the column and could be eluted only with the use of a salt gradient. No enzyme peak beyond the first was detected. About 80% of the activity applied to the column was recovered in fractions 3-6 inclusive.

A summary of the outcome of this purification of peroxidase from garbanzo seedlings is recorded in Table 2.

Active fractions from the DEAE-Sephadex column were kept separately at 0 - 4^{o} and the purest used normally within a week as a source of enzyme in detailed studies of the reaction with chalcone. Further samples of the enzyme were purified as required from either a freeze-dried preparation, previously purified through the Sephadex G-75 step, or a fresh batch of seedlings.

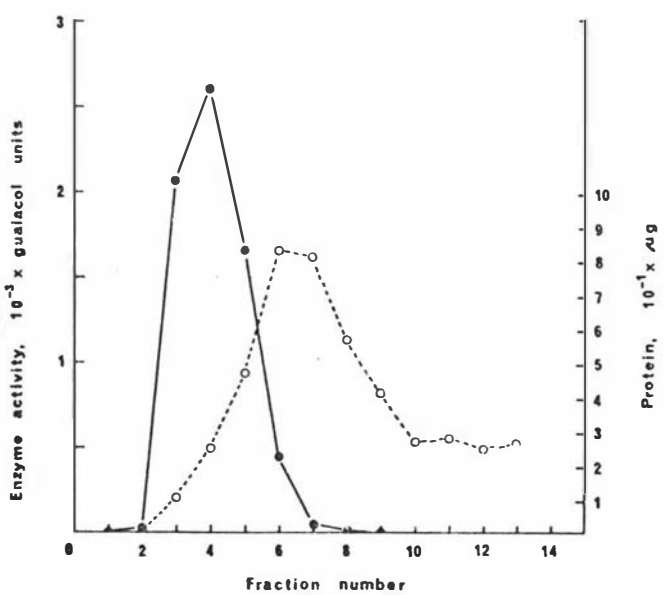
While the final yield of purified enzyme was just 30% of the activity extracted in the cell-free preparation, the amount which could be quickly purified adequately met requirements. Polyacrylamide gel electrophoresis of samples of the enzyme at various stages of purification showed that some differential losses occurred at the isoenzyme level. At the most, 3 acidic and 4 basic isoenzymes were noted.

2A-7 OC as major, unstable product

Preliminary work had established OC as the major chromatographically isolated product of chalcone oxidation catalysed by either garbanzo or horseradish peroxidase. As a prelude to more detailed studies, an investigation of

Figure 14

DEAE A-50 Sephadex chromatography of enzyme from Sephadex G-75 filtration (See Fig. 13).



●—● Peroxidase activity per 2.5ml fraction, 10^{-3} x guaiacol units.

o---o Protein per fraction, 10^{-1} x µg.

Table 2

Purification of peroxidase enzyme from garbanzo seedlings

Preparation	Volume ml	Concn. units/ml	Total units x 10^{-3}	Protein mg/ml	Sp. Act. units/mg	Yield %	Purity
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[x]	34	1720	59	1.19	1445	41	54
DEAE-Sephadex[xx]	49.5	865	43	0.011	77300	30	2880
DEAE-Seph., frn 3	16	880		0.004	204500		7620
" " frn 4	16.5	1060		0.010	105000		3920
" " frn 5	17	660		0.019	35300		1320

[x] Values recorded are for fractions 15-18 pooled (see Figure 13)

[xx] Values are those calculated for fractions 3-5 collectively

methods for the production of maximum yields and preparative amounts of this compound was undertaken.

Small scale reactions with purified garbanzo enzyme, in a total volume of 4 ml of 0.05 M tris buffer incubated at room temperature (20-25^{0}) were employed in the selection of workable conditions and methods. Evaluation of relative yields of OC was done by measurement of the absorbance at 325 nm (λ max.) of 85% ethanol eluates of OC spots taken from 2-D chromatograms of products. Visual inspection of these chromatograms also permitted an assessment of side reactions and residual chalcone to be made.

From the procedures tested, the best yield of OC was obtained on the addition of hydrogen peroxide (4 μmoles) to chalcone (3.6 μmoles, 1 mg) in the presence of enzyme (100-200 units) in 4 ml total volume 0.05 M tris buffer pH 8.5. The reaction went to completion in about 4 minutes. Quantitative extraction of products into 2 x 5 ml volumes of ether was realised after acidification of the buffer to pH 7.5. Strong acidification to at least pH 1 resulted in drastically reduced recovery of OC and the appearance of many background spots on the chromatogram which indicated instability of product under such conditions. The Y' spots (Figure 11) appeared regularly on chromatograms when reactions were carried out and product extracted at pH 6.8, but this phenomenon, much less marked and occasionally absent under pH 7.5 conditions of reaction and extraction, was avoided under the conditions selected of reaction at pH 8.5 and extraction at pH 7.5.

Preparative quantities of OC were successfully obtained when the small-scale reaction mixture given above

was scaled up fifty-fold. For each 50 mg batch of chalcone incubated, yields of OC were determined spectrophotometrically on an aliquot of the total ether-extracted product after removal of the ether solvent and dissolution of the solid in a standard volume of 85% ethanol. In the calculation of yields, the absorbance of crude product at 325 nm (λ max. of OC) was taken as a direct measure of OC content since the u.v. spectrum of this crude product closely matched that for OC purified with minimum delay by 2-D chromatography. In the four separate preparative incubations, the yield of OC ranged from 70 - 76% of the chalcone consumed.

Examination of quantitative 2-D chromatograms of the product from each preparative run confirmed the conclusion from u.v. spectroscopy that OC was the only quantitatively significant component of the crude product. Besides traces of the known additional products, namely, flavonol, aurone and Y' spots, a number of minor background spots were present on the chromatograms. Collectively, these spots, along with some further amounts found to still remain in the aqueous reaction medium after quantitative ether extraction of OC, were thought to account for the difference between chalcone consumed and estimated OC formation in the preparative runs.

Estimation of OC eluted from the quantitative 2-D chromatograms of the preparative runs provided the first direct evidence of suspected instability of OC under the conditions of chromatography. Thus on the basis of OC eluted from these chromatograms, yields ranged from 20 - 24% of chalcone consumed in contrast to values of 70 - 76%

determined directly by u.v. absorption measurements for the same samples. Confirmation of this extent of loss of OC on chromatography and recovery was obtained from the results of chromatography of known amounts of the purified compound. Losses of the compound were apparently caused by chemical change rather than through tailing on chromatograms or inefficient elution. Further tests indicated losses could be reduced very considerably and up to about 80% of the OC sample applied could be recovered if the time for which chromatograms were 'dried' in air at room temperature was minimised both between solvent directions in 2-D chromatography and before elution for quantitative estimation. Purity of the eluted compound was also enhanced by the more rapid handling as judged by changes in the shape of the u.v. spectrum.

Purification of the crude OC from the four preparative runs above was achieved by crystallisation from ethanol/water (1:1), to yield orange-yellow solids (56 mg). The mother liquor contained much impurity and proved unsuitable as a source of more OC solids. Further purification of the solids (56 mg) was achieved upon slow concentration of the solution in acetone under an air jet to yield a light yellow precipitate of OC solid (17 mg) and an orange-brown mother liquor This solid OC was recovered, washed with cold solvent and retained at -10^{o} for characterisation.

Manipulation of OC in solution, associated with the preparation of this purified sample, led to noticeable darkening of the colour of the solution and rather extensive instability of the OC as revealed by 2-D chromatography of samples of mother liquors. A number of competing pathways

of OC loss appeared to operate since many unknown spots were present on chromatograms and none was exceptionally prominent quantitatively.

From the several lines of evidence available on the instability of OC it was therefore also considered probable that yields of crude OC, measured after solvent extraction, would be depressed to an extent. Studies on a spectrophotometric method were consequently undertaken so that the extent of OC formation in the reaction might be determined directly and accurately and the background provided for more detailed biochemical work.

2A-8 Spectrophotometric evidence for the presence of an initial reaction product

Enzyme reactions were run in 0.05 M tris buffer pH 8.0 in 2 ml total volume at 25°, using cuvettes of 1 cm light path. Under the reaction conditions selected, all chalcone was consumed in the reaction. Reference spectra (Figure 15) were determined for chalcone and OC in pH 8.0 tris buffer and for OC in this medium acidified to pH 2-3.

The absorption spectrum of the product of a rapid reaction catalysed by either garbanzo or HRP enzyme is presented in Figure 16, together with details of the reaction mixtures employed. In the next 2 hours this product spectrum was observed to undergo a complex pattern of change. Appreciable alteration occurred in even the first five minutes. Included in Figure 16 are some examples of the spectrum at the post-reaction times noted. At no time did the changing spectrum match or closely approach that for OC

Figure 15

Reference spectra of chalcone and OC in tris buffer.

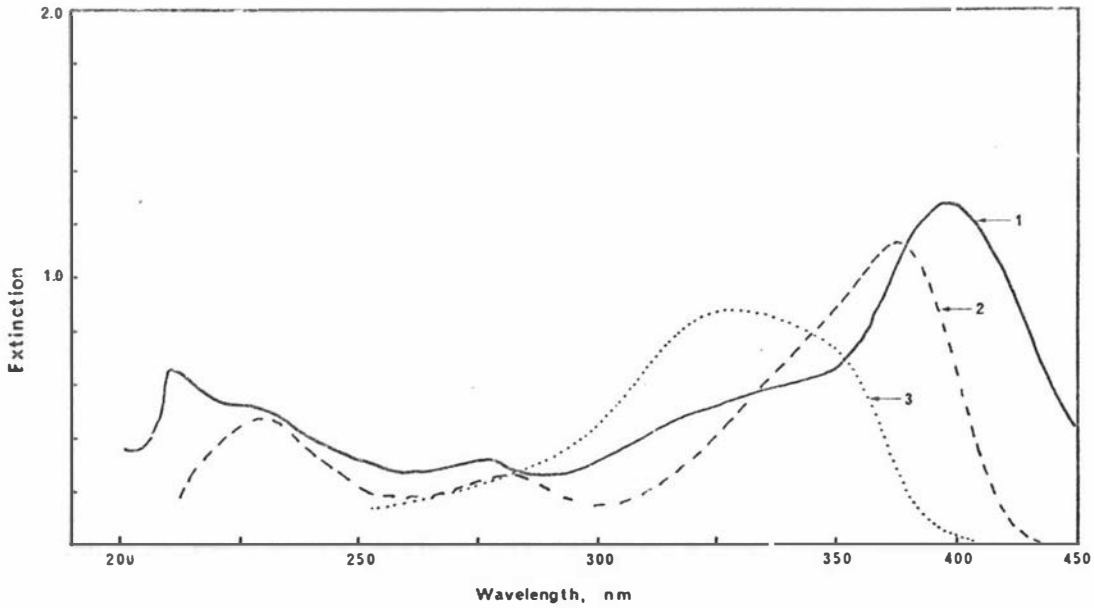
1 = Chalcone in 0.05 M tris buffer pH 8.0
2 = OC in 0.05 M tris buffer pH 8.0
3 = Sample as in spectrum 2, buffer acidified to pH 2-3

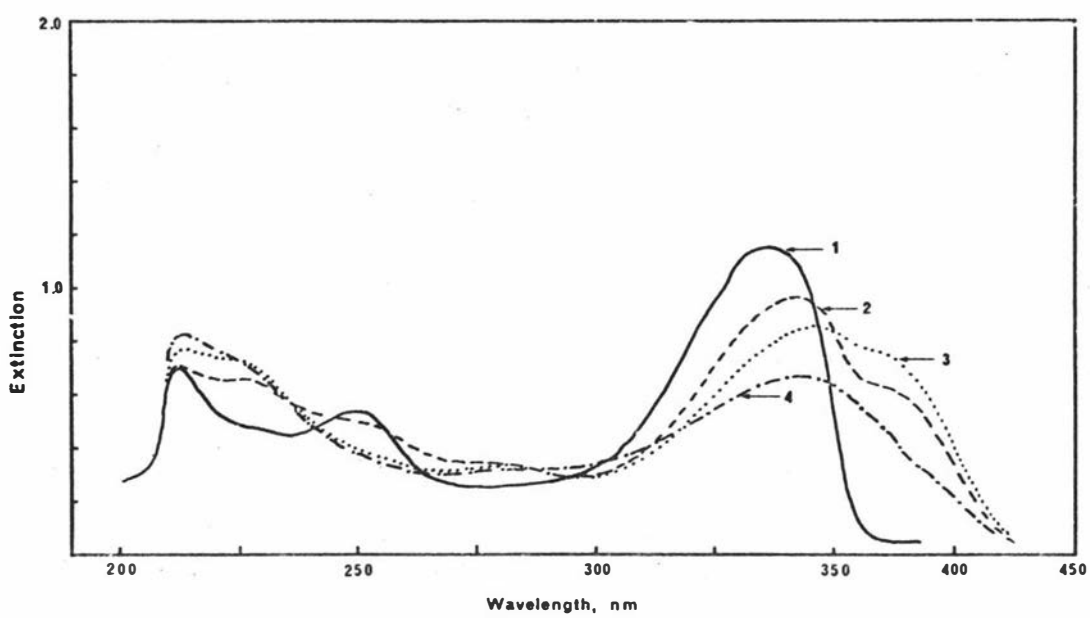
Figure 16

Spectrum of initial product of peroxidase-catalysed reaction of chalcone and subsequent changes therein with time in pH 8.0 tris buffer.

1 = Spectrum of initial product recorded immediately after complete chalcone consumption in reaction mixtures which contained initially chalcone (0.09 μmole), enzyme (either 1 unit HRP or 3 units garbanzo) and H_2O_2 (0.11 μmole) in 0.05 M tris pH 8.0, total volume 2ml, at 25^{o}.

2, 3 and 4 = Spectra observed after initial product held in buffer for 12, 24 and 120 minutes respectively.


2.0
1.0
Extinction
200
250
300
350
400
450
Wavelength, nm
1
2
3


2.0
1.0
Extinction
200
250
300
350
400
450
Wavelength, nm
1
2
3
4

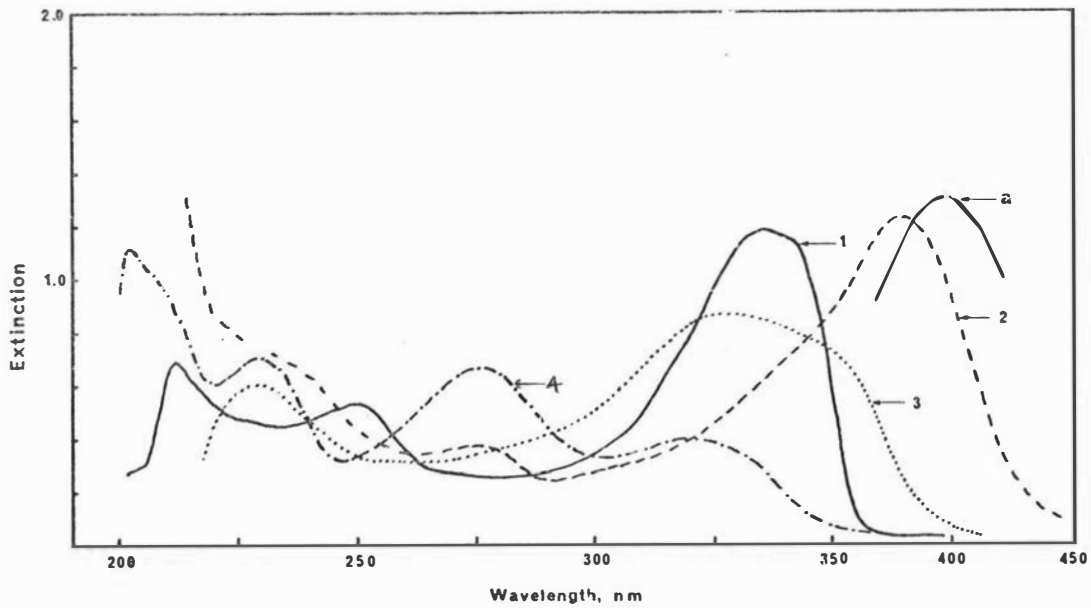
in pH 8.0 buffer. However, some OC formation could have been indicated by the observed increase in absorption at 380 nm (Figure 16). From this behaviour it was clear that the initial reaction product was not OC, a fact which gave potential support to earlier chromatographic indications that precursors of OC may exist in the Y' spots.

The search for conditions under which a high amount of OC could be derived from the initial product revealed that controlled addition of alkali (25 µl of 2.5 N NaOH/2 ml reaction volume) rapidly transformed the initial product to OC. This quantity of alkali was critical for good results in terms of OC formation. In Figure 17, the spectra associated with this transformation are presented; in the alkaline medium, OC appeared in the ionised form and the characteristic OC spectrum (Figure 15) was observed on acidification. The effect of acidification on the spectrum of the initial product is also shown in Figure 17; back titration to pH 8 restored the initial spectrum while additional alkali, as above, resulted in the anticipated OC formation. Thus the spectrum of the initial product at pH 8 was held to represent the ionised form and that at acid pH the unionised form.

Delay beyond a few minutes between the rapid production of the initial product and addition of alkali to the cuvette resulted in the appearance of a shoulder or even a second peak around 340 nm on the alkali spectrum of OC (compare Figure 18). This phenomenon was attributed to impurities formed spontaneously from the unstable initial product on standing in pH 8 buffer. These impurities caused much more significant distortion in the ionised than in the

Figure 17

Spectral evidence of the transformation of initial product of reaction to largely OC upon controlled addition of alkali.



1 = Initial product in pH 8.0 tris buffer, from chalcone (spectrum a, 0.09 μmole) using reaction mixture and conditions given in Figure 16.

2 = Ionised OC formed on immediate addition of 25 μl 2.5 N NaOH to initial product.

3 = OC converted to unionised form on addition of 75 μl 1.44 N HCl to the product observed in 2.

4 = Unionised form of initial product observed on addition of 30 μl 1.44 N HCl to initial product in pH 8.0 tris buffer (spectrum 1).

unionised spectra of both the initial product and the derived OC. In subsequent work on the isolation of the initial product, spectral assessment of purity was therefore based on the degree of distortion of the ionised OC spectrum which formed on addition of alkali.

2A-9 Isolation of the initial product

Spectrophotometric observation showed the initial product was most stable in acidified buffer, pH 2 - 3, and quite unstable at pH 7.5 as previously used for solvent extraction in the isolation of OC.

Successful recovery of freshly-formed initial product was achieved by ether extraction from buffer first acidified to pH 2 - 3. The reaction mixture used to prepare the initial product contained chalcone (0.75 µmole) garbanzo enzyme (47 units) hydrogen peroxide (0.22 µmole) and 0.05 M tris buffer pH 8.0 to make 4 ml. Immediately after rapid decolorisation (less than 1 minute), the mixture was acidified with 75 µl 1.44 N HCl and the initial product quantitatively extracted into 2 x 5 ml volumes of ether which were combined. The quantity of aqueous phase present in this ether extract was not removed by any drying procedure since it served a vital role of providing a lasting, slightly acidic, environment which stabilised the initial product in the course of subsequent manipulations. Following removal of the ether close to 0^{o}, the extracted product was taken up in a small volume of ethanol and aliquots used to check spectral properties against those known for the initial product as directly formed in aqueous media. In 85% ethanol, the spectrum was approximately that of

initial product in acidified buffer (Figure 17, spectrum 4). Addition of alkali, (5 µl 1.25 N NaOH/2 ml cuvette) resulted in the rapid formation of ionised OC and on acidification (10 µl of 1.44 N HCl), typical OC absorption was recorded. Further, an initial product like spectrum was noted when an aliquot of the extracted product was added to tris buffer pH 8.0. Formation of OC on appropriate addition of alkali to this buffer solution also occurred. Based on the correspondence in spectral properties and behaviour in alkaline media, the extracted product was deemed identified with the initial product observed directly in solution. Purity of the extracted product was diminished considerably by delays in the extraction process or by exposure to temperatures above 0^{o} for significant periods of time thereafter. Thus, rapid extraction after formation of the initial product was demanded and limited warming during ether removal by rotary evaporator was practised to good effect. The recovered product was held successfully on ice for a number of hours.

Ether extraction of initial product from buffer acidified to pH 7 rather than to pH 2 - 3, provided evidence of spontaneous generation of OC, following the extraction of initial product, when acidic conditions were not maintained. While the spectrum taken of the wet ether extract itself closely matched that of the unionised initial product in ethanol, the spectrum in ethanol of the product obtained immediately after evaporation of the ether showed that already a partial conversion of initial product to OC had occurred. With extraction against a more alkaline pH 7.5 buffer medium, the carry-over of the aqueous phase in the

wet ether extract would provide a slightly basic environment which would be expected to promote even greater instability of the initial product. From earlier experience of OC recovery under such conditions of extraction, the conversion of initial product to OC apparently occurred rapidly and almost completely in the environment provided during the isolation of the crude solid product.

2A-10 Chromatographic identification of the initial product as largely OC precursors

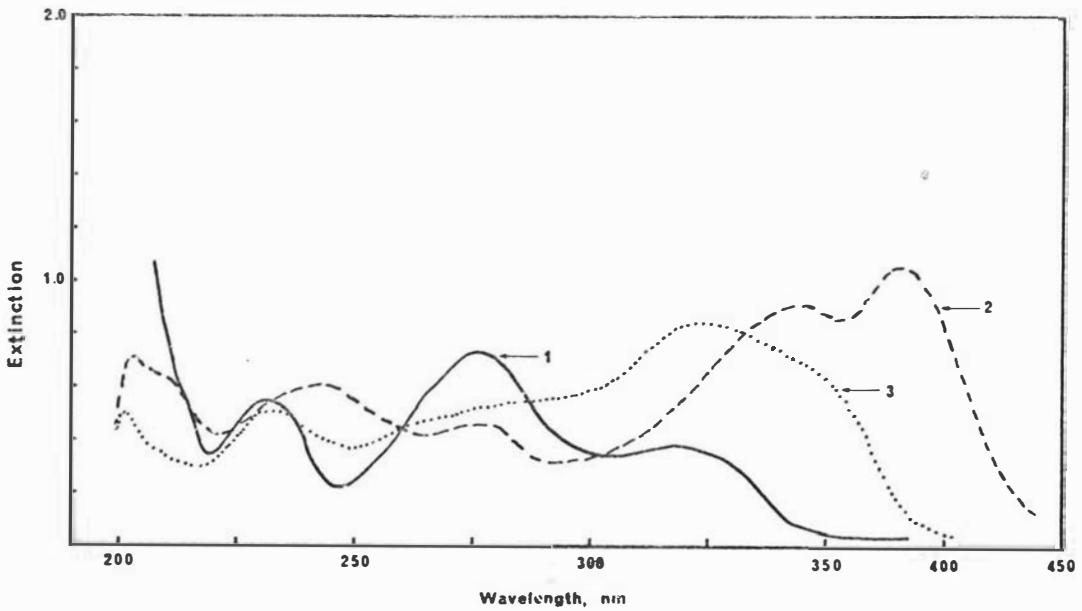
Two-dimensional chromatography of the initial product isolated by ether extraction, gave rise to two major spots located in the positions of the previously noted Y' spots (Figure 11). The eluate of each of these spots was found (see below) to yield mainly OC, on controlled alkali treatment. The compounds present in the Y'_1 and Y'_2 spots which gave rise to OC are hereafter referred to as $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ respectively. From this work it was concluded that the initial product consisted largely of a mixture of the two compounds, $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$.

On account of the known instability of the initial product, rapid one-dimensional chromatography was used to define its nature. Thus, samples of initial product which were prepared and isolated as described in the previous section, were first chromatographed for 1 hour in BeAW (benzene:acetic acid:water), without prior equilibration of the chromatogram. After drying for 1 hour, the chromatogram was inspected under u.v. light and a light blue spot eluted from low R_f, equivalent to that of spots Y'_1 and Y'_2 (coincident in BeAW). The spectrum determined after 10 minutes

elution in 85% ethanol, was similar to that of the unionised initial product; addition of minimum alkali (determined empirically on account of the acetic acid present from chromatography) indicated the formation of OC contaminated with much impurity (Figure 18). Similar chromatography in 10% HA (acetic acid in water) revealed two major spots, both light blue-grey in u.v.light, at R_fs equivalent to spots Y'_1 and Y'_2. Each spot was eluted and found to have an absorption spectrum similar to the other and to the eluate from the BeAW chromatogram; alkali treatment provided spectral evidence of similar OC formation, again in the presence of much impurity. Rapid 2-D chromatography, with 15 minutes drying between solvents, then directly demonstrated that the initial product chromatographed largely as the two Y' spots and that these had been eluted separately and in combination from the 10% HA and BeAW chromatograms respectively. The initial product sample, as applied to these chromatograms, was observed spectrophotometrically to give rise to clean OC on controlled alkali treatment. Therefore, the recovery in eluates of the Y' spots of only impure samples of precursors of OC was interpreted as evidence of partial breakdown of these precursors, $\not Y_1$ and $\not Y_2$, following chromatography and prior to spectrophotometric examination. The extent of this breakdown, best observed spectrophotometrically as distortion of the derived OC anion spectrum, indicated these precursors were very labile compounds even under the relatively mild, necessarily acidic, conditions employed in chromatography. In addition to the Y'_1 and Y'_2 spots on the 2-D chromatograms of initial product, a number of other minor spots were noted, including

Figure 18

Spectra for Y' spot eluted from 1-D chromatogram of initial product run in BeAW.



1 = Eluate in 85% EtOH of Y' spot

2 = Impure OC anion formed upon addition of 5/ul 1.25 N NaOH to Y' spot eluate; impurities are represented by 345 nm peak.

3 = Unionised form of impure OC; sample for spectrum 2 plus 10/ul 1.33 N HC1.

traces of OC and flavonol but not of aurone. Unidentified minor spots were considered to arise mainly from breakdown of $\emptyset Y_1$ and $\emptyset Y_2$ prior to chromatography.

From these chromatographic studies the most direct conclusion was drawn, namely, that the $\emptyset Y_1$ and $\emptyset Y_2$ precursors of OC present in the respective Y' spots actually constituted the spectrophotometrically observed initial product in the main. The basis for this conclusion was the lack of spectrophotometric evidence for any change in the precursors of OC between the spectrophotometrically detectable initial product and chromatographic phases of the observation, with due allowance being made for the presence of impurities (expected) in the chromatographic eluates. All subsequent results were found to support this assessment.

2A-11 Chromatographic studies of other precursor compounds in initial product

Earlier, it was recorded that rechromatography of the Y' spots provided evidence for the existence therein of precursors, not only of OC but also of flavonol and aurone (see section 2A-5). To further investigate this finding, samples equivalent to each Y' spot were prepared by 1-D chromatography of initial product in 5% HA. The required initial product was prepared from 4 mg of chalcone with the reaction mixture as detailed in section 2A-9 above scaled up 20-fold.

This product was resolved into Y_1' and Y_2' bands (equivalent to the respective Y' spots) on a full size* chromatogram run for 3 hours and dried for 1.5 hours. Each Y' band detected on the chromatogram under u.v. light was excised and eluted rapidly by immersion in ethanol. On removal of the solvent at low temperature, the product obtained from each band was taken up in a small volume of ethanol and a sample rechromatographed in 2-D not only in the recovered state but also after treatment with alkali as required for OC formation. The amount of alkali added was determined empirically on account of the acetic acid present in the samples but was restricted to the minimum required to ensure rapid, complete formation of OC.

The resulting 2-D chromatograms showed that direct rechromatography of each Y' preparation resulted in retention of that Y' preparation as the main spot. None of the other Y' preparation was detected. Many background spots were also present together with traces of OC but not of aurone or flavonol. Where alkali treatment had been given prior to rechromatography, OC was the main product, along with some aurone and flavonol; the result was the same for both Y' preparations. Again, many background spots were present. From these results a number of significant points were confirmed. Firstly, alkali-labile precursors of OC ($\emptyset Y_1$ and $\emptyset Y_2$), aurone (Y_1 and Y_2) and flavonol existed in two forms in each case. Secondly, the flavonol

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* This size chromatogram permitted a distance of up to 40 cm from origin to solvent front whereas the commonly employed chromatogram provided half of this distance (see Experimental).

precursors, like Y_1 and Y_2, appeared quantitatively minor. Thirdly, interconversion of the quantitatively important OC precursors, $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$, had apparently not occurred in the processes associated with recovery and direct rechromatography.

2A-12 Effect of pre-chromatographic treatment on product pattern

From the preceding spectrophotometric, product isolation and limited chromatographic studies, it was clear that the chromatographic pattern of reaction products observed would be affected dramatically by the treatment the preparation received prior to chromatography. It was therefore deemed essential to establish the effects of certain pre-chromatographic treatments on a standard initial product preparation so that such secondary effects could be readily distinguished from any primary changes in product composition during chromatographic assessment of products from reactions carried out under a variety of conditions. Thus, for both garbanzo and HRP enzyme-catalysed reactions, 2-D chromatograms were prepared using an originally standard preparation of initial product which had then been exposed to various different treatments prior to uniform chromatography. The standard reaction mixture used to provide the preparation for each chromatogram contained chalcone (0.75 μmole), enzyme - either garbanzo (19 units) or HRP (12 units) and hydrogen peroxide (0.22 μmole) in 0.05 M tris pH 8.0, 4 ml total volume. After the rapid, complete consumption of chalcone, three treatments were applied prior to ether

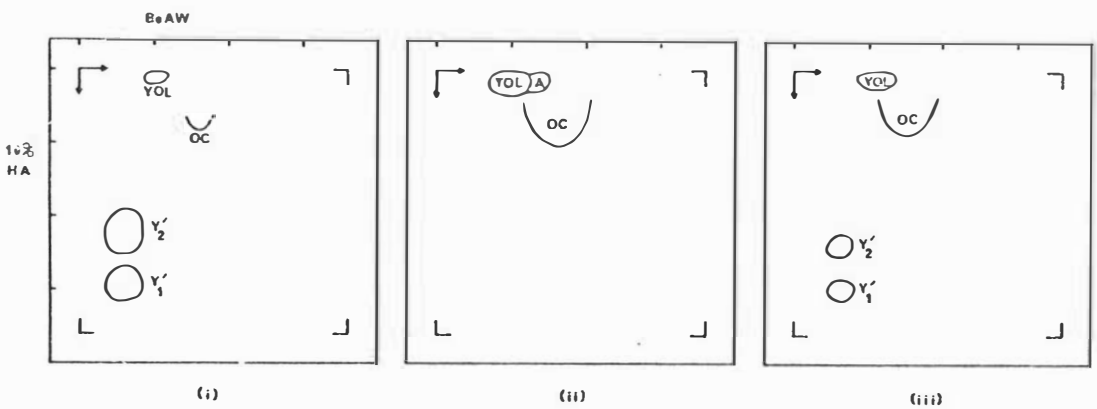
extraction of products; (i) acidification to pH 2-3, (ii) addition of alkali required to transform $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to OC, then acidification to pH 2-3, (iii) acidification to pH 7.

Results were found to be independent of the enzyme used in the reaction. Direct extraction at pH 2-3 (treatment (i)) gave the expected pattern of Y' spots and traces only of OC, very faint flavonol and no aurone (see Figure 19). With alkali treatment, however, the major product was OC (Figure 19) as expected on previous spectrophotometric evidence. No Y' spots remained. Both aurone and flavonol spots were present, though quantitatively minor, with the former largely concealed under the latter. An intermediate product pattern was noted (Figure 19) for direct extraction at pH 7. With this treatment, the Y' spots were always present together with a strong OC spot. Considerable variation in Y' spot intensity was noted between replicate chromatograms so that the visual pattern oscillated somewhat in the range intermediate between treatment (i) and (ii) patterns. A flavonol spot was invariably prominent, however, but an aurone spot was not normally clearly detectable since the Y_1 and Y_2 precursors remained largely intact. This indicated that the flavonol precursors were, akin to $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$, more labile than Y_1 and Y_2. On chromatograms where the Y' spots were weak, direct evidence of the presence of Y_1 or Y_2 in the corresponding Y' spot could be seen once the aurone artifact had formed.

In an extension of the above work, an additional chromatographic product pattern was detected and the cause

Figure 19

Product patterns associated with various treatments of initial product, formed in pH8 tris buffer, prior to extraction and 2-D chromatography.



(i) = Acidification to pH 2-3 and direct extraction.

(ii) = First basidification, then acidification to pH 2-3 and extraction.

(iii) = Neutralization to pH 7 and direct extraction.

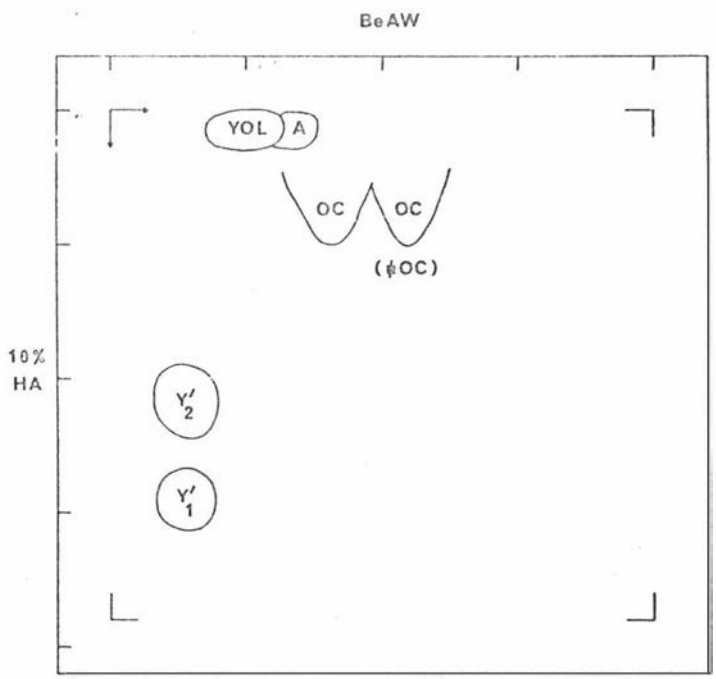
See Figure 10 and Table 1 for identification of symbols.

attributed to the presence of an additional OC precursor probably derived from $\emptyset Y_1$ and $\emptyset Y_2$. The pattern in question (Figure 20) was typified by the second OC spot which displayed an R_f higher than true OC in the first direction solvent (BeAW). The intensity of the additional OC spot was variable but could exceed that of the true OC and Y' spots. Experiment showed that this new pattern was best generated when samples of initial product which had been ether extracted from pH 2-3 solution were held as moist solid, after removal of the ether, for an hour or two at room temperature prior to chromatography. With suitably prepared sample material, 1-D chromatography in BeAW revealed a faster-running dull blue spot just clear of the OC spot. This dull blue spot was eluted after minimal drying of the chromatogram and found to have a u.v. spectrum different to OC and $\emptyset Y$ compounds. However, addition of alkali (5 μl 1.25 N per 2 ml) rapidly transformed the compound irreversibly into clean OC, observed as the anion. On 2-D chromatograms, this conversion to OC probably occurred between the first and second direction chromatography and therefore, in the second direction, only an additional OC spot was seen. This new compound ($\emptyset OC$) was thought to arise from one or both of the $\emptyset Y$ compounds in the interval the sample of product, initially a mixture of $\emptyset Y_1$ and $\emptyset Y_2$, was held prior to chromatography.

On similar handling of product directly extracted at pH 7, only faint $\emptyset OC$-derived spots of OC could be detected, which suggested that under less acidic holding conditions prior to chromatography, either the $\emptyset OC$ compound was formed to only a very minor degree or that it converted to OC as

Figure 20

Chromatographic pattern associated with the presence of ϕOC, the source of the second OC spot



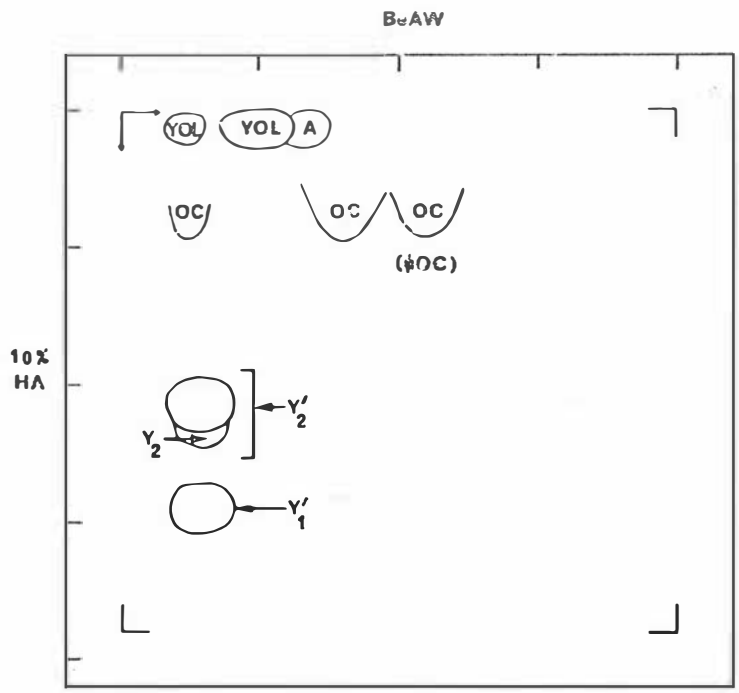
See Figure 10 and Table 1 for identification of symbols

fast as it was formed.

The most complex chromatographic pattern derived from a sample extracted in the initial product form may now be appreciated (Figure 21). This was best observed when a sample equivalent to 1 mg chalcone and treated as described above to promote ØOC formation, was chromatographed on a full-size 2-D chromatogram which was dried between solvent directions overnight, rather than a few hours at most as previously. Evidence was present for the occurrence of both OC and ØOC, the latter as the derived second OC spot. Small amounts of aurone and flavonol, present at the outset of chromatography were also noted in the expected positions. The additional flavonol and OC spots in line with the Y' spots, represented compounds produced from the combined Y' spot in the interval between first and second direction chromatography. On keeping the chromatogram, partial separation of the Y_2 compound from the Y_2' spot was recorded. Such separation was enhanced by the low amount of $ØY_2$ which remained during second direction chromatography and the full-scale dimensions to which the chromatogram was run. Uniform development of aurone colour in the Y_1' spot indicated co-chromotography of Y_1 in the Y_1' mixture. No evidence for even a partial separation of flavonol and OC precursors on this chromatogram could be detected. The presence of flavonol precursors in the Y' spots could be directly observed on the chromatograms, after keeping for a day or two, by the appearance of characteristic flavonol fluorescence in the spots.

Figure 21

Complex chromatographic pattern associated both with the occurrence of ϕOC and of extended drying time between solvent directions.



See Figure 10 and Table 1 for identification of symbols.

2A-13 Summary of products and their interrelationships

From a combination of the foregoing spectrophotometric and chromatographic studies, the immediately detectable products of chalcone utilisation in the reaction catalysed by peroxidase, either from garbanzo or horseradish, were found to include precursors of no less than three distinct compounds, OC, flavonol and aurone (Figure 22). For each of these compounds, two alkali-labile precursors were detected, with chromatographic properties akin to the previously known aurone precursors, Y_1 and Y_2.

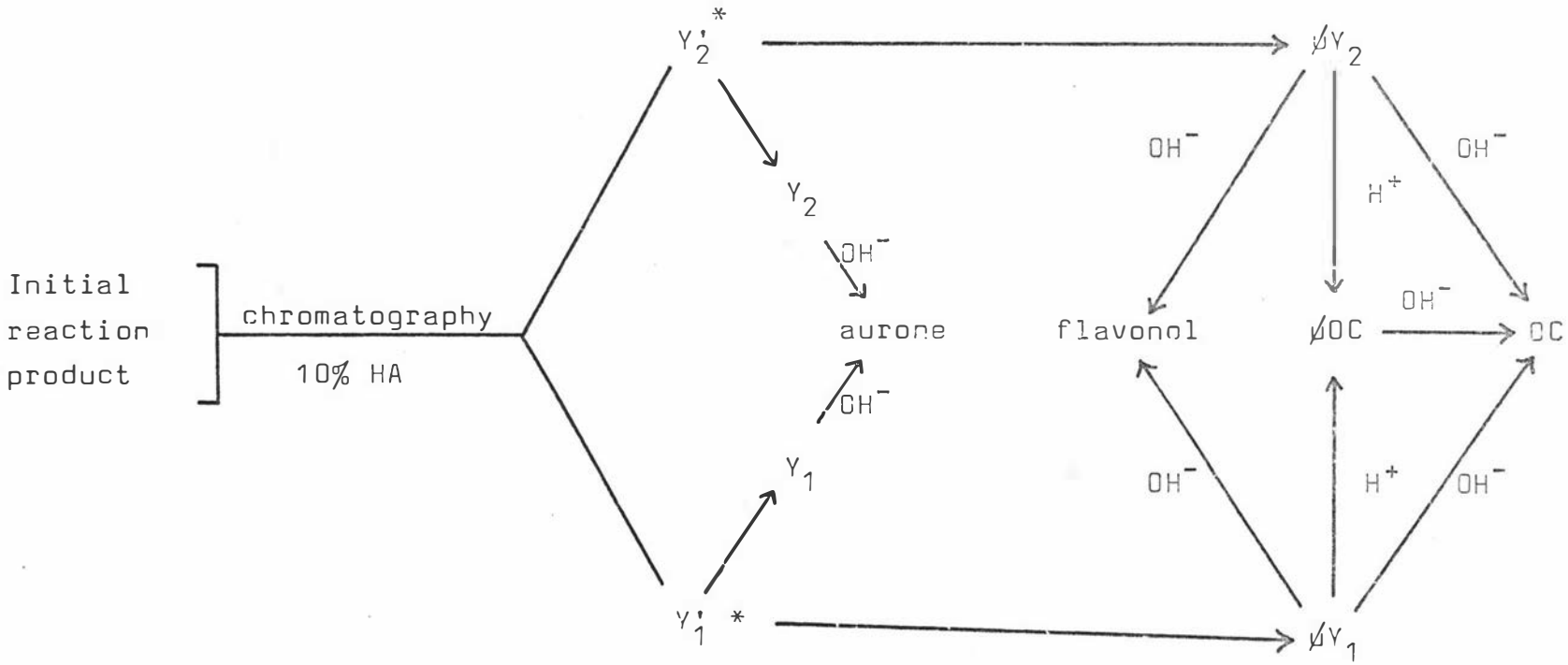
The precursors of OC were never resolved chromatographically from the flavonol precursors. However, both flavonol precursors were distinct in chromatographic properties from the dihydroflavonol, garbanzol, a previously expected biosynthetic precursor of flavonol (see Introduction, section 1A-3 and Figure 3). While the relationship of OC and flavonol precursors was not clear at this stage, it was later discovered (see section 2C-3) that $\acute{\emptyset}Y_1$ and $\acute{\emptyset}Y_2$ could give rise, under alkaline conditions, to either OC or flavonol. Thus, as summarised in Figure 22, $\acute{\emptyset}Y_1$ and $\acute{\emptyset}Y_2$ were both common precursors of OC and flavonol and no evidence was obtained that each Y' spot represented other than the appropriate $\acute{\emptyset}Y$ and true Y compounds. The additional chromatographically isolated product, $\acute{\emptyset}OC$, was apparently formed from $\acute{\emptyset}Y$ compounds under slightly acidic conditions and was considered to represent an intermediate compound, not necessarily obligatory, in the formation of OC from $\acute{\emptyset}Y$ compounds (Figure 22).

Quantitatively, the initial product was indicated to

Figure 22

Products of peroxidase-catalysed reaction of chalcone, their transformations and interrelationships.

* Chromatographically inseparable mixture of (Y_1 + $\emptyset Y_1$) or (Y_2 + $\emptyset Y_2$). Refer to text for identification of the symbols.



be essentially a mixture of $\emptyset Y_1$ and $\emptyset Y_2$. Only small amounts of Y_1 and Y_2 were formed. Controlled alkali transformation, or spontaneous transformation of $\emptyset Y_1$ and $\emptyset Y_2$ after ether extraction from pH 7.5 buffer, resulted chiefly in OC formation and little flavonol production from these common precursors.

From a biochemical standpoint, therefore, the reaction could be simplified for study to one of $\emptyset Y_1$ and $\emptyset Y_2$ formation from chalcone. However, from the chemical aspect, a number of new compounds, $\emptyset Y_1$, $\emptyset Y_2$, OC and $\emptyset$OC, were encountered and consequently study was required to elucidate their interrelationships and structures.

2B BIOCHEMISTRY OF THE MAIN REACTION

In this part, the results are recorded of studies of the biochemistry of $\emptyset Y_1$ and $\emptyset Y_2$ formation from chalcone, catalysed by peroxidase either from garbanzo or horseradish. Aspects of the reaction which were investigated include the effect of reaction pH and chalcone and hydrogen peroxide concentration on reaction rate. Further, the requirement for molecular oxygen is shown and, in addition, the consumption of catalytic rather than stoicheiometric amounts of hydrogen peroxide. The first sections which follow detail the outcome of preliminary studies which were required in order to develop an accurate spectrophotometric method for monitoring the reaction.

2B-1 Spectrophotometric method for monitoring chalcone disappearance

A spectrophotometric method was developed to

continuously monitor the progress of the enzymic reaction of chalcone over time in order to facilitate biochemical studies. In the method adopted, the time course of chalcone disappearance was recorded at the wavelength of the absorption maximum of this compound, which varied with the pH of the reaction buffer from 371 nm at pH 6.2 to 418 nm at pH 8.9. At the selected wavelength, the initial product showed negligible absorption, irrespective of the pH of the reaction buffer in the range pH 5.8 - 8.9 (compare absorption spectra for chalcone and initial product in tris buffer pH 8.0, Figure 17). Reaction rate, taken as the slope of the progress curve of chalcone disappearance at the steepest part, was the initial rate except in a minority of reaction conditions where progress curves were sigmoidal, e.g. suboptimal pH. Sections of progress curves used for the measurement of reaction rates were always recorded within short times (10 - 60 seconds) of the start of the reaction and were consequently not distorted by instability of the initial product contributing absorption at the monitored wavelength (compare Figure 16).

2B-2 Effect of preincubation of chalcone on reaction rate

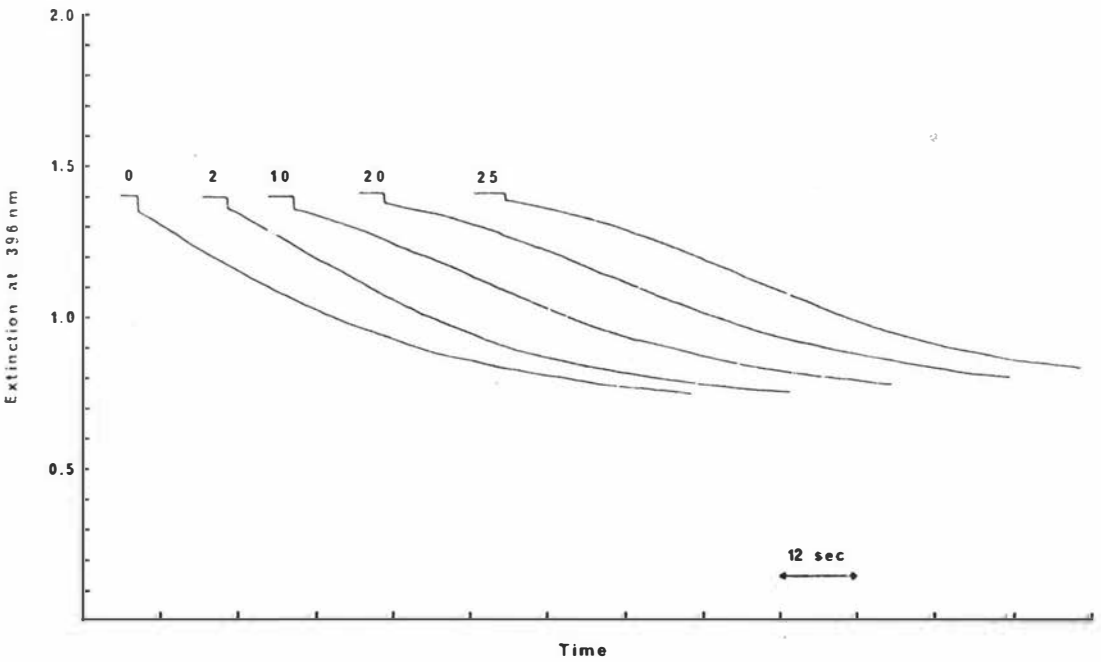
For the HRP-catalysed reaction in 0.05 M tris buffer pH 8.0, preincubation of chalcone in this buffer, prior to the addition of enzyme and hydrogen peroxide to start the reaction, caused changes to occur in the progress curve of chalcone disappearance. The two changes noted in response to this pre-incubation were development of a sigmoidal progress curve and an associated decrease in

maximum reaction rate (Figure 23). Both the degree of sigmoidal character and the decrease in reaction rate intensified gradually as the duration of chalcone preincubation increased. With the reaction mixture given in Figure 23, the observed maximum rate decreased steadily with increase in preincubation time up to 10 minutes. At this point the observed rate had fallen to 75% of the rate obtained for zero preincubation time. Extended preincubations of up to 30 minutes had little further effect on reaction rate. Additional studies showed that the sigmoidal character and decreased reaction rate occurred only when chalcone was preincubated with buffer; preincubation of enzyme and hydrogen peroxide with buffer followed by addition of chalcone, for example, gave a progress curve typical of rapid sequential addition of chalcone then enzyme and hydrogen peroxide. When the reaction mixture used above was altered by increasing the HRP enzyme to 0.2 and 0.4 unit, coupled with hydrogen peroxide additions of 0.4 and 0.04 µmole at the lower enzyme activity and 0.02 µmole only at the higher activity, the previously noted effects of chalcone preincubation on the progress curves were always evident qualitatively. These results indicated the general validity of the phenomena associated with preincubation of chalcone in pH 8.0 tris buffer using HRP enzyme. Hence in this HRP system, delay between addition of chalcone and the start of the reaction would have a significant effect on observed reaction rate.

With phosphate buffer pH 8.0 in place of tris, progress curves which were always slightly sigmoidal were

Figure 23

Effect of preincubation of chalcone in tris buffer pH8 on progress curve of HRP-catalysed reaction



The reaction mixture contained chalcone (0.09 μmole), HRP enzyme (0.05 unit) and H_2O_2 (0.4 μmole) in 0.05 M tris buffer pH 8.0, total volume 2ml, temperature 25°. Curves record the time course of chalcone disappearance at fixed wavelength, 396 nm. Numbers refer to time (minutes) chalcone was preincubated in buffer before the reaction was started by addition of enzyme and H_2O_2.

made more so upon preincubation of chalcone. Reaction rate, however, was not affected. In contrast, at pH 7 in both tris and phosphate buffers and at pH 8.9 in tris only, preincubation of chalcone was without effect, when the reaction components given in Figure 23 were again used. With both buffers at pH 7, progress curves were always sigmoidal. At pH 8.9 in tris, however, the curves were never sigmoidal.

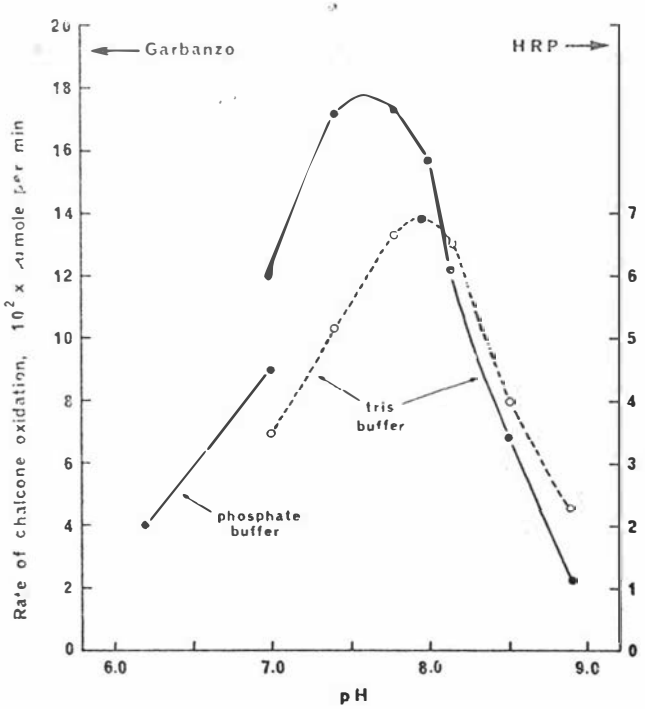
Thus the progress curve of the reaction was specifically affected by chalcone preincubation when the reaction was run in 0.05 M tris pH 8.0. Since this buffer was used in most biochemical investigations (see section 2B-3), the above studies showed that it was necessary to adopt a procedure to avoid the effect of chalcone pre-incubation when rate data were required. The adopted procedure was to add chalcone to buffer immediately before the addition of enzyme, followed by hydrogen peroxide to start the reaction. This same procedure was used with garbanzo enzyme, although with this enzyme progress curves became slightly sigmoidal only for reaction at pH 7 and below.

2B-3 pH - activity profiles

Activity as a function of pH with chalcone substrate was found to be as shown in Figure 24 for garbanzo and HRP enzymes. Both enzymes displayed definite optima at slightly alkaline pH values. Appreciable reaction rates were observed even at the extremes of pH tested. In further studies with either enzyme, reactions were generally run in pH 8.0 tris buffer.

Figure 24

Rate of peroxidase-catalysed chalcone consumption as a function of pH



o--o HRP enzyme ●—● Garbanzo enzyme

For garbanzo enzyme, the reaction mixture contained chalcone (0.09µmole), enzyme (1 unit) and H_2O_2 (0.6 µmole) in 0.05 M buffer as indicated, total volume 2ml. temperature 25°.

For HRP enzyme the reaction mixture consisted of chalcone (0.09µmole), enzyme (0.1 unit) and H_2O_2 (0.5µmole) in 0.05 M buffer pH indicated, 2 ml total volume, temperature 25°.

A check was made to ensure that the reaction product was unchanged in the pH range 6 - 9 and that the rates measured on the basis of chalcone disappearance therefore reflected changes in the rate of the same reaction. Consequently, a reaction was run in duplicate in a series of 0.05 M buffers, which ranged from pH 5.8 - 9.0 at 0.4 unit interval. Phosphate buffers were used in pH 5.8 - 7.0 range and tris buffers in pH 7.0 - 9.0 range. The reaction mixture at each pH contained chalcone (0.75 μmole), hydrogen peroxide (1.5 μmole), HRP enzyme (2.5 units) and buffer to make 4 ml total volume. Reactions at pH 7 and above were completed in about 2 minutes and products were extracted without delay after the buffer had been acidified to pH 7. Reactions at acid pH were left longer until the chalcone which remained in solution was consumed and the products were then extracted at the reaction pH. Products viewed after 2-D chromatographic separation were readily identified as having originated from the ØY compounds, mainly, at each pH when the patterns shown in Figures 19 and 20 were used as guides. Thus pattern (iii) of Figure 19 was typical of the products extracted at pH 7 while some movement toward pattern (ii) was evident where the reaction conditions had approached pH 9. However, for products extracted below pH 7, pattern (i) of Figure 19 was approached more strongly as the reaction pH fell, while at the two lowest pH values, a small second OC spot, indicative of ØOC formation (Figure 20), was also present.

This conclusion from chromatographic examination that the ØY compounds were consistently the main product

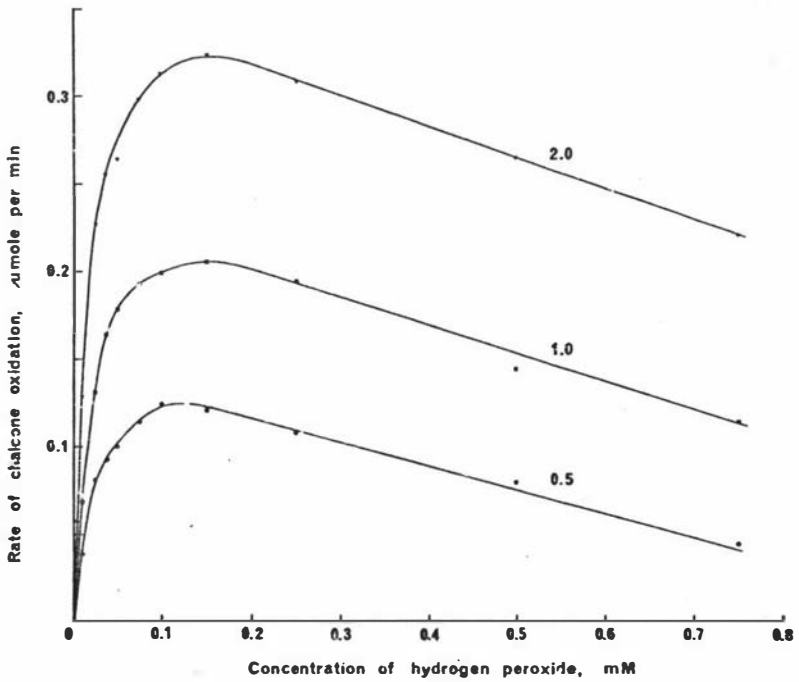
at each reaction pH tested was also supported by direct spectrophotometric observation of the initial product in the same pH range. Thus, the spectrum observed at each reaction pH was the same as that given at that pH by a standard sample of ØY compounds recovered by solvent extraction as previously described (section 2A-9). The spectrum observed at pH 7 and above closely resembled spectrum 1 Figure 16, while between pH 7 and 5.8, the spectrum gradually changed to finally resemble spectrum 4 Figure 17. Rapid, complete reactions of chalcone (0.09 µmole) were obtained for these spectrophotometric observations with high enzyme activity (HRP, 10 units) in the presence of hydrogen peroxide (0.5 µmole) in buffer, 2 ml total volume.

2B-4 Effect of hydrogen peroxide concentration on reaction rate

As the concentration of hydrogen peroxide was increased in the reaction mixture, the rate of chalcone disappearance also increased to a maximum and then gradually decreased. This effect was observed with both garbanzo enzyme (Figure 25) and HRP enzyme (Figure 26). The curves resembled substrate concentration curves with inhibition by higher substrate concentrations. Differences were apparent between the two enzymes, firstly in relation to the optimal concentration of hydrogen peroxide (about 0.15 mM and 0.30 mM for garbanzo and HRP enzymes respectively) and secondly, in their activity towards chalcone per unit of peroxidatic activity assayed with guaiacol. The garbanzo enzyme was also inhibited somewhat more strongly than the

Figure 25

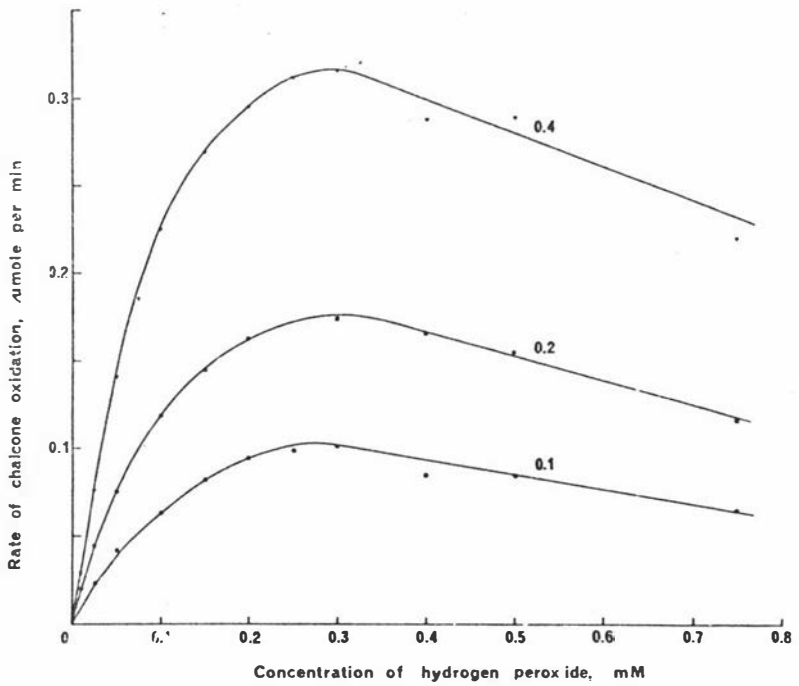
Effect of concentration of H_2O_2 on the rate of chalcone consumption in the presence of garbanzo peroxidase



The reaction mixture contained chalcone (0.09 μmole), H_2O_2 as shown and enzyme (units indicated by numbers on the curves) in 0.05 M tris buffer pH 8.0, total volume 2 ml, temperature 25°. Chalcone disappearance was monitored at 396 nm.

Figure 26

Effect of concentration of H_2O_2 on the rate of chalcone consumption in the presence of HRP enzyme.



The reaction mixture contained chalcone (0.09 µmole), H_2O_2 as shown and enzyme (units indicated by numbers on the curves) in 0.05 M tris buffer pH 8.0, total volume 2 ml, temperature 25°. Chalcone disappearance was monitored at 396 nm.

HRP enzyme at higher hydrogen peroxide concentration. Maximum reaction rates were not strictly proportional to enzyme concentration. In fact it was usual to find that maximum rates were not highly reproducible when fresh dilutions of reaction components and particularly enzyme, were used as, for example, between days.

Control reactions were run in which enzyme was not only omitted but also replaced by an equal amount of heat-inactivated enzyme (15 minutes at 100^{0}). The chemical reaction with enzyme omitted, as measured by chalcone disappearance, was slow and increased from 0.001 /umole/min with 0.15 mM hydrogen peroxide to 0.002 /umole/min with 0.75 mM hydrogen peroxide. This rate was 1% or less of the enzymic rate except when higher hydrogen peroxide and lower enzyme concentrations were combined in which case the comparative figure could then approach 4%. With heat-inactivated enzyme present, reaction rates approximately 5% of the enzymic rates were found with the HRP preparation, while the garbanzo preparation gave rates about 1 - 1.5% of the enzymic ones. These values with heat-inactivated enzyme were consistent over the range of enzyme and hydrogen peroxide concentrations in Figure 25 and 26. Products were extracted (at pH 7) from a series of these control reactions after 2 minutes incubation time and were assessed on 2-D chromatographic separation. The presence of small amounts of OC and flavonol in the products were noted.

2B-5 Effect of chalcone (substrate) concentration on reaction rate

A comparable result was obtained for garbanzo (Figure 27)

and HRP enzyme (Figure 28) when chalcone concentration in the reaction mixture was varied within the upper and lower limits imposed respectively by the absorbance of the compound and reaction rates at highest enzyme levels. Typical substrate concentration curves were again apparent. Both enzymes responded similarly and appeared to be saturated at chalcone concentrations a little above 40 μM. Reaction rates were determined for chalcone concentrations up to 60 μM but the values were unchanged for concentrations above 50 μM.

Control reactions with boiled enzyme gave rates about 5% and 1.5% of the active enzyme rates for HRP and garbanzo enzymes respectively, throughout the range of chalcone concentrations.

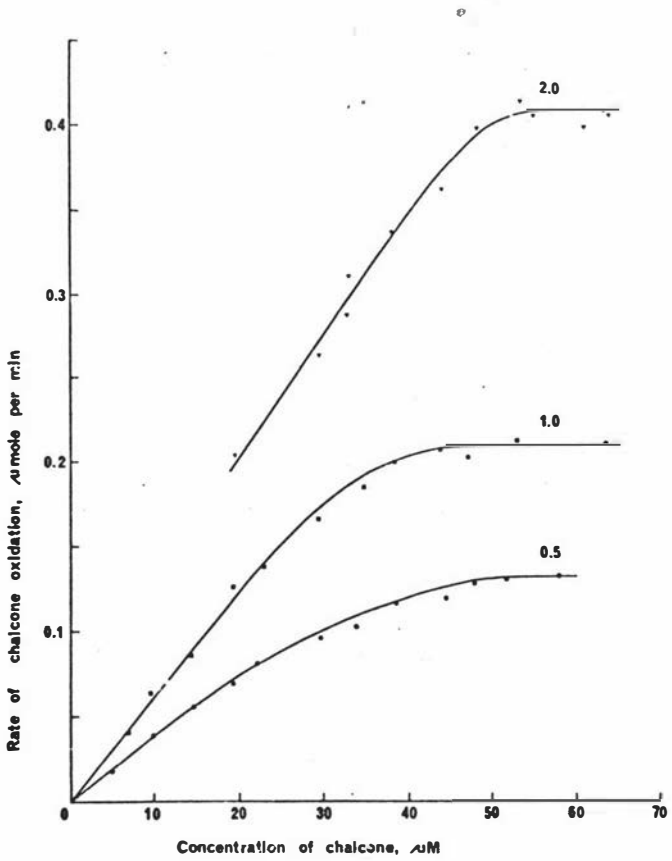
2B-6 Oxygen consumption in the reaction

Manometric experiments showed that oxygen was consumed in stoicheiometric amount in the reaction (Table 3). With garbanzo enzyme, the molar ratio of oxygen consumed to chalcone added was about 0.85 on average, while for HRP enzyme, it was close to 1.0. The reason for the lower ratio with garbanzo enzyme was apparently the incomplete consumption of chalcone added since reaction mixtures were completely decolorised only with HRP enzyme. It was not clear why the series of reactions catalysed by garbanzo enzyme did not proceed nearer to completion. The results in Table 3 thus indicated that one mole of oxygen was consumed in the conversion of one mole of chalcone to βY compounds.

Results in Table 3 further showed that the stoicheiometry of oxygen uptake in the reaction was independent of

Figure 27

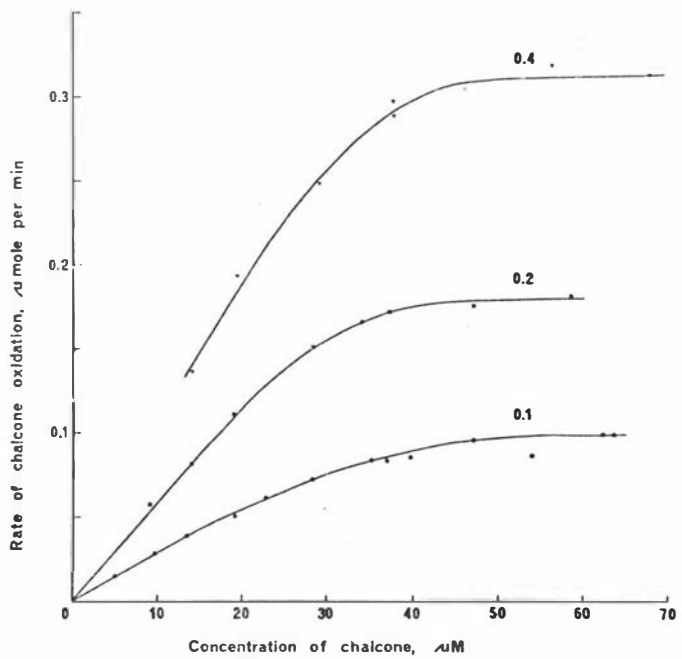
Effect of chalcone substrate concentration on reaction rate in presence of garbanzo peroxidase.



The reaction mixture contained chalcone as shown, enzyme (units indicated by numbers on the curves) and H_2O_2 (0.3 µmole) in 0.05 M tris buffer pH 8.0, total volume 2 ml, temperature 25°. Chalcone disappearance was recorded at 396 nm.

Figure 28

Effect of chalcone substrate concentration on reaction rate in presence of HRP enzyme.



The reaction mixture contained chalcone as shown, enzyre (units indicated by numbers on the curves) and H_2O_2 (0.6 ∧mole) in 0.05 M tris buffer pH 8.0, total volume 2 ml, temperature 25^{o}. Chalcone disappearance was recorded at 396 nm.

Table 3

Oxygen consumption in peroxidase-catalysed reaction of chalcone[x]

H_2O_2 (μmoles)	1.0	1.0	0.5	1.0	0.5	0.5	0.25	3.0	3.0	3.0	1.5
Chalcone (μmoles)	2.15	1.90	1.59	1.52	1.16	0.94	0.90	2.66	2.47	1.87	0.90
O_2 uptake (μmoles)	1.79	1.71	1.34	1.32	0.96	0.78	0.75	2.74	2.52	1.80	0.88
$\frac{O_2 \text{ uptake}}{\text{chalcone}}$	0.83	0.90	0.84	0.87	0.83	0.83	0.84	1.03	1.02	0.96	0.98
Enzyme (units) Garbanzo	53	26	26	53	26	26	10	-	-	-	-
HRP	-	-	-	-	-	-	-	13	13	13	13

[x] See Experimental for details.

the concentration of hydrogen peroxide added within the limits used in the experiments with garbanzo enzyme. In addition, no strict stoicheiometric requirement for hydrogen peroxide was indicated. Additional manometric experiments with HRP enzyme provided further evidence in support of both of these features of the reaction. Thus, consumption of a fixed amount of chalcone (1.5 μmoles) when the hydrogen peroxide added to the reaction mixture was reduced from 3.1 to 0.22 μmole, through three intermediate levels, was always complete and was matched by stoicheiometric oxygen uptake.

28-7 Consumption of hydrogen peroxide in the reaction

Manometric results (section 28-6) had pointed to a stoicheiometric requirement for oxygen rather than hydrogen peroxide in the enzymic formation of ØY compounds from chalcone. However, the reaction rate was affected by the hydrogen peroxide concentration in a manner compatible with this compound being a co-substrate in the reaction (Figures 25 and 26). The role of hydrogen peroxide was therefore further investigated in experiments which measured its net consumption in the reaction. These experiments showed that a catalytic rather than stoicheiometric net consumption of hydrogen peroxide occurred.

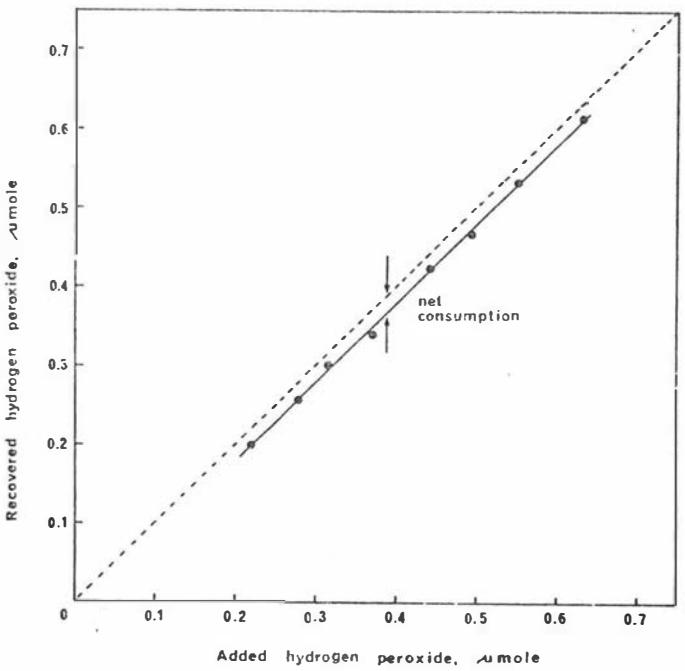
To facilitate these experiments, a rapid spectrophotometric method was developed (see Experimental) to estimate hydrogen peroxide in the concentration range 0.1 - 0.5 mM. This method was based on measurement at 470 nm of the oxidation product of guaiacol, referred to as tetraguaiacol, formed upon quantitative utilisation of hydrogen peroxide

in a rapid enzyme-catalysed peroxidatic reaction. A calibration curve was constructed in the presence of products of the chalcone reaction. This was necessary to correct for tetraguaiacol formation being significantly lower under these conditions than in the straight buffer system (see Experimental). This method was then applied to estimate the residual amount of hydrogen peroxide after the reaction of chalcone and, from the known amount of hydrogen peroxide added to start the chalcone reaction, the net hydrogen peroxide consumption in the reaction was determined by difference.

Using the samples of hydrogen peroxide which were also employed in the construction of the calibration curve, the complete reaction of chalcone (0.37 μmole) was carried out in the presence of each concentration of hydrogen peroxide in turn, under otherwise standard conditions (see Figure 29). Under the conditions selected, a rapid reaction of chalcone was always ensured. On account of the relatively high concentration of chalcone used, its disappearance could be conveniently monitored spectrophotometrically at the wavelength (470 nm) used subsequently to record tetraguaiacol formation in the estimation of residual hydrogen peroxide. This estimation was made immediately the chalcone had disappeared. Tetraguaiacol formation observed on estimation of hydrogen peroxide after the chalcone reaction and for an identical aliquot of the same hydrogen peroxide preparation in the calibration step, gave an accurate measurement, by difference, of the net consumption of hydrogen peroxide in the reaction of 0.37 μmole of chalcone. Thus net consumption of hydrogen peroxide

Figure 29

Net consumption of H_2O_2 in the chalcone oxidation catalysed by HRP enzyme.



The reaction mixture contained chalcone (0.37 µmole), HRP enzyme (1 unit) and H_2O_2, as indicated, in a total volume of 2 ml 0.05 M tris buffer pH 8.0, temperature 25°. See Experimental and Figure 62 for details of the estimation of H_2O_2.

was found to be uniformly small at about 0.02 μmole when 0.37 μmole of chalcone was reacted in the presence of HRP enzyme (Figure 29). When the same experiment was repeated with chalcone reduced to 0.27 and 0.18 μmole, the average net consumption of hydrogen peroxide was consistently low at 0.02 and 0.01 μmole respectively. Comparable results were also obtained when garbanzo enzyme (9.5 units) was substituted for HRP enzyme. In this case the average net consumption of hydrogen peroxide was 0.03, 0.01 and 0.02 μmole for reaction of 0.37, 0.27 and 0.18 μmole of chalcone respectively.

On account of the low amount of hydrogen peroxide consumed it was not possible from the above data to detect any meaningful relationship between the consumption of hydrogen peroxide and chalcone. However, another line of evidence suggested that hydrogen peroxide consumption varied with reaction conditions. Since the minimum amount of hydrogen peroxide which had to be added for the reaction to go to completion decreased as the enzyme concentration increased, it was found that in the reaction system given in Figure 29, hydrogen peroxide added could be reduced to 0.04 μmole minimum when HRP enzyme was increased to 5 units (compare minimum hydrogen peroxide near 0.22 μmole with 1 unit of enzyme). Net consumption of hydrogen peroxide in the reaction with this higher amount of enzyme fell to virtually zero under conditions where the hydrogen peroxide added ranged from 0.04 to 0.74 μmole.

Thus, these direct measurements established a net requirement for catalytic amounts of hydrogen peroxide in the reaction, a result which was in agreement with not only the

demonstrated consumption of 0.37 μmole of chalcone upon addition of only 0.04 μmole of hydrogen peroxide in the system just mentioned, but also earlier findings of a stoicheiometric oxygen requirement which was independent of hydrogen peroxide concentration.

2B-8 Effect of anaerobic conditions

Experiments were run under anaerobic conditions to test whether oxygen was obligatory for the formation of the ØY compounds from chalcone or whether under these conditions stoicheiometric amounts of hydrogen peroxide could substitute in the reaction. The results proved an obligatory requirement for oxygen.

In a typical experiment, chalcone (1.46 μmoles, 400 μg) and HRP enzyme (200 units) were added to 4 ml of 0.05 M tris buffer pH 7, in a Thunberg tube and hydrogen peroxide (4 μmoles in 0.1 ml water) was placed in the stopper. The system was then made anaerobic (see Experimental), the tube warmed to 25° in a water bath and the contents of the assembly mixed. After incubation for 30 minutes, during which time the solution remained yellow, hydroquinone (20 μl of 0.04 M solution in ethanol) was added rapidly and mixed with the contents of the tube to prevent aerobic reaction of chalcone in the extraction preliminaries. (Aerobic controls showed all chalcone was consumed within 30 seconds; hence aeration in the extraction step would probably have permitted a rapid reaction of chalcone). The material recovered by ether extraction at pH 7 was subjected to 2-D chromatography and found to consist largely of unchanged chalcone. A very small amount of OC

was present (1.7 and 3.1 µg in duplicate runs; that is, less than 1% of chalcone added) but ØY compounds did not persist in detectable amounts. This OC was thought to represent ØY compounds formed in the very slow reaction which would have occurred when the chalcone and HRP enzyme were originally mixed in the aerated buffer and possibly also because traces of oxygen may have been available in the anaerobic incubation.

Spectrophotometric observations of the inhibition of the reaction under anaerobic conditions were also made using Thunberg-type cuvettes. The complete reaction mixture contained, chalcone (0.73 µmole), HRP enzyme (1 unit) and hydrogen peroxide (0.55 - 1.5 µmoles) in 0.05 M tris buffer pH 8.5, total volume 3 ml, temperature 25°. Once the system had been made anaerobic, the hydrogen peroxide was mixed in as before. In order that the cuvette would fit into the spectrophotometer the Thunberg stopper was replaced under a stream of nitrogen by a low-rise stopper, to the under end of which further hydrogen peroxide (0.88 µmole in 5 µl) had been added. Then, when a very slight reaction which scavenged the last available oxygen in the system had subsided, as shown by the then steady chalcone absorbance at 450 nm, the extra hydrogen peroxide on the stopper was added by shaking the cuvette. No further decrease in chalcone absorbance was noted which indicated that in the fully anaerobic system the reaction was inhibited completely and did not respond to increments in hydrogen peroxide concentration. Aeration of the reaction mixture, by bubbling with air, resulted in immediate consumption of chalcone, in amount related as expected to

the duration of aeration and therefore to the oxygen supplied.

Limitation of the extent of the reaction by oxygen supply was alternatively observed at 470 nm when HRP enzyme (14 units) and hydrogen peroxide (0.22 - 2.2 μmoles) were added to an open cuvette which contained chalcone (1.1 μmoles) in normally-aerated 0.05 M tris buffer pH 8.5, 2 ml total volume. The reaction, rapid with the lower and very rapid with the higher hydrogen peroxide concentrations used, ended abruptly after a fixed quantity of chalcone disappeared and could be restarted repeatedly by aeration for a few seconds at a time.

28-9 Extent of reaction and evidence for product inhibition

The composition of the reaction mixture had a marked effect, not only on the rate of reaction as previously noted, but also on the extent of chalcone consumption before the reaction virtually ceased. Only with certain reaction mixtures was chalcone consumption complete or nearly so. The extent of reaction as revealed spectrophotometrically, was sensitive to levels of each component under certain conditions. For example, as the concentration of hydrogen peroxide increased, the extent of reaction, minor in absence of added hydrogen peroxide, increased rapidly before being curtailed again at higher concentrations in a manner akin to reaction rate (Figure 26). The extent also increased with enzyme level; in a reaction mixture which contained chalcone (0.37 μmole), hydrogen peroxide (0.2 μmole) and HRP enzyme in 0.05 M tris buffer pH 8.0, 2 ml total volume, consumption of chalcone continued to

about 45, 70 and 90% completion with enzyme levels of 0.05, 0.1 and 0.2 unit respectively.

Under a range of chalcone concentrations at a low enzyme level, the reaction stopped after a fixed amount of chalcone was consumed provided that the chalcone concentration did not first become limiting and stop the reaction sooner. Hence, when the reaction mixture contained chalcone (range 0.09 - 0.13 μmole), HRP enzyme (0.05 unit) and hydrogen peroxide (0.4 μmole) in 0.05 M tris buffer pH 8.0, 2 ml total volume, the reaction always virtually ceased after 0.048 μmole of chalcone had been consumed. With less chalcone initially added (0.055 μmole), the quantity of chalcone consumed before the reaction stopped was less. The reaction could be restarted, after the fixed quantity of chalcone had been consumed, on addition of further enzyme. Aeration and hydrogen peroxide were not limiting when the reaction first stopped. Restoration of chalcone to the initial level had apparently no effect, but exact measurements were difficult to make since, through instability of the initial product, absorption was increasing in the stopped reaction at the wavelength used to observe chalcone disappearance (compare Figure 16). The rate of the restarted reaction was, however, very slow in comparison with that expected for the extra quantity of enzyme added and the levels of chalcone and hydrogen peroxide present. It was concluded that product inhibition was the most likely cause of the observed initial cessation of the reaction when a fixed quantity of product had been formed and a variable quantity of chalcone remained. Further, such

inhibition explained the limited reaction with additional enzyme. This limited reaction was also taken as evidence that the reaction was not stopped initially through gradual enzyme destruction only, since in that case the additional enzyme would have been expected to be fully active at first.

2B-10 Effect of various additives on the reaction

Using the established spectrophotometric methods to follow chalcone disappearance, the effects of a selection of additives on the rate of reactions catalysed by garbanzo and HRP enzymes were found to be as summarised in Table 4. Where progress curves were sigmoidal, maximum rather than initial rates were used in calculating the values presented. The reaction mixtures used (Table 4) were selected to give maximum reaction rates in the absence of additives (see Figures 25 and 26) except for those with the last three additives in the Table. Here, the hydrogen peroxide added was cut to one-sixth of previous amounts to reduce the control reaction rates and make the system better suited to testing for possible stimulatory effects of the three additives. As an additive, cyanide could not be used satisfactorily since a rapid chemical reaction was observed when KCN was added to chalcone in the pH 8.0 buffer.

Cupric and manganous ions were inhibitory, the latter strongly so when added at a concentration which approached that of the added chalcone. Sigmoidal progress curves were observed in the presence of cupric ion, except at the lower concentration with garbanzo enzyme. In the

Table 4

Effect of additives on the rate of enzymic reaction of chalcone

Additive	Final Conc^{n}	Reaction rate as % of control	
		Garbanzo enz.	HRP enz.
Cu^{2+} (as Cu_2SO_4)	1 mM	34	54
	0.2 mM	49	42
Mn^{2+} (as $MnCl_2$)	0.2 mM	3	4.5
	80 µM	9	13
EDTA[y]	1 mM	81	87
SEDC[z]	4 µM	12	40
	2 µM	48	60
2-Mercaptoethanol	20 µM	10	13
	8 µM	25	30
Hydroquinone	0.1 mM	0.3	0.4
	20 µM	1.3	2.0
	2 µM	14	16
Catechol	50 µM	0.3	0.4
Pyrogallol	50 µM	0.3	0.4
p-Phenylenediamine	50 µM	0.1	0.2
Phenol[x]	0.1 mM	100	92
Resorcinol[x]	0.1 mM	27	29
p-Cresol[x]	0.1 mM	64	63

Basic reaction mixtures contained, enzyme (garbanzo 1 unit, HRP 0.2 unit), chalcone (0.09 µmole - 45 µM) and H_2O_2 (0.3 µmole with garbanzo and 0.6 µmole with HRP enzyme) in 0.05 M tris buffer pH 8.0, 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 minutes; chalcone was then added followed immediately by H_2O_2 to complete the system.

[x] With these additives, the H_2O_2 added was cut to one-sixth of that given in the basic reaction mixtures above. Chalcone and 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.

[y] Ethylenediaminetetraacetic acid, disodium salt.

[z] Diethyldithiocarbamate, disodium salt.

HRP-catalysed system, the reaction showed a more pronounced lag phase but attained a greater rate at the higher compared with the lower cupric ion concentration. Thus the values recorded in Table 4 summarise but one feature of the response of the system to cupric ion. With manganous ion, however, the initial rates were maximum rates. EDTA was slightly inhibitory and was without effect on the shape of the progress curves. On the other hand, SEDC induced in all cases strongly sigmoidal progress curves, the very marked lag phase of which lasted for about 50 seconds for the lower and about 150 seconds for the higher concentration of the compound. Mercaptoethanol, like SEDC, was a very potent inhibitor, yet progress curves were not sigmoidal.

The redogenic donors, hydroquinone, catechol, pyrogallol and p-phenylenediamine, were all found to be strongly inhibitory at a concentration similar to the chalcone. When these donors were incubated separately in the reaction mixture with only the chalcone omitted, the pyrogallol reaction was found to give rise to absorption at 396 nm, the wavelength used to monitor chalcone disappearance. The rate of reaction found with chalcone in the presence of pyrogallol was corrected accordingly. The reaction in the presence of the lowest concentration of hydroquinone was still strongly inhibited but progressed without any lag.

No stimulatory effect was recorded for the oxidogenic donors tested, phenol, resorcinol and p-cresol. Phenol was without effect but the other compounds, particularly resorcinol, were inhibitory. Progress curves retained their

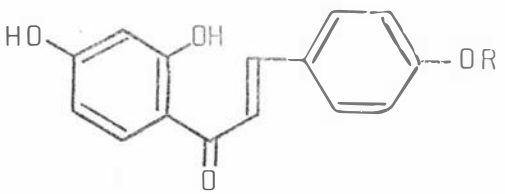
normal shape. Resorcinol and p-cresol may have been inhibitory intrinsically or because of unsuspected impurities present in the chemicals used. However, the known high purity of phenol used would permit the definite conclusion that this oxidogenic donor was not stimulatory. Results as a whole suggested that oxidogenic donors in general would not be stimulatory.

2B-11 Chalcone substrate specificity

Eight chalcones (Figure 30) were tested in the spectrophotometric system to determine which compounds would serve as substrates in a peroxidase-catalysed reaction. Products were not isolated; only disappearance of chalcone was monitored. All the chalcones except (II) and (V) were found to undergo enzymic reaction. No difference was apparent in the specificities of HRP and garbanzo enzymes.

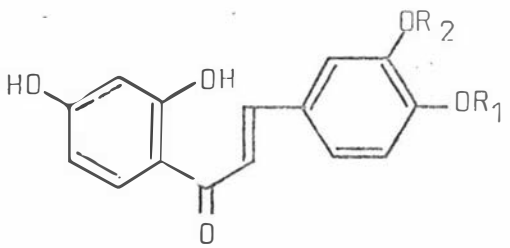
Since only very small amounts of a number of these chalcones were available a sample of each compound tested, except (VI) and(VII), was purified by paper chromatography (see Experimental). Despite the use of specially washed paper, however, the prepared compounds were contaminated with impurities eluted from the paper. This was shown by the occurrence of sigmoidal progress curves and reduced rates when the control sample of isoliquiritigenin, which had been through this purification, was reacted in the presence of either garbanzo or HRP enzyme. As the effect of impurities would have varied between samples, on concentration differences alone, no attempt was made to measure quantitatively differences in reaction rates between the chalcones.

Figure 30. Chalcones tested as substrates in the peroxidase-catalysed reaction.



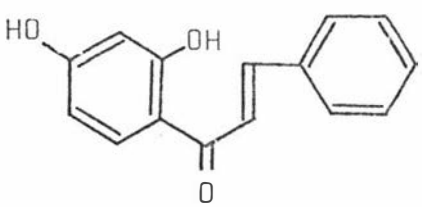
(I) R = H

(II) R = CH_3

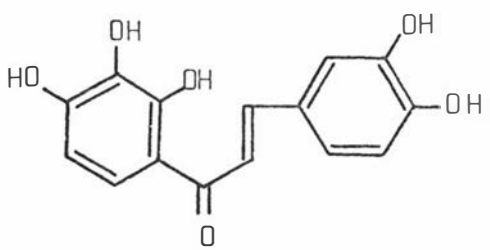


(III) R_1 = H R_2 = CH_3

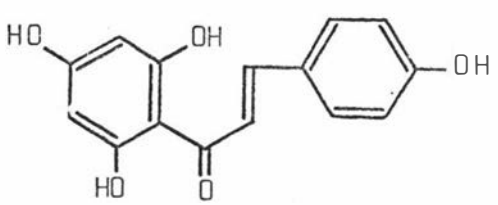
(IV) R_1 = CH_3 R_2 = H



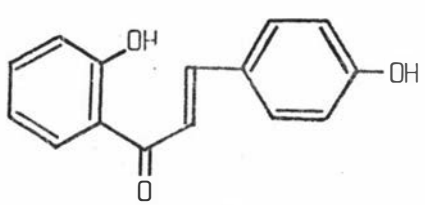
(V)



(VI)



(VII)



(VIII)

Reaction mixtures used contained chalcone (about 0.09 µmole for each compound) enzyme (HRP 0.2 or 34 units, garbanzo 1.0 or 25 units) and hydrogen peroxide (0.6 µmole with HRP and 0.3 µmole with garbanzo enzymes) in 0.05 M tris buffer, pH 8.0, 2 ml total volume, temperature 25°. The higher enzyme activities were used with all chalcones except (I) and (II). Controls were always run, both with boiled enzyme and with enzyme omitted.

None of the chalcones was found to react as rapidly as isoliquiritigenin (I). Chalcone (III) reacted readily but at a rate roughly 25% of that of (I), with both garbanzo and HRP enzymes. Another to react rapidly was (VIII). However, the product in this case was extremely unstable and absorption rapidly reappeared at 275 nm, the wavelength monitored with this compound. Hence the high enzyme activities were needed to clearly show enzymic reaction. Chalconaringenin (VII) was used directly from the bottle and reacted readily in the enzymic reaction. The marked instability of this chalcone in tris buffer precluded the effective use of the low enzyme activities. Considerably less effective than the foregoing as a substrate was chalcone (VI), while (IV) reacted but slowly. Compounds (II) and (V) failed to react at all.

These results suggested that a free phenolic hydroxyl group in the B ring, preferably in the para position (compare (III) and(IV)) was required for activity in the enzyme-catalysed reaction. It should be pointed out however, that since spectrophotometric evidence only was available, it cannot be assumed that the enzymic reaction observed for the additional chalcones was necessarily equivalent to that

undergone by the isoliquiritigenin chalcone.

2C BIOCHEMISTRY OF THE FORMATION OF MINOR PRODUCTS

While the reaction was justifiably simplified to one in which chalcone is converted into the ØY compounds for the purpose of conducting the biochemical studies recorded in the foregoing sections, it nevertheless remained that the additional minor products (section 2A-13 and Figure 22) were of equal interest in a qualitative sense. This was largely because recognised compounds of two distinct flavonoid classes, aurone and flavonol, were being generated apparently in the form of alkali-labile precursors. In addition, the question of the place of the dihydroflavonol, garbanzol, in this system became of interest. Further studies on all of these minor products were pursued therefore, either in conjunction with work on the ØY compounds reported in previous sections or in new experiments designed to provide more specific information. Various observations were made on the formation of Y_1 and Y_2 while the precursors of flavonol were eventually recognised to be identical with the established precursors of OC, namely, $ØY_1$ and $ØY_2$. Evidence for the formation of trace amounts of garbanzol in the reaction was also obtained.

2C-1 Fluorimetric estimation of aurone and flavonol

The only satisfactory way in which the specific alkali-labile precursors could be estimated quantitatively was after their conversion to aurone or flavonol, since no effective chromatographic separation of the precursors from

the enzymic reaction product could be achieved. As aurone and flavonol both fluoresced in the visible (Table 1) and absorbed maximally in the u.v., a fluorimetric method of estimation was developed to enable the expected quantities, too low for absorption measurements and possibly contaminated with u.v.-absorbent but non-fluorescent materials, to be measured (see Experimental).

2C-2 Observations on Y_1 and Y_2 formation

Qualitative information on Y_1 and Y_2 formation in the reaction could be obtained only after 2-D chromatographic separation of the products and then only if overlapping $\not{\alpha}Y$ compounds were sufficiently weak for the fluorescent aurone artifact to be visible in the Y' spots after storage of the chromatograms. Under conditions where the Y' spots were strong (i.e. much $\not{\alpha}Y_1$ much $\not{\alpha}Y_2$ had persisted), elution followed by alkali treatment and rechromatography to check for aurone formation would have been necessary to demonstrate the presence of Y_1 and Y_2. On account of the low amounts generally formed, quantitative estimates of Y_1 and Y_2 could only be made fluorimetrically, after the compounds had been converted to aurone by alkali treatment. Therefore, in view of the labour this involved, few quantitative measurements of Y_1 and Y_2 formation were made. Instead, numerous visual observations of a qualitative nature were routinely made after the 2-D chromatograms had been held for up to several weeks and aurone artefact had formed in the Y' spots. Frequently, the conditions of product extraction (e.g. from pH 7 or 7.5 buffer - see section 2A-12) ensured that only minor amounts of the $\not{\alpha}Y$ compounds remained to appear on the

chromatograms while most of the much more stable Y_1 and Y_2 persisted and hence aurone artifact formation in the Y' spots was often readily detectable. The presence of even sub-microgram amounts of Y_1 and Y_2 in the product could be detected when ΔY compounds were virtually absent on chromatograms. Where product had been treated with alkali prior to extraction and chromatography the Y_1 and Y_2 precursors had been largely converted to aurone before chromatography and these chromatograms were of no interest in the present context.

(a) The enzymic nature of Y_1 and Y_2 formation was demonstrated on 2-D chromatographic comparison of the products of reactions carried out in the presence of active enzyme (garbanzo and HRP), heat-inactivated enzyme (15 minutes at 100°) and with enzyme omitted. The complete reaction mixture contained in a total volume of 4 ml of 0.05 M tris buffer pH 8.0, chalcone (0.18 μmole), hydrogen peroxide (0.6 or 0.3 μmole with HRP enzyme (0.4 unit) and garbanzo enzyme (2 units) respectively) and enzyme (as given). Complete and control reaction mixtures were incubated at room temperature for 2 minutes then acidified to pH 7 and the ether-extracted products suojected to chromatography. Inspection of the stored chromatograms clearly showed that Y_1 and Y_2 formation was greatest in the complete systems and that very low amounts were also formed in all the control reactions. Chalcone consumption was almost complete in the presence of active enzyme, but was slight in the control reactions.

(b) Ubiquity of Y_1 and Y_2 in reaction product. Inspection of a large number of 2-D chromatograms of

products of the reaction carried out under a considerable range of enzyme (both garbanzo and HRP), chalcone and hydrogen peroxide concentrations showed that Y_1 and Y_2 were invariably formed as minor products when the main chromatographic product was UC. Further, as an example of the consistent presence of Y_1 and Y_2 in the reaction products, the absence of an observable effect of reaction pH on the occurrence of these products may be specifically cited. From the 2-D chromatograms of products of a standard reaction run in a buffer range pH 6 - 9 approximately (see section 2B-3 for details). it was possible to detect clearly Y_1 and Y_2 occurrence from pH 6.6 upwards. At lower pH the Y' spots were heavy and direct evidence of Y_1 and Y_2 content was not so definite, but presumably alkali treatment of the Y' spot eluate followed by rechromatography would have confirmed the presence of the aurone precursors. A noticeable relative increase in aurone and decrease in Y_1 and Y_2 spot intensities was evident on the chromatograms of products from particularly reaction at pH9. This was interpreted as reflecting accelerated chemical formation of aurone from Y_1 and Y_2 in the course of the enzymic reaction due to the high reaction pH. Coupled with the earlier experience that no aurone spot was observed when the major reaction products were preserved and chromatographed as the ØY compounds (section 2A-12 and Figure 19), it was concluded that Y_1 and Y_2 were initially formed in the reaction at all pH levels and that all aurone found on chromatograms was subsequently derived from these precursors.

(c) The combined yield of Y_1 and Y_2 in the reaction under typical conditions with both garbanzo and HRP enzymes was measured fluorimetrically, as aurone, and was found to account for about 1% of the chalcone which reacted. Details of the reaction mixtures used and procedures adopted for the isolation and fluorimetric estimation of Y_1 and Y_2 as aurone are contained in the Experimental.

(d) Evidence for an effect of oxygen on the formation of Y_1 and Y_2 was obtained from the results of the anaerobic reactions previously detailed (section 2B-8) in connection with the major reaction product. Thus, compared with the aerobic control, levels of Y_1 and Y_2 were considerably reduced but still detectable under anaerobic conditions. Similar low levels were formed when boiled enzyme was present in anaerobic controls but when enzyme was omitted levels decreased almost to the limit of detection.

A prominent additional feature of these anaerobic reactions was the formation of aurone which occurred presumably by a direct, and possibly non-enzymic, pathway from chalcone and not by way of Y_1 and Y_2. This aurone was recovered from 2-D chromatograms and was estimated fluorimetrically by the procedure outlined in the Experimental for the estimation of Y_1 and Y_2-derived samples. This ensured that the small amounts of flavonol included in the extremities of the aurone spot, together with some chalcone (non-fluorescent), did not interfere. Results, corrected for losses in isolation and sample preparation, indicated an average anaerobic formation of 21 µg aurone in the complete reaction mixture but only about 1 µg when

enzyme was omitted. However, in the presence of heat-inactivated enzyme the average anaerobic aurone formation of 19 µg was similar to that with active enzyme. In contrast to these anaerobic results the level of directly recovered aurone in aerobic controls was always very low at approximately 1 µg for the chemical reaction and about 2 µg in the presence of either active or heat-inactivated enzyme. Further studies with the complete anaerobic reaction mixture showed that most of the observed aurone formation apparently occurred in the first few minutes of incubation, since incubation times of from 5 to 100 minutes resulted in similar observed aurone levels. A four-fold increase in enzyme concentration (to 800 units of HRP) resulted surprisingly in no aurone formation and the appearance of at least one unknown product of now extensive chalcone consumption. In view of the apparent complexity of the anaerobic reaction, no further study of this aspect was undertaken.

That anaerobic aurone formation was catalysed with about equal effectiveness by both active and heat-inactivated enzyme would indicate chemical rather than enzymic catalysis. However, more work would be necessary to fully substantiate this conclusion. Nevertheless, the results clearly showed that in both the active and heat-inactivated enzyme systems, an anaerobic mechanism of aurone formation from chalcone existed in contrast to the alternative pathway through Y_1 and Y_2 of the aerobic reaction.

(e) Effect of additives. The effects of the presence in the reaction mixture of certain additives on the formation of Y_1 and Y_2 were briefly investigated with results as

indicated below. The reaction mixture contained, chalcone (1.8 μmoles), garbanzo enzyme (95 units), hydrogen peroxide (8 μmoles) and additive (see below) in 4 ml total volume of 0.05 M tris buffer pH 8.5, at room temperature. Additive was combined with enzyme in buffer and then, after 2 minutes, chalcone was added followed by hydrogen peroxide to complete the system. After incubation, the buffer was acidified to pH 7.5 prior to extraction of the products into ether. Extraction at this pH ensured that ØY compounds were virtually absent on the 2-D chromatograms of products.

When the redogenic donors hydroquinone and catechol were separately added to the reaction system above at a final concentration of 1 mM, product chromatograms showed that complete inhibition of Y_1 and Y_2 and OC formation had occurred during the 10 minute incubation period. Donor concentrations of 0.5 and 0.2 mM permitted small amounts of both Y_1 and Y_2 and OC to be formed; in the case of hydroquinone the reaction was noticeably more extensive. However, Y_1 and Y_2 formation was always much reduced compared with that observed in the control reaction which, in the absence of any additive, went to completion.

Mercaptoethanol and dithiothreitol each had a similar effect on the reaction at a final concentration of 1 mM. Product chromatograms for a 5 minute incubation time were very similar in pattern to those originally observed when chalcone was incubated with crude cell-free garbanzo enzyme in the absence of added hydrogen peroxide. Thus Y_1 and Y_2 were present in low amount and OC was absent. Further, much chalcone was recovered unchanged on account of the reaction being strongly inhibited. Despite the absence of

OC on these chromatograms, formation of OC precursors probably occurred since a number of unknown background spots were present, presumably as a consequence of chemical reaction at the precursor level with the thiols. Thus there was considered to be no definite evidence in these results for the separation of the Y_1 and Y_2- and $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$-forming enzyme reactions. The extent of reaction previously measured spectrophotometrically in the presence of low concentrations of mercaptoethanol (section 2B-10) was further indirect evidence for the presumed formation of OC precursors in addition to Y_1 and Y_2.

While sodium diethyldithiocarbamate strongly inhibited the reaction of chalcone overall, a significant effect on product distribution between Y_1 plus Y_2 and OC was noted on the chromatograms, namely a proportionate increase in Y_1 and Y_2 formation. The levels of Y_1 and Y_2 observed were in excess of (probably at least double) those for the control reaction despite much chalcone being recovered unchanged after an incubation time which extended to 25 minutes.

Finally, the overall reaction of chalcone was inhibited very strongly by manganous ion (1 mM final concentration) during incubation for 5 minutes and the levels of Y_1 and Y_2 were much lower than in the controls. Use of cupric ion in an identical manner to manganous ion also inhibited the overall reaction somewhat, with an equivalent effect on the levels of Y_1 and Y_2.

(f) Promotor(s) of Y_1 and Y_2 formation in cell-free garbanzo enzyme. The capacity of supernatant of boiled garbanzo cell-free enzyme (boiled supernatant) to promote

Y_1 and Y_2 formation, while also inhibiting OC formation and thereby sparing chalcone, has been mentioned earlier (section 2A-2) in relation to the system in which garbanzo enzyme, partially purified by precipitation, was incubated with chalcone in the absence of added hydrogen peroxide. This activity in the boiled supernatant was further investigated briefly in a series of fractionation experiments which demonstrated that the compound(s) responsible was highly water soluble, probably non-ionic and non extractable by organic solvents such as ether and ethyl acetate. Since it was apparently valid that the Y_1 and Y_2-promoting activity of interest was causally associated with the inhibition of chalcone consumption, fractions prepared from boiled supernatant were conveniently assayed on the basis of their capacity to inhibit chalcone consumption. The assay reaction mixture contained the minimum of hydrogen peroxide needed for the reaction to go to completion in the absence of any inhibition. When aliquots of the fractions from boiled supernatant were added, any significant inhibition was then readily detected visually by persistence of the chalcone colour after a reaction time of a few minutes. This assay procedure indicated that the activity was not extracted into ether or ethyl acetate from pH 7.5 tris buffer solution, either directly or after acidification. Following chromatography in 5% HA of a sample of boiled supernatant which had been evaporated to dryness, the activity was detected in the heavily contaminated zone at the solvent front. Preliminary runs indicated that the activity passed directly through columns of both anion and cation exchange resins. All evidence indicated that the

active compound(s) possessed properties characteristic of simple sugars.

2C-3 Studies on flavonol formation

Preliminary work had established that precursors of flavonol, present in the Y' spots on 2-D chromatograms, were formed in the enzymic reaction of chalcone (section 2A-11). These precursors were considered to be the source of recovered flavonol, just as Y_1 and Y_2 were the source of aurone in the aerobic reaction. The presence of the dihydroflavonol, garbanzol, as an expected precursor of flavonol was not noted on chromatograms. This compound would be clearly resolved from the Y' spots under the 2-D chromatographic conditions used (19).

Further work on the precursors of flavonol which occurred in the Y' spots came to a satisfactory conclusion from a biochemical view point when it was discovered (see below) that these precursors were in fact the same ØY compounds which gave rise to OC. Measurement of the various small amounts of flavonol which were formed on product chromatograms under the normal conditions of reaction and extraction was therefore of no biochemical significance.

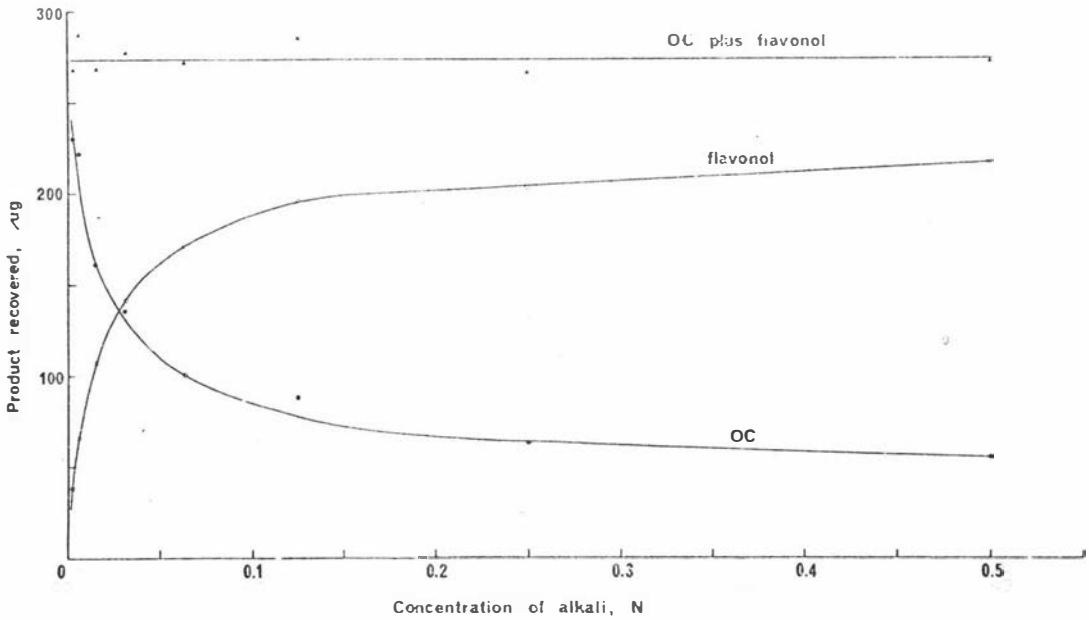
(a) $ØY_1$ and $ØY_2$ as precursors of flavonol as well as OC. The effective transformation of ØY compounds into OC upon treatment with a low concentration of alkali was recorded earlier (section 2A-8). However, in a more extensive investigation, it was discovered that the alkali concentration exerted a profound effect on the transformation of the ØY compounds. Thus, as the alkali concentration increased, the established product at low alkali concentration,

OC, gradually gave way to another product, which was identified as flavonol (see Experimental). Figure 31 shows the quantitative relationship between these mutually exclusive transformation products as a function of the concentration of the aqueous alkali to which a standard mixture and amount of ØY compounds was added (see Experimental for the preparation of ØY compounds in bulk). The recovery of OC and flavonol combined, corresponded on average to about 75% of that amount of OC which was spectrophotometrically measured as equivalent to the quantity of ØY compounds transformed. When allowance was made for the losses noted in separate manipulations involving the products, this recovery was in agreement with OC and flavonol being the only quantitatively significant products of transformation in alkali, a conclusion which was substantiated on 2-D chromatographic examination of a portion of each of the product mixtures. Spectrophotometric examination of the crude product of transformation of ØY compounds throughout the alkali concentration range showed a gradual change from an OC spectrum to that of flavonol, through a range of obviously composite spectra (see Experimental, Figure 63 for reference spectrum of flavonol). From the quantitative aspects of product formation it was immediately clear (Figure 31) that both $ØY_1$ and $ØY_2$ were transformed in a parallel manner in alkali.

Direct comparison of the transformation of these two compounds by repetition of the above experiment with separate samples of $ØY_1$ and $ØY_2$ (see Experimental for method of preparation), within a somewhat curtailed pH range, demonstrated convincingly (Figure 32) that for any given pH within

Figure 31

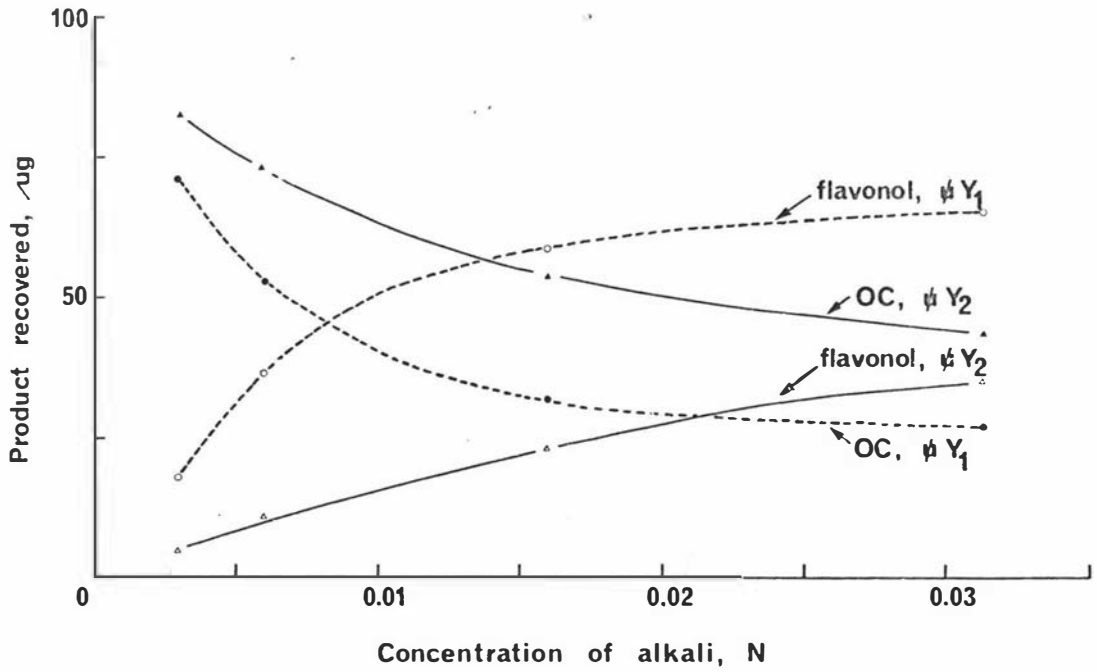
Competitive formation of OC and flavonol from ØY compounds as a function of the concentration of alkali.



In the alkali transformation, an aliquot of an enzyme product mixture of ØY compounds, dissolved in 85% EtOH (100 µl, equivalent to 350 µg of OC), was added with rapid mixing to 3.9 ml of freshly prepared NaOH, (final concentration given in Figure) at room temperature. After 30 seconds, the solution was acidified with a minimum of HCl and then ether extracted (2 x 5 ml). Product recovered from the combined ether extract was examined chromatographically for each sample. OC and flavonol were separated by 1-D chromatography of a portion of each product mixture in BeAW. The quantities measured spectrophotometrically on elution of these products in 85% EtOH were used, uncorrected for losses which would have been similar for all samples, in the preparation of the figure.

Figure 32

Comparison of the transformation of ϕY_1 and ϕY_2 in a range of alkali concentrations in relation to the distribution of product between OC and flavonol.



In the transformation reaction, the chromatographically prepared ϕY compound (in 50 µl 85% EtOH and equivalent to 116 µg of OC) was treated as detailed in Figure 31 for the mixture of ϕY compounds, using only the lower concentrations of alkali given in the Figure. At the lowest alkali concentration, 2 minutes elapsed before the solution was acidified. As before, products were estimated spectrophotometrically following chromatographic separation.

the range, $\emptyset Y_1$ gave rise to flavonol in relatively greater quantity than did $\emptyset Y_2$. As required by the result for the mixture of $\emptyset Y$ compounds, however, the OC and flavonol products were always mutually exclusive and therefore the essential difference apparent in behaviour of $\emptyset Y_1$ and $\emptyset Y_2$ was the more facile reaction of the former.

The formation of OC and flavonol from $\emptyset Y$ compounds was shown to be by mutually exclusive routes rather than by a sequential one through OC as an intermediate, in an experiment which investigated the effect of making a two-stage addition of the total alkali. In the first stage, the minimum alkali required for the transformation of $\emptyset Y$ compounds essentially to OC was added. After two minutes at this low alkali concentration, the second stage addition of alkali was made to achieve a range of final concentrations as before. Isolation and estimation of the products by the usual procedures showed that there had been little effect of the second stage addition of alkali since in all samples OC was the major and flavonol the minor component.

(b) Effect of cupric ion on flavonol formation. It has already been observed that in the presence of added cupric ions the enzymic reaction was inhibited (Table 4). In a separate study in which the reaction products rather than rates were monitored, chromatographic evidence for a strong, apparently specific, inhibition of flavonol formation was observed when copper was present in the enzymic reaction mixture. For example, flavonol was virtually absent on 2-D chromatograms of products when cupric ion at a final concentration of 1 mM was included in the reaction

mixture (given in section 2C-2(e)) and the products extracted from buffer acidified to pH 7.5. Since the conditions of product extraction ensured virtually no precursors of flavonol and OC persisted to appear on product chromatograms, the added copper had seemingly inhibited flavonol precursor and therefore flavonol formation. This result was obtained before the flavonol precursors were known to be the ØY compounds. Since the product chromatograms displayed a prominent OC spot, the existence of common flavonol and OC precursors was not at all evident, in fact the superficial indications were of distinct precursors for these two compounds. However, when the amount of OC product on chromatograms was measured spectrophotometrically it was found that the yield in terms of chalcone consumed was much reduced when copper was present in the enzymic reaction mixture. Thus, the wider effect of copper, beyond the visually obvious suppression of flavonol was recognised. Subsequent studies (see below) revealed that in the presence of copper the enzymic reaction products, the ØY compounds, underwent extensive chemical degradation. This post-enzymatic chemical effect was found to be responsible for the originally observed chromatographic product pattern in which flavonol was virtually absent and the quantity of the still prominent OC much reduced. Hence the effect of added cupric ion was extended in this direction beyond simple inhibition of the reaction rate.

Evidence that added copper caused destruction of the ØY compounds first came from a 2-D chromatographic examination of the products of an enzymic reaction which was run in the presence of a high concentration of copper. Thus the

product extracted directly at pH 2 - 3 following the enzymic reaction of chalcone (1.8 μmoles) at pH 8.0 in the presence of added cupric ion (5 mM final concentration) was found to contain neither ØY compounds nor flavonol and only traces of OC. Normally the product would have been observed chromatographically as predominantly the Y' spots (ØY compounds mainly) under the conditions employed. Instead, many background spots were present, in agreement with the proposed extensive copper-promoted breakdown of the usual enzymic reaction product. This result showed that both $ØY_1$ and $ØY_2$, if formed as expected in the enzymic reaction, had been destroyed in the presence of copper.

The contention that ØY compounds were still formed normally in the enzymic reaction in the presence of copper and that their absence in extracted product was due to a subsequent copper-promoted destruction was supported by the results of the following experiment which directly investigated the chemical destruction of ØY compounds in the presence of copper. In this experiment ØY compounds which had been recovered from a large scale enzymic reaction were incubated with added copper in 0.05 M tris buffer pH 8.0. The incubation mixture in 4 ml total volume contained ØY compounds formed from 0.5 mg chalcone (added in 0.1 ml 85% ethanol) and cupric ion (as 0.2 M $CuSO_4$ in water) at a final concentration of 1,3 or 5 mM. A control mixture had copper omitted. Each reaction mixture was incubated for 10 minutes at room temperature. Then the unchanged ØY compounds present were stabilised for extraction and chromatography by transformation to OC, predominantly, with a minimum addition of alkali (50 μl of 2.5 N NaOH per sample). Each sample was

then acidified (150 µl of 1.44 N HCl) two minutes later and ether extracted. From the 2-D chromatograms of the ether-extracted compounds it was clear that the presence of copper during the incubation at pH 8 had resulted in an accelerated destruction of øY compounds since in those cases there was a very marked decrease in the amount of OC recovered and hence in the øY compounds remaining at the end of the incubation. The extent of destruction of øY compounds increased with increasing copper concentration. The amount of flavonol recovered, already low in the control by virtue of the controlled alkali transformation, decreased gradually as the copper concentration increased to become almost nothing at the highest copper level. This more intense decrease in flavonol than in OC recovery was most noticeable and consistent. A contributing cause was undoubtedly the effect which the presence of increasing amounts of copper had on the pH attained when a fixed amount of alkali was added after the incubation to transform residual øY compounds. Thus the final pH would have been lower the higher the concentration of copper present and this would favour OC formation at the expense of flavonol formation in the transformation of øY compounds (Figure 31). An independent check of the validity of this lowering of pH was the greater persistence of Y_1 and Y_2 noted in the products of transformations at the higher copper concentrations. However, the pH attained was always adequately high to ensure efficient and complete transformation of the remaining øY compounds. An additional explanation for this strong and at times absolute suppression of flavonol formation in the course of transformation of øY compounds in the presence of

copper is considered in the Discussion.

A further experiment indicated that a product of the extensive copper-promoted destruction of ØY compounds demonstrated in the foregoing investigations was p-hydroxybenzaldehyde. Here the enzymic reaction was run in the presence of increasing amounts of added cupric ion (0.5 - 4.0 mM final concentration) to ensure an increasing amount of product destruction. The extent of this was again assessed by alkali transformation of the residual ØY compounds to OC which was isolated and estimated. Table 5 shows the results of spectrophotometric estimations of p-hydroxybenzaldehyde and OC recovered from the product chromatograms in relation to the concentration of cupric ion added to the reaction mixture. Details of the reaction mixtures are given with the Table. The increase in p-hydroxybenzaldehyde as the destruction of the ØY compounds increased (decrease in OC recovered) suggested this was one compound formed as a consequence of the copper-promoted destruction. The initially low level of flavonol recovered was completely suppressed when the concentration of added cupric ion exceeded 3 mM. Identification of p-hydroxybenzaldehyde rested on a comparison of u.v. spectral and chromatographic properties of eluted and reference samples.

(c) The possibility of dihydroflavonol being a flavonol precursor. Despite the findings described above on the origin of flavonol in the peroxidase-catalysed system, it remained of interest to investigate the possibility of a peroxidase-catalysed reaction of the dihydroflavonol, garbanzol, to also form flavonol under the conditions optimum for chalcone reaction. Thus, garbanzol was found not to

Table 5

Effect of addition of cupric ion to the enzymic reaction mixture on the chromatographic recoveries of OC and p-hydroxybenzaldehyde

Compound recovered (µg)	Final concentration of Cu^{2+} added (mM)					
	0	0.5	1.0	2.0	3.0	4.0
p-hydroxy-benzaldehyde	2.7	25.2	29.3	31.2	35.8	36.7
OC	239	178	155	99	76	59

The reaction mixture contained chalcone (1.8 µmoles), HRP enzyme (6 units), cupric ion (as 0.2 M $CuSO_4$, see above) and hydrogen peroxide (4 µmoles) in 4 ml total volume 0.05 M tris buffer pH 8.0. Incubation was for 15 minutes at room temperature (6 minutes for reaction with cupric ion omitted) in which time the chalcone consumption was complete. Alkali, (50 µl 2.5 N NaOH) was added and then 2 minutes later, acid (150 µl 1.44 N HCl), prior to ether extraction and 2-D chromatography of the products.

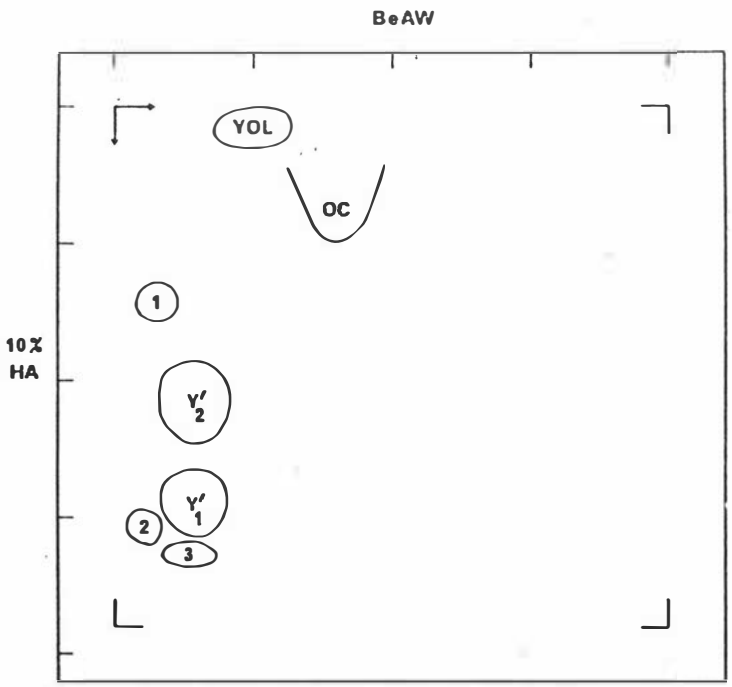
function as a hydrogen donor in the peroxidatic reaction at pH 8. The reaction mixture contained garbanzol (0.09 µmole), HRP enzyme (50 units) and hydrogen peroxide (0.5 µmole) in 2 ml total volume of 0.05 M tris buffer pH 8.0, temperature 25°. During the incubation time of several minutes, the spectrum of garbanzol in buffer (similar to that of the initial product of the chalcone reaction, Figure 17) remained unchanged. Extraction of the neutralised reaction mixture with ether recovered unchanged garbanzol as determined by subsequent 2-D chromatography. Addition of garbanzol to the chalcone reaction system was found to induce a lag phase in the reaction and cause a decrease in the maximum rate of chalcone consumption. The reaction mixture to which garbanzol was added contained chalcone (0.065 µmole), HRP enzyme (0.1 unit) and hydrogen peroxide (0.5 µmole) in 2 ml total volume of 0.05 tris buffer pH 8.0. Additions of garbanzol (prior to hydrogen peroxide) of 0.022, 0.044, 0.066 and 0.088 µmole reduced maximum reaction rate to 71, 55, 42 and 35% respectively of the control rate in the absence of garbanzol. Extraction of the reaction mixtures as before (duplicates combined prior to extraction) followed by 2-D chromatography of the products showed unchanged garbanzol to be present in all cases. Elution and u.v. absorbance measurement indicated between 50 and 65% of the initially added garbanzol was recovered. These results indicated garbanzol is not a precursor of flavonol in this peroxidase-catalysed system.

(d) Occurrence of additional flavonol-producing substances in trace amounts. Signs of the existence of precursors of flavonol other than the well-established ØY

compounds were repeatedly noted on re-inspection of 2-D chromatograms of enzyme reaction product after several days in storage. After this time traces of flavonol were detected in u.v. light by the appearance of characteristic fluorescence in up to three spots on the chromatogram, located as depicted in Figure 33. This delayed appearance was taken as evidence for the existence in the mixtures chromatographed of trace amounts of distinct new flavonol precursors. Elution and rechromatography demonstrated that flavonol had been definitely formed in each precursor spot. The amounts of flavonol formed and presumably therefore also of the precursors, were very low; detection was largely due to the intense fluorescence of flavonol which premitted ready location of less than 0.1 µg of the compound on a chromatogram. No specific study of the origin of these trace precursors of flavonol was made, but one or more of them were present after most extraction treatments which were applied to the reaction product. All were perhaps test detected on the one chromatogram after product extraction at pH 7. Spot 1 occurred even after extraction at pH 2-3 and appeared to be absent from many alkali-treated preparations. However, for the samples on which Figure 31 was based, it was noted that a definite increase in intensity of spot 2 occurred as the alkali concentration used in the transformation of ØY compounds increased and along with it the yield of flavonol. Rechromatography of spot 1 suggested that this precursor could give rise to that in spot 2, in addition to flavonol. Particularly spots 1 and 3 were also readily observed in the products when ØY compounds, isolated by solvent extraction, were then incubated

Figure 33

Location of trace flavonol-producing substances on standard 2-D chromatograms



Spots 1, 2 and 3 are the trace precursors of flavonol which were located after spontaneous formation of flavonol had occurred on the stored chromatograms. See figure 10 and Table 1 for explanation of the other symbols.

with added cupric ion in buffer pH 8 (see above), despite the mild alkali treatment prior to final extraction. The possibility that these three precursor substances were derived from ØY compounds is considered in the Discussion.

2C-4 Evidence for garbanzol formation in the enzymic reaction

The biosynthesis of garbanzol from chalcone in trace amounts with cell-free enzyme preparations from garbanzo seedlings has been well established (10,14,19). Since in the present work a peroxidase enzyme has been implicated in the formation of Y_1 and Y_2 which also occurred in the above cell-free preparations, it became of much interest to discover whether garbanzol formation too could possibly be accounted for by peroxidase catalysis.

The aim of this investigation was solely to obtain a result which would provide a qualitative answer. Two approaches were adopted to check the involvement of peroxidase in garbanzol formation. Firstly a check was made for garbanzol as a product in the normal reaction catalysed by HRP enzyme in the presence of hydrogen peroxide and secondly, the effect of specific inhibition of peroxidase activity in the garbanzo cell-free enzyme preparation was investigated. The results of both these lines of investigation supported garbanzol formation as a very minor product of peroxidase-catalysed reaction of chalcone and strongly suggested this enzyme was responsible for garbanzol formation previously recorded with cell-free enzyme systems.

The reaction mixture used to firstly check for garbanzol formation in the purified system contained chalcone-(carbonyl-^{14}C) (0.9 µmole), HRP enzyme (5 units) and hydrogen

peroxide (2 μmoles) in 4 ml total volume tris buffer, pH 7.5. In the control, enzyme in buffer was heated (15 minutes at 100°) and allowed to cool to room temperature before the other components were added. After the rapid reaction in the complete system at room temperature, the products were extracted directly into ether and chromatographed in 2-D on full-size papers, with carrier garbanzol (50 μg) present. Radioautography showed the clearly resolved garbanzol spot to be weakly active in the enzyme run. Counting of the eluted garbanzol on planchettes gave activities of 1270 and 450 cpm for the enzyme and control runs respectively. Since the specific activity of the labelled chalcone was 727 cpm/μg (23.5% counting efficiency) the yield of garbanzol was very low and the product would not be detected visually on chromatograms.

In the second experiment with garbanzo cell-free enzyme and no added hydrogen peroxide, typical of the conditions previously used to observe garbanzol formation, the inhibitors hydroquinone and diethyldithiocarbamate were used separately at 5 mM final concentration. The reaction mixture contained cell-free enzyme (4 ml), inhibitor (5 mM final concentration, in 0.1 ml) and radioactive chalcone-(3,5 - T_2) (0.9 μmole) in pH 7.5 tris buffer, 5 ml total volume. The inhibitor was mixed with the enzyme before addition of chalcone and the mixture was incubated for 20 minutes at 37°. Inhibitor was omitted from the controls with both active and heat-inactivated (15 minutes at 100°) enzyme. Products were ether extracted from the pH 7.5 buffer after removal of protein (see Experimental) and subjected to 2-D chromatography on full-size papers in

the presence of carrier garbanzol (50 µg) as previously. The garbanzol spot, clearly separated near the Y_2' spot position, was eluted and the activity determined by liquid scintillation counting. Total activity in the garbanzol spot was calculated as 3680, 155, 733 and 92 cpm respectively for active enzyme with no inhibitor, with hydroquinone inhibitor, with diethyldithiocarbamate inhibitor, and boiled enzyme with no inhibitor. Specific activity of the chalcone was 5.13×10^3 cpm/µg and the counting efficiency 30%.

2D CHARACTERISATION STUDIES ON ϕY_1, ϕY_2, OC and ϕOC

The studies reported in the preceding parts contributed to the development of a general understanding of the requirements for the enzymic reaction of chalcone and for the isolation of major products either as the precursors ϕY_1 and ϕY_2 or as the derived OC. Emphasis was then diverted from biochemical to chemical aspects of the reaction to provide information on the structures of the new compounds previously detected on preliminary mapping of the assortment of reaction products. This approach was also expected to provide data invaluable in the interpretation of the biochemistry of the reaction and in the elucidition of interrelationships between the range of compounds formed during or subsequent to the enzymic reaction. In the present part, therefore, results of efforts to prepare compounds for characterisation are recorded followed by the findings on the properties of these compounds from which proposed structures have been advanced.

2D-1 Preparation and purification of individual compounds

(a) OC preparation and purification. The preliminary

preparative method of OC production (section 2A-7) was developed, of necessity, before biochemical and spectrophotometric studies had been conducted to reveal the oxygen requirement for the reaction and the existence of ØY compounds as alkali-labile precursors of OC. Improved yields of OC were therefore sought not only by bubbling air through the reaction mixture in the course of incubation but also by controlled addition of alkali immediately the chalcone had been consumed in order to maximise the efficiency of conversion of ØY compounds to OC. Acidification with HC1,2 minutes after this addition of alkali, preceded recovery of the OC product by ether extraction. For added convenience, HRP enzyme was now used exclusively in the preparative scale reactions (see Experimental). With this improved method, yields of OC, measured spectrophotometrically on the ether-extracted product, were 85 - 90% of the chalcone consumed compared with 70 - 76% for the preliminary method.

It was difficult to purify OC by conventional recrystallisation because of the instability of the compound. The OC solid obtained from the preparative reaction was found to be best purified with the maximum recovery (about 60%) by precipitation from acetone solution which was being concentrated under an air jet (see Experimental). Solids deposited were always in the form of fine angular yellow particles, not crystals. Melting was not sharp; for the spectroscopic sample (see section 2A-7) it began at 223° and was virtually complete at 234°.

(b) Preparation and purification of ØY compounds.

Application of the technique which had been previously

developed for small-scale samples of extracting ØY compounds without delay after the reaction buffer had been acidified to pH 2 - 3 (section 2A-9) was successful with preparative-scale runs. Thus the reaction was run as in OC production above, except that acid and not alkali was added immediately the reaction was over and the product, preserved as ØY compounds, was immediately extracted into ether. Yields of ØY compounds, measured as OC after controlled alkali addition to the spectrophotometric samples, matched those reported above for direct isolation of OC from the same reaction system.

The high instability of the ØY compounds meant that an appreciable amount of development work was required before satisfactory preparations of the individual compounds could be obtained from the reaction product mixture. Eventually a paper chromatographic separation using 0.5% acetic acid (HA) solvent at low temperature (about 4^{o}), coupled with specific techniques of recovery of the compounds, was developed for the isolation of mg amounts of $ØY_1$ and $ØY_2$ in acceptable yield and at quite high purity, considering the nature of the compounds (see Experimental). Acetone was used to elute the compounds from the strips of still-wet chromatography paper. Drying of the chromatograms before elution was avoided since it resulted in partial degradation of the ØY compounds and impure preparations. The organic solvent was removed at 0^{o} from the acetone eluate and the small amount of aqueous acidic solution (wet chromatography paper eluted) which remained was bulked up with distilled water if necessary and was twice extracted with an equal volume of ether. The total ether extract was

evaporated around 0^{o} to provide the recovered compound. The 0.5% HA chromatographic solvent was selected not for any feature of the separation of the ØY compounds achieved, but to supply automatically from the moist chromatography paper eluted, small amounts of acid found to be vital for the preservation of the ØY compounds throughout the separation and recovery processes. Inadequate acid led not only to appreciable spontaneous OC formation but also to general breakdown of the ØY compounds and sample contamination as the eluate was manipulated and the recovered sample submitted to further investigation. By this method, $ØY_2$ was prepared as a whitish solid apparently finely crystalline, which slowly developed a definite yellow coloration on standing at 0^{o}. However, it kept well at -10^{o}. For $ØY_1$ on the other hand, a slightly yellow, glassy solid was always obtained and crystallisation never developed.

(c) Preparation and purification of ØOC. A method for the preparation of ØOC (see Experimental) was discovered when a solution of a reaction product mixture of $ØY_1$ and $ØY_2$ in aqueous acetone or ethanol was concentrated under an air jet and placed in ice for a few hours. The white solid which precipitated was a somewhat impure sample of ØOC. Yields of ØOC from this procedure were low, being no more than 12% of that amount of OC equivalent to the quantity of ØY compounds taken. In earlier work (section 2A-12) µg amounts of the same compound, a precursor of OC, were detected spectrophotometrically following 1-D chromatography in BeAW of a suitably pre-treated sample of ØY compounds. A problem associated with attempted purification of ØOC by repeated deposition from hot solvent was the tendency,

sometimes very marked, for the compound to be converted largely to OC. Evidence that ØOC was formed from both $ØY_1$ and $ØY_2$ was obtained when chromatographically isolated samples of these compounds, which had been held for some time in storage of 0°, were chromatographed in BeAW and found to contain small amounts of ØOC.

2D-2 Studies on $ØY_1$ and $ØY_2$

(a) Detection and resolution of two forms of $ØY_2$.

The existence of $ØY_2$ in two chromatographically distinct forms was first observed when a reaction product mixture was chromatographed in 1-D in 5% HA on a full-length chromatogram. With a low loading of ØY compounds, equivalent to about 100 µg of OC, applied as a short band (2 cm), this chromatography resolved the normally somewhat elongated, uniformly dense, $ØY_2$ spot into two distinct but partially overlapping zones. Heavier loadings obscured evidence of this separation. Evidence was subsequently obtained (see below) that both chromatographic components of $ØY_2$ exhibited identical chemical and spectral properties. These findings indicated that stereochemical modifications of the $ØY_2$ compound had been chromatographically resolved, a conclusion which was further supported by optical activity measurements. The form of the compound in the more mobile zone was designated $ØY_2a$ and the other $ØY_2b$. Complete separation of these two forms was achieved in two ways. Firstly, a reaction product mixture of ØY compounds, equivalent to 250 µg of OC, was run on a full-size 2-D chromatogram developed with the 5% HA solvent in both directions. On this chromatogram, the $ØY_2a$ and $ØY_2b$ forms appeared as contiguous spots. Secondly,

full-length 1-D chromatography of further portions of the same mixture, applied as separate 2 cm long bands, in 0.5% HA solvent at 4° for 16 hours (solvent had run off for at least 10 hours), also effectively separated the two forms. No evidence of any separation of the $\not{\alpha}Y_1$ component into two forms was ever observed.

(b) Optical activity of the $\not{\alpha}Y$ compounds. While not only the mixture of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ which constituted the enzymic reaction product but also $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ as generally isolated were found to be virtually optically inactive, the $\not{\alpha}Y_2$a and $\not{\alpha}Y_2$b forms of $\not{\alpha}Y_2$ were however optically active, the former being dextrorotatory and the latter levorotatory at the wavelength of the sodium D line. Transformation of these forms to OC with alkali resulted in the loss of this optical activity.

Preparative chromatography of the reaction product in 0.5% HA (section 2D-1(b)) separated $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ as expected, but in the band of the latter compound the two component forms were not visually resolved on account of the high loading. However, some resolution had occurred, since the band on being halved into higher and lower R_f portions, provided separate eluates which were found to possess optical activity indicative of an enriched $\not{\alpha}Y_2$a and $\not{\alpha}Y_2$b content respectively. Repeated preparative chromatography of each of these eluates gradually enhanced the optical purity of the predominant form in the remaining sample as indicated in Table 6. The minor quantities of the other form which separated on rechromatography were always discarded on account of diminished chemical purity. Limitations were imposed on the extent of this chromatographic purification

Table 6

Optical activity of ϕY_2

Specific rotations, $[\alpha]_D$, with progressive chromatographic resolution of ϕY_2a and ϕY_2b forms

Compound and form	Specific rotation $[\alpha]_D$ after chromatographic purification step			
	1	2	3	4
ϕY_2a	(+) 72°	(+) 170°	(+) 192°	(+) 241°
ϕY_2b	(-) 143°	(-) 195°	(-) 224°	-

Enzymic reaction product constituted the mixture originally chromatographed in 0.5% HA. See text for details of the origin of ϕY_2a and ϕY_2b samples. In the second and third purifications, chromatograms were run for 5 hours (full-length development, no solvent run-off) but in the fourth purification, development was extended to 16 hours to remove the last of the ϕY_2b form from the ϕY_2a preparation. Measurement of the quantity of ϕY compound was based on the OC content of the sample, determined spectrophotometrically upon alkali transformation (see Experimental).

by the losses of compound in each cycle and by the difficulty in avoiding an increase in impurities which co-chromatographed more particularly with the ϕY_2a form. Analytical 1-D chromatography indicated that the final samples of ϕY_2a and ϕY_2b forms were each essentially free of the other form. Thus the finally observed specific rotations were assumed to represent those for the optically pure forms, within the limits of chemical purity imposed by the nature of the compound. These results, together with the chemical and spectral evidence (see below) thus characterised ϕY_2a and ϕY_2b as enantiomers of ϕY_2.

Elution of the band of ϕY_1 obtained on initial chromatography of reaction product, either in its entirety or in separate halves on an R_f basis, as in the case of ϕY_2 compound, failed to uncover significant optical activity in the compound. Successful rechromatography was effictively prevented by the ready occurrence of impurities which co-chromatographed with ϕY_1.

(c) Absence of interconversion of ϕY compounds.

Preliminary work indicated that ϕY_1 and ϕY_2 did not interconvert in the course of elution and rechromatography in 5% HA solvent. Of equal interest was the possibility of interconversion of the compounds in 0.05 M tris buffer pH 8.0; that is, under enzymic reaction conditions. Samples of ϕY_1 and of ϕY_2 (as the ϕY_2a form) were dissolved in this buffer at room temperature and 4 ml samples, which contained initially ϕY compound equivalent to about 200 μg of OC, were withdrawn at various intervals up to 30 minutes later, acidified to pH 2 - 3 and ether extracted in preparation for full-length 1-D chromatography in 5% HA. This

developed chromatogram, when dry, showed no evidence of any interconversion of ϕY_1 and ϕY_2 or of $\phi Y_2 a$ to the $\phi Y_2 b$ form. The effect of this incubation of ϕY compounds in pH 8 buffer had been to promote the gradual conversion to a number of compounds including the known product OC. In the absence of any detectable interconversion therefore, ϕY_1, $\phi Y_2 a$ and $\phi Y_2 b$ can all be considered equally likely products of the enzymic reaction.

(d) Proportions of ϕY compounds formed in the enzymic reaction. Using 1-D chromatography in 0.5% HA and all of the techniques required to ensure recovery of the compounds at the best possible purity, the proportions of ϕY compounds were determined in the products of a series of enzymic reactions (Table 7). Each sample of product was twice chromatographed at 4^{o}; on one occasion for 5 hours to separate ϕY_1 and ϕY_2 and on the other for 16 hours to separate the latter compound into its $\phi Y_2 a$ and $\phi Y_2 b$ forms. In this separation, ϕY_1 was largely lost in the solvent run-off. Compounds were quickly visualised on the wet chromatograms by minimal u.v. irradiation (to develop a blue fluorescence in the location of the originally invisible spots - see Experimental) and the marked spot areas were excised immediately and eluted for 30 minutes in standard volumes of 85% ethanol. Relative quantities of the compounds were obtained directly from the absorbance readings at 275 nm, the common wavelength of the absorption maximum. This was possible since $E^{1\%}_{1cm}$ values at this wavelength were all practically the same (see Experimental) on the basis of spectrophotometric measurement of the absorbance of each ϕY compound at 275 nm and of the derived OC at 325 nm.

Table 7

Proportions of ØY compounds in enzymic reaction product

No.	Reaction pH	Added chalcone (µmoles)	Enzyme	Proportions in Products	
				$\frac{ØY_1}{ØY_2}$	$\frac{ØY_2 \text{ a form}}{ØY_2 \text{ b form}}$
1	8.0	1.8	HRP	0.85	1.30
2	8.0	1.8	G[×]	0.81	1.30
3	7.0	1.8	HRP	0.80	0.92
4	7.0	1.8	G	0.60	0.94
5	8.0	3.6	HRP	0.81	1.12
6	8.0	3.6	G	0.60	1.13
7	7.0	3.6	HRP	1.00	0.93
8	7.0	3.6	G	0.69	0.93

× Garbanzo enzyme

Reaction mixtures contained, in a total volume of 4 ml of 0.05 M tris buffer (pH as above), chalcone (given above), enzyme, (HRP 34 units, garbanzo 66 units) and H_2O_2 (2 µmoles with HRP and 1 µ mole with garbanzo enzyme). Reaction time was 3 minutes at room temperature and the higher chalcone concentration was aerated during this time. Products were extracted with ether after acidification of the reaction medium to pH 2-3. Appreciable residual chalcone was recovered from runs number 7 and 8.

These results showed that while some variation was possible, there was no great preponderance of either $\not{\alpha}Y_1$ or $\not{\alpha}Y_2$ in the reaction product under generally typical reaction conditions. However, it was apparently usual for a small but definite excess of $\not{\alpha}Y_2$ to be found. For $\not{\alpha}Y_2$, the two forms $\not{\alpha}Y_2a$ and $\not{\alpha}Y_2b$ were quite closely balanced, with reaction conditions, chiefly pH, influencing the preponderance of one or other of these enantiomers.

(e) Rates of transformation of $\not{\alpha}Y$ compounds to OC under alkaline conditions. In the pH 9 - 11 range where OC was the only significant transformation product, the rates of transformation of $\not{\alpha}Y$ compounds could be conveniently determined spectrophotometrically by monitoring the appearance of OC anion at 375 nm and 380 nm at pH 8.9 and pH about 11, respectively (Table 8). The rates of OC formation were thus greater for $\not{\alpha}Y_1$ than for $\not{\alpha}Y_2$ and both forms of the latter displayed similar rates. Below pH 8.9 the $\not{\alpha}Y$ compounds not only transformed more slowly but also gave rise to extraneous products in significant quantities which distorted the u.v. spectrum of the transformation product. Above pH 11, the rate of transformation increased rapidly and at pH 12 was too fast to measure. In addition, at pH 12 significant amounts of flavonol were formed particularly from the $\not{\alpha}Y_1$ sample and the rate of product appearance could no longer be monitored meaningfully at any one wavelength.

(f) Product distribution from separate $\not{\alpha}Y_2$ forms treated with alkali. For $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$, the comparative distribution of product between OC and flavonol as a function of the alkali concentration at which the transformation

Table 8

Maximum rates of formation of OC from ØY compounds in alkaline buffers

Buffer medium	Rates of OC formation (µg/min) from:		
	$ØY_1$	$ØY_2a$	$ØY_2b$
0.05 M tris pH 8.9	4.20	3.44	3.56
0.05 M tris base pH 11 approx.	16.20	11.40	11.70

The ØY compound, in 50µl EtOH, was rapidly mixed with 1.95 ml of buffer in a cuvette at room temperature and the time course of OC anion formation was monitored at 375 nm (pH 8.9) or 380 nm (pH 11). The above sample of each ØY compound generated 27µg of OC on controlled transformation with alkali in EtOH. Progress curves recorded were sigmoidal and maximum rates were used in calculating the values recorded in the Table. For the purpose of this calculation, the $E^{1\%}_{1cm}$ value of OC anion was determined as 1.1×10^3 at the monitored wavelength in each buffer. Samples of $ØY_2a$ and $ØY_2b$ were prepared directly from enzyme reaction product by chromatography in 0.5% HA for 17 hours at 4^o.

occurred has already been established (Figure 32). Thus the reaction of these two compounds, though qualitatively similar, was more facile for $\not{o}Y_1$.

With the discovery and availability of separate $\not{o}Y_2a$ and $\not{o}Y_2b$ forms of $\not{o}Y_2$, the products of transformation of these separate forms at low (0.003N) and at high (0.05 N) alkali concentrations were determined and compared with the results similarly obtained for $\not{o}Y_1$ (Table 9). At low alkali concentration the product distributions from both forms of $\not{o}Y_2$ were similar, yet distinctly different from that for $\not{o}Y_1$, in agreement with the result of the previous comparison of $\not{o}Y_1$ and $\not{o}Y_2$ (Figure 32). Predictably, at high alkali concentration, the product pattern was common for all three precursor preparations. Thus, the $\not{o}Y_2a$ and $\not{o}Y_2b$ forms, in addition to displaying similar rates of formation of OC at lower pH (see above), were also found to give rise to similar balances in the competing products, OC and flavonol, upon transformation at low alkali concentration.

(g) Copper-promoted destruction of $\not{o}Y$ compounds in buffer pH 8. In the course of biochemical studies, copper was found to not only inhibit the enzymic reaction (Table 4) but also to function as an apparently specific inhibitor of flavonol formation (section 2C-3(b)). However, this virtuall; complete flavonol inhibition was accompanied by much reduced recoveries of OC. Subsequently it was shown that the reductions in both flavonol and OC recoveries were linked effects caused by the copper-promoted destruction of $\not{o}Y$ compounds in a chemical reaction. It was further apparent from some results that this destruction affected both $\not{o}Y_1$ and $\not{o}Y_2$ (see section 2C-3(b) for details). In the course

Table 9

Distribution of product between OC and flavonol on transformation of ØY compounds with low and high concentrations of alkali

Alkali concentration	% Yield × of products (OC : flavonol)		
	$ØY_1$	$ØY_2a$	$ØY_2b$
0.003 N	54 : 15	63 : 3	59 : 4
0.5 N	5 : 64	4 : 59	4 : 63

× Yields were calculated from the quantities recovered after 1-D chromatographic separation of the product mixture, expressed as a percentage of the spectrophotometrically measured OC content of the ØY sample transformed; (250, 210, and 240 µg of OC for the samples of $ØY_1$ and $ØY_2a$ and $ØY_2b$ forms of $ØY_2$ respectively). Samples were treated as outlined in Figure 31.

of this work it was also concluded that one product of the copper-promoted destruction of ØY compounds was p-hydroxybenzaldehyoe.

(h) Attempted methylation of ØY compounds. Attempted methylation of the ØY compounds with diazomethane at 0^{o} was unsuccessful since the compounds appeared to be unstable to the treatment.

2D-3 Some reactions of OC

Attempts were made to prepare a derivative of OC which would be considerably more stable than the parent compound and which could therefore be used to advantage in characterisation studies.

(a) Attempted formation of acetyl derivative. Acetylation of OC in acetic anhydride containing a drop of pyridine, overnight at room temperature was not successful on account of difficulties encountered in working up the product and the low yield of crystalline material (not characterised) finally obtained.

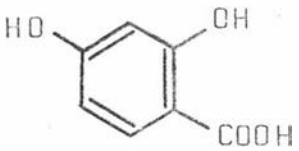
(b) Preparation of stable methyl ether. Methylation with diazomethane at 0^{o}, however, was successful and a stable methyl ether of OC was formed. The crystalline product was obtained in 83% yield in a typical preparation from 90 mg of OC and further purified by recrystallisation from acetone-ethanol as fine light-yellow needles. NMR and mass spectra (see section 2D-6) indicated that the compound was a mono-methyl ether of OC. TLC of the original crystalline preparation on silica gel layers in the solvent system benzene-absolute ethanol, 10:1, revealed only one spot, brown-purple in u.v. light, colour unaffected on fuming

with ammonia, R_f 0.48, compared with OC, R_f 0.35. Charring the sulphuric acid-sprayed plate revealed only the same spot. The purified crystals melted about 210-220° on rapid heating only; with normal rates of heating they gradually gave rise, between 205-225°, to a sublimate of colourless needles and some charred material. The colourless needles melted at 270-273°. These same needles could also be observed to form if the melt obtained by rapid heating of OC-methyl ether through 220° was held at 220-230° for a few minutes.

(c) Hydrogenolysis of OC and OC-methyl ether.

Catalytic reduction of OC in 95% ethanol over a 10% palladium on charcoal catalyst (see Experimental) was found to proceed very rapidly and to result in very extensive hydrogenolysis of the compound. Uptake of the first mole equivalent of hydrogen occurred in the first three minutes. After the uptake of at least a further mole equivalent of hydrogen in the next few minutes, examination of the product by 2-D chromatography revealed numerous compounds, all quantitatively minor, and no residual OC. Among the most significant product quantitatively was hydroquinone, which occurred in about 11% yield. Other products mainly exhibited chromatographic properties and colour reactions with diazotised sulphanilic acid typical of low molecular weight phenolic compounds. Small amounts of 2,4-dihydroxybenzoic acid were detected, for example.

Hydroquinone

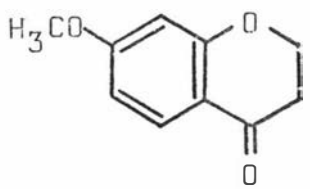


2,4-dihyroxybenzoic acid

On account of this extensive degradation of OC, a second sample was hydrogenated until only one mole equivalent of hydrogen had been taken up and the catalyst was quickly filtered off. The product, on 2-D chromatographic examination, showed that hydrogenolysis had been again extensive, despite the presence of considerable unchanged OC. With this result, no further attempt was made to achieve controlled catalytic reduction of the OC molecule.

When OC-methyl ether was hydrogenated in a similar system till in excess of one mole equivalent of hydrogen had been taken up, the product showed, on TLC on silica gel layers (developed with benzene-absolute ethanol, 10:1) that hydrogenolysis had again occurred. From a portion of the product mixture, small samples of two of the most prevalent compounds were separated by preparative TLC, using the above system. The first compound, which was the slowest running component in the above solvent system, was isolated as colourless needles and was identified as hydroquinone. The second, isolated as an oil, was the fastest moving component, R_f 0.9 approximately, and appeared on the layer as a light, bright blue, band in u.v. light This compound displayed

prominent u.v. absorption maxima in 85% ethanol at 230, 270 and 315 nm and the spectrum was unchanged upon addition of alkali (0.003 N final concentration). Mass spectrometry revealed an empirical formula $C_{10}H_{10}O_3$ for the molecular ion at m/e 178 and indicated the compound was possibly 7-methoxychroman-4-one.



7-methoxychroman-4-one

(d) Hydroquinone as a common degradation product of OC. A number of examples of the formation of small amounts of hydroquinone in the course of manipulation of OC were noted. For example, the compound appeared in OC which had been recovered from chromatograms run in 10% HA. It also appeared to be formed during chromatography of OC on polyamide columns eluted with aqueous ethanol.

2D-4 Further observations on ØOC

The observed behaviour of purified ØOC under various experimental conditions (essentially pH effects) was as follows. Alkali treatment was shown to transform ØOC rapidly and efficiently to OC in both u.v. spectral and chromatographic studies. The latter indicated that flavonol was not formed from ØOC. Thus, on addition of alkali (NaOH, final concentration 0.003 N) to a solution of ØOC in 85%

ethanol in a cuvette, the rapid formation of OC anion was observed. Transformation of ØOC to OC proceeded slowly in 0.05 M tris buffer, pH 8.9. At a lower alkaline pH, other reactions became significant and their products distorted the OC anion spectrum on further addition of alkali. To check chromatographically on the product of transformation, ØOC (equivalent to 130 µg OC in 50 µl of ethanol) was added to 4ml of sodium hydroxide, concentrations 0.006, 0.03 and 0.125 N. After 45 seconds, each mixture was acidified and the product extracted into ether. Chromatography in BeAW showed the product was OC in all cases and that no flavonol had been formed.

The ready conversion of ØOC to OC was experienced on a number of occasions when the compound was being manipulated in solution in acetone or alcohol. In some cases most of the sample was converted, especially if the solution was heated in an attempt to increase solubility.

Chromatography of ØOC in BeAW could be carried out and much of the compound recovered unchanged if the entire process was performed with a minimum delay and only partial drying of the chromatogram. Mobility in BeAW was slighly greater than that of OC (see Figure 21). On chromatography in 5% HA the compound barely moved from the origin. When a sample was submitted to 2-D chromatography in the above solvent pair, with 30 minutes drying time between solvents, a weak streak extended to about R_f 0.6 from the ØOC spot in the final 5% HA solvent. Within this streak, some weak spots were evident, including OC. The streak was thought to represent breakdown of ØOC after first direction chromatography.

2D-5 Trimethylsilyl derivatives of ØY compounds, OC and flavonol

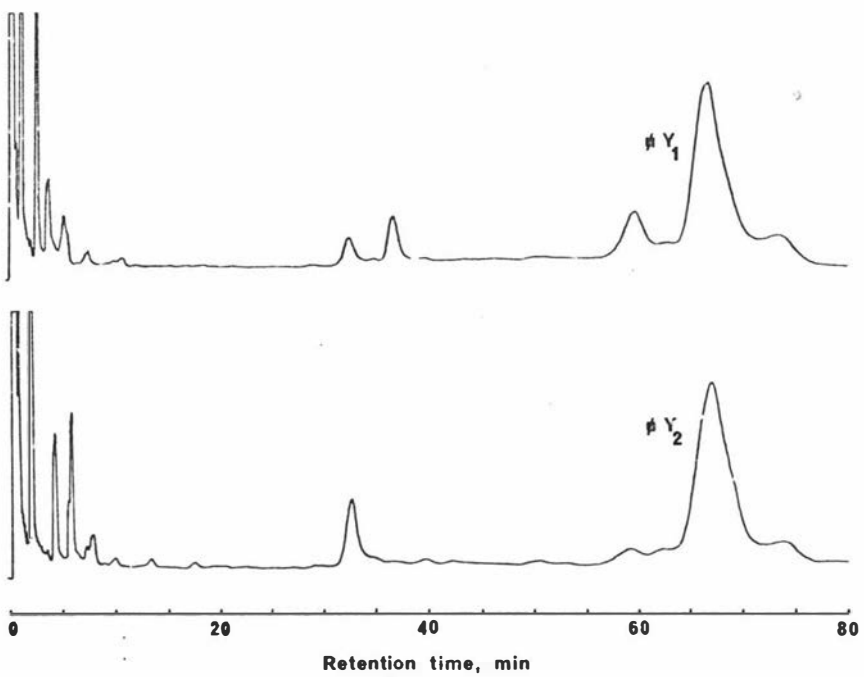
An attempt was made to stabilise the ØY compounds by the preparation of the volatile trimethylsilyl (TMS) ethers and thereby facilitate characterisation of these unstable compounds by mass spectrometry. Solid samples of the separate ØY compounds were treated with the silylating reagent, N,O,-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and after the reaction had occurred, as indicated by solution of the solid, the solution was analysed for products by GLC (see Experimental). One major and several minor peaks were present on the chromatograms of the reaction product for each ØY compound (Figure 34). The major peak, if not a mixture, indicated therefore that one predominant TMS-ether product had been formed from each ØY compound.

OC and flavonol were also separately treated with BSTFA and the resulting products analysed by GLC. Again, one major TMS-ether product was indicated in each case and the chromatograms, by the absence of additional minor peaks (except those from the reagent), indicated that, in contrast to the results with the ØY compounds, clean reactions had occurred.

Further GLC in which mixtures of the TMS derivatives of the different compounds were examined under the previous conditions (Figure 34), revealed that the major TMS-ether product from each of the ØY compounds was chromatographically indistinguishable from that of flavonol. However, the product from OC had a slightly lower relative retention time and was reasonably clearly resolved from these other TMS-ethers. In Figure 34, its presence in small amount,

Figure 34

Gas-liquid chromatograms of the products of reaction of ϕY_1 and ϕY_2 with the silylating reagent BSTFA[x].



[x] BSTFA - N,O-bis-(trimethylsilyl)-trifluoroacetamide

The glass column, 4mm internal diameter and length 2 metres was packed with 80-100 mesh Gas Chrom Q which was coated with 10% SE-30 stationary phase. The N_2 carrier gas flow rate was 50 ml/min. Column temperature was programmed to rise from an initial 200° at sample application to 240° at a rate of 1° per min.

especially in the $\not{\alpha}Y_2$- derived product, may be indicated in the small peak which emerged immediately prior to the main one.

A sample of the major TMS-ether product formed from each of the four compounds above was collected in a glass capillary, which was then immediately sealed, by sampling the effluent as the main part of the peak of interest was coming off a GLC column (see Experimental). These samples were then examined by mass spectrometry which indicated further that the main TMS product from each $\not{\alpha}Y$ compound was identical with the TMS derivative of flavonol.

2D-6 Physical properties of compounds characterised

(a) The pK'_a of $\not{\alpha}Y$ compounds, OC and flavonol.

Values of pk'_a were determined for the above compounds in 0.05 M tris buffers, from spectrophotometric data and are presented in Table 10.

(b) U.v. absorption spectra of $\not{\alpha}Y$ compounds, OC, $\not{\alpha}$OC and OC-methyl ether. The u.v. absorption spectra of solutions of these compounds in 85% ethanol are presented in the Figures listed below. Further, the spectra observed on addition of solid sodium acetate and of alkali (NaOH, final concentration approximately 0.003 N) to the ethanolic solutions of the compounds are presented. Neither boric acid nor aluminium chloride proved useful diagnostic reagents with any of these compounds.

Similar spectra were observed for chromatographically prepared samples of $\not{\alpha}Y_1$ (Figure 35) and of both forms of $\not{\alpha}Y_2$; $\not{\alpha}Y_2$a (Figure 36) and $\not{\alpha}Y_2$b (Figure 37). Comparable spectra were also recorded, for the solvent-extracted enzyme

Table 10

pK'_a of ØY compounds, OC, ØOC and flavonol

Compound	$ØY_1$	$ØY_2a$	$ØY_2b$	$ØY_1 + ØY_2^x$	OC	ØOC	flavonol
pK'_a	6.45	6.65	6.60	6.45	6.58	6.39	7.14

[x] Solvent-extracted enzyme product mixture was used.

product mixture prior to chromatography and separation of the ØY compounds. Spectra in sodium acetate were recorded immediately after the solution was quickly shaken with a small excess of solid, since the compounds were unstable in this medium. The spectra recorded on addition of alkali are of OC anion which appeared rapidly as a result of the induced transformation of the compounds. This stable spectrum was recorded a few minutes after the alkali had been added and when transformation was complete, as indicated by no further increase in absorbance at 380 nm. In this transformation, an intermediate absorption with maximum similar to that recorded on the addition of sodium acetate, was noted on rapid manual scanning of the appropriate wavelength region, immediately alkali was added. However, the initial rate of transformation was such that the intermediate spectrum (presumably that of ionised ØY compound) could not be recorded.

Spectra are presented in Figure 38 for OC and in Figure 39 for OC-methyl ether. For OC, spectra in sodium acetate and in alkali were stable. No significant spectral change occurred on addition of sodium acetate to OC-methyl ether and therefore the spectrum is omitted from Figure 39. Addition of alkali however, caused very rapid degradation of the sample; the spectrum shown was recorded without delay and was still changing somewhat.

The spectrum of ØOC is recorded in Figure 40. On addition of alkali, the compound was rapidly transformed into OC anion by way of an intermediate (probably ionised ØOC), fleeting evidence of which was noted by the transient absorption peak at about 340 nm.

(c) I.r. spectra of ØY compounds, OC, OC-methyl ether and ØOC. The i.r. spectra (KBr disc) of chromatographically isolated samples of $ØY_1$ and $ØY_2$ (the latter as $ØY_2a$ and $ØY_2b$ forms) are presented in Figures 41 and 42. A spectrum almost identical to those in Figure 42 was recorded for the sample of $ØY_2$ which aggregated in granular form within an enzyme reaction product mixture of ØY compounds on overnight storage and which was subsequently recovered without recourse to chromatography (see Experimental). This granular solid provided the most directly isolated sample of $ØY_2$. A sharper spectrum of $ØY_1$ could not be obtained. After the spectrum of each ØY sample had been recorded, a portion of the KBr disc was immediately eluted in 85% ethanol and the purity of the recovered compound checked spectrophotometrically. A detectable drop in purity had invariably occurred during the handling for i.r. spectroscopy, but in all cases the i.r. spectrum recorded was that of a sample which had remained largely in its designated form.

Spectra (KBr disc) of OC, OC-methyl ether and ØOC are reproduced in Figures 43, 44 and 45 respectively.

(d) Mass spectra. The mass spectrum of OC is depicted in Figure 46; ions of very low relative intensities have been omitted. Mass spectral data which are presented in Figure 47 and Table 11 showed that OC-methyl ether is a mono-methyl ether of OC. Empirical formulae determined by mass measurement of the prominent respective molecular ions were OC, $C_{15}H_{10}O_5$ and OC-methyl ether, $C_{16}H_{12}O_5$. A stronger spectrum, more suited to mass measurement was obtained from OC-methyl ether than from OC. A small quantity of a dimethyl-eher product (m/e 298) was noted in the spectrum

Table 11

Empirical formulae of ions in mass spectrum of OC-methyl ether

ion m/e	Relative Abundance	Measured Mass	Exact Mass	Empirical formula
298[x]	4.1	298.0847	298.0841	$C_{17}H_{14}O_5$
284[xx]	100.0	284.0675	284.0685	$C_{16}H_{12}O_5$
267	4.1	267.0652	267.0657	$C_{16}H_{11}O_4$
255	3.5	255.0676	255.0657	$C_{15}H_{11}O_4$
241	2.4	241.0508	241.0501	$C_{14}H_9O_4$
227	1.8	227.0712	227.0708	$C_{14}H_{11}O_3$
191	71.0	191.0348	191.0344	$C_{10}H_7O_4$
151	8.8	151.0406	151.0395	$C_8H_7C_3$
135	9.4	135.0441	135.0446	$C_8H_7O_2$
134	21.2	134.0365	134.0368	$C_8H_6O_2$
121	14.1	121.0289	121.0290	$C_7H_5O_2$
119	21.8	119.0497	119.0497	C_8H_7O
110	51.7	110.0377	110.0368	$C_6H_6O_2$
94	39.4	94.0422	94.0419	C_6H_6O

[xx] Molecular ion

[x] Impurity, dimethyl ether

of OC-methyl ether.

The spectrum recorded for ØOC (Figure 48) changed with time and became more like that of OC, indicating isomerisation. The sample was also somewhat impure as indicated by the large number of weak ions (not shown in Figure 48) up to about m/e 500.

Spectra recorded for the main TMS-ether product prepared from $ØY_1$, $ØY_2$, flavonol and OC and isolated by GLC (see section 2D-5) are recorded in Figures 49, 50, 51 and 52.

(e) NMR spectra. The NMR spectrum of OC in hexadeuteroacetone, in which all the proton signals were located downfield in the aromatic region between δ6.7 and 7.9, is shown in Figure 53. The spectrum in Figure 54, which was recorded after the addition of deuterium oxide, has been integrated.

The aromatic region of the spectrum of OC-methyl ether is shown in Figure 55. Limited solubility of the compound in acetone restricted the intensity of this spectrum. The only observed signal not shown in Figure 55 was a singlet at δ4.02 for which the integral was one-third approximately of that for the entire aromatic region. This integral is in agreement with the finding by mass spectrometry that the compound is a mono-methyl ether of OC.

The spectrum of ØOC showed the presence of some impurity but was mainly complicated by the transformation of the sample to OC which occurred in the course of manipulation. This conversion had reached significant proportions before a spectrum was recorded and was virtually complete, as revealed by NMR and subsequent u.v. spectra, before any

expanded-scale NMR spectrum of the aromatic region could be obtained. However, the initial spectrum recorded, sweep width 0 - 500 Hz downfield from TMS, revealed two doublets (δ5.16, J=6.3 Hz and δ3.96, J=6.3 Hz) upfield of the complex pattern in the aromatic region. The aromatic region was especially complicated by the presence of an appreciable amount of OC in the sample since the spectra of ØOC and OC, though similar, did not coincide.

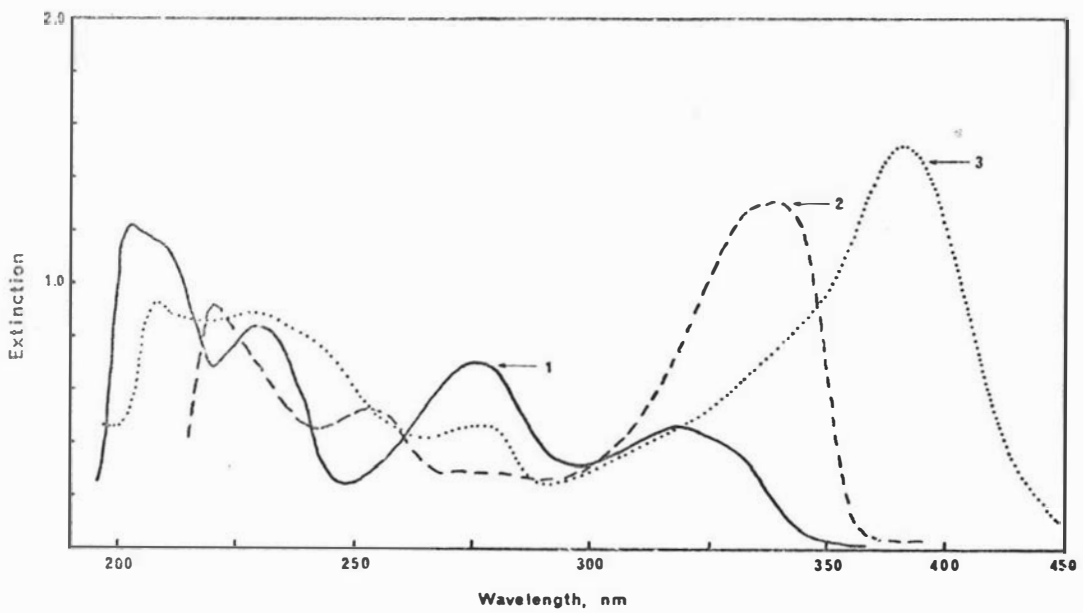
While the above spectra were recorded at room temperature the spectra for ØY compounds were determined with the samples cooled to 0^{o}. Results are presented in Figures 56 ($ØY_1$), 57, 58 ($ØY_2$a form of $ØY_2$), 59 and 60 ($ØY_2$b form of $ØY_2$). In addition to the signals in the aromatic region, the spectra of these compounds characteristically displayed an upfield pair of doublets.

2D-7 Proposed structures for OC, ØOC and ØY compounds

The data presented above are consistent (see Discussion) with the formulation of these compounds as the structures presented in Figure 61. Thus, OC is identified as 8-hydroxybenzo (2,1-b)-4-hydroxy-2H-oxepin-5-one-2-spiro-1'-cyclohexa-2',5'-dien-4'-one, and ØOC as the α-diketone tautomer of this enol. OC-methyl ether is the 8-O-mono-methyl ether of OC. $ØY_1$ and $ØY_2$ are formulated as diastereoisomers of 8-hydroxybenzo (2,1-b)-3,4-dihydroxy-2H-oxepin-5-one-2-spiro-1'-cyclohexa-2',5'-dien-4'-one. $ØY_2$a and $ØY_2$b differ from one another in being enantiomers of one of these diastereoisomers and $ØY_1$ is a mixture of the enantiomers of the other diastereoisomer.

Figure 35

U.v. absorption spectra for $\not{\alpha}Y_1$.



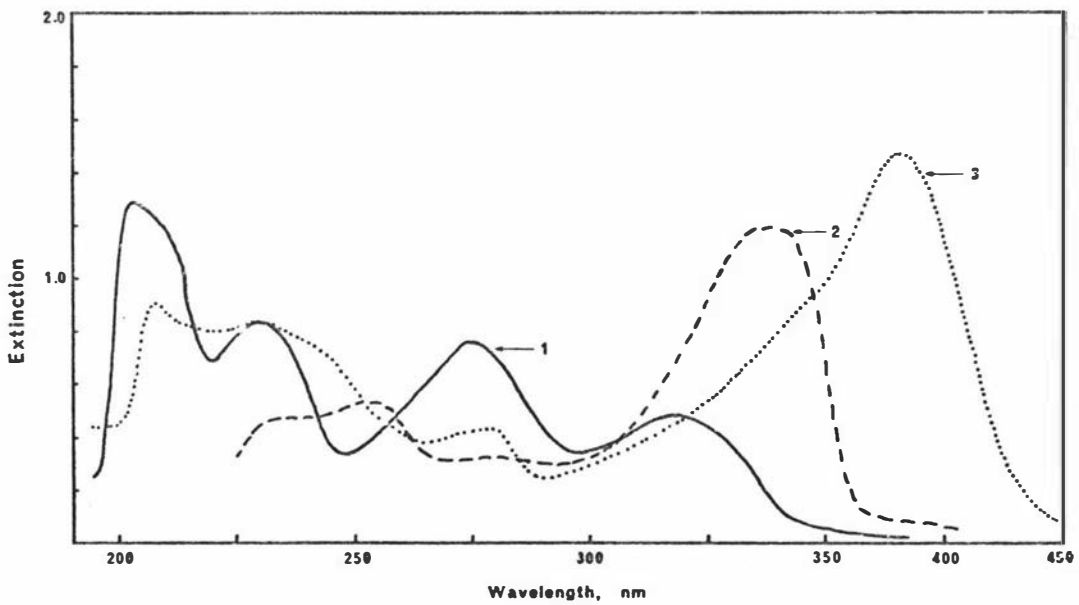
1 = $\not{\alpha}Y_1$ in 85% EtOH, λmax 275, 318 nm

2 = As 1, with solid NaOAc added, λmax 339 nm

3 = As 1, with NaOH added, final concentration 0.003 N, λmax 382 nm (spectrum is that of OC anion)

Figure 36

U.v. absorption spectra for $\not{\alpha}Y_2$a form of $\not{\alpha}Y_2$



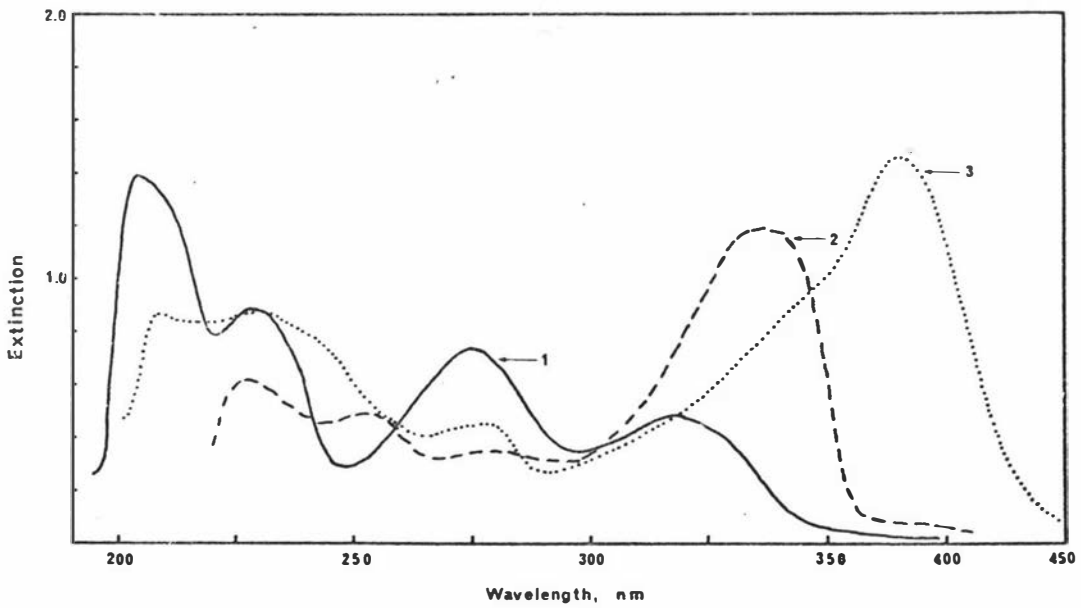
1 = $\not{\alpha}Y_2$a form in 85% EtOH, λmax 275, 317 nm

2 = As 1, with solid NaOAc added, λmax 335 nm

3 = As 1, with NaOH added, final concentration 0.003 N, λmax 381 nm (spectrum is that of OC anion).

Figure 37

U.v. absorption spectra for $\not{\alpha}Y_2b$ form of $\not{\alpha}Y_2$



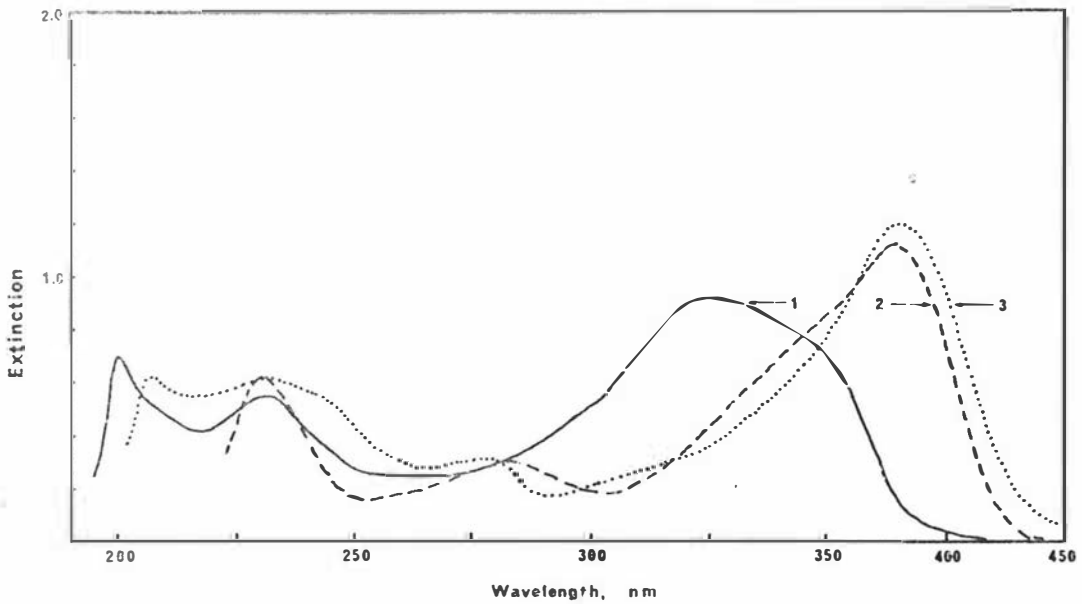
1 = $\not{\alpha}Y_2b$ form in 85% EtOH, λ max 275, 317 nm

2 = As 1, with solid NaOAc added, λmax 336 nm

3 = As 1, with NaOH added, final concentration 0.003 N, λ max 382 nm, (spectrum is that of OC anion).

Figure 38

U.v. absorption spectra for OC



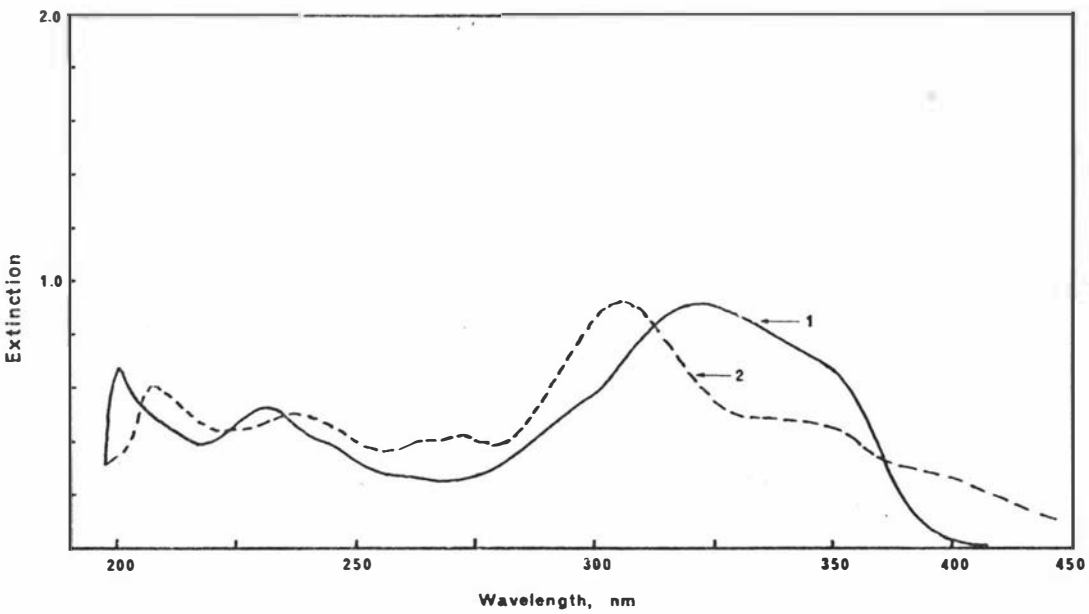
1 = OC in 85% EtOH, λ max 325 nm, $E^{1\%}_{1cm} = 0.845 \times 10^3$

2 = As 1, saturated with NaOAc, λ max 378 nm

3 = As 1, NaOH added, final concentration 0.003 N, λ max 381 nm

Figure 39

U.v. absorption spectra for OC-methyl ether

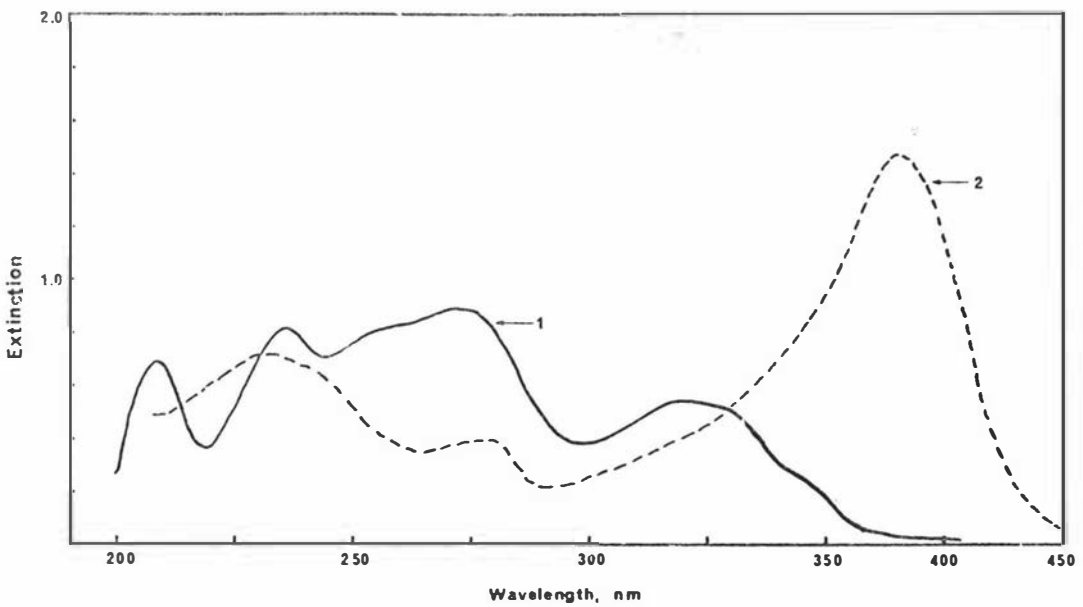


1 = OC-methyl ether in 85% EtOH, λ max 322 nm, $E^{1\%}_{1\,cm} = 0.086 \times 10^3$

2 = As 1, NaOH added, final concentration 0.003 N, (sample decomposes)

Figure 40

U.v. absorption spectra for ⌀OC



1 = ⌀OC in 85% EtOH, λ max 273, 320 nm

2 = As 1, NaOH added, final concentration 0.003 N,
(spectrum is that of OC anion)

Figure 41

I.r. spectrum (KBr disc) of $\not{\alpha}Y_1$

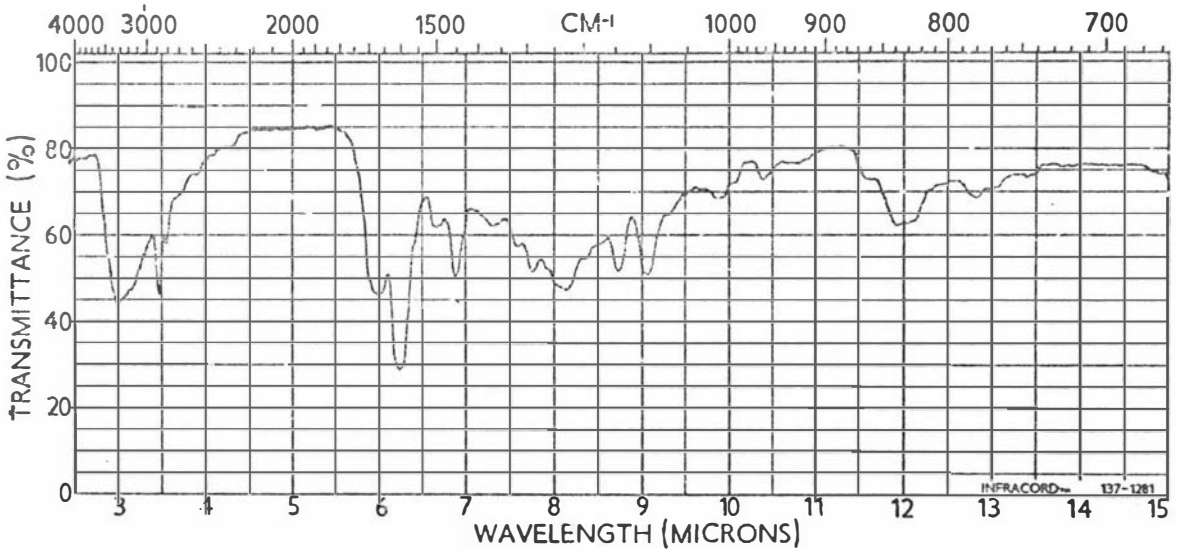
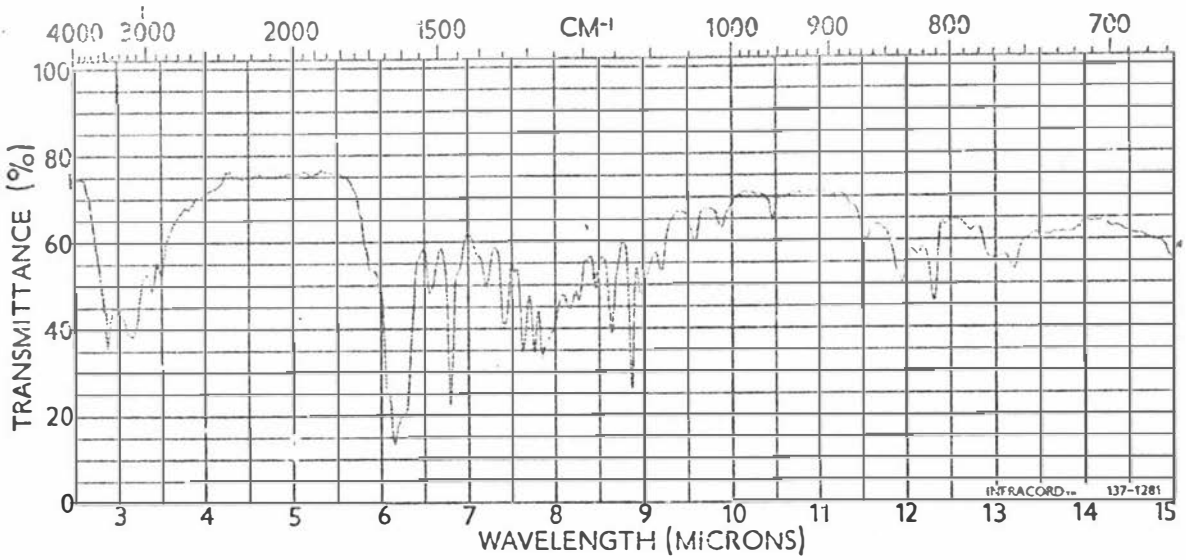
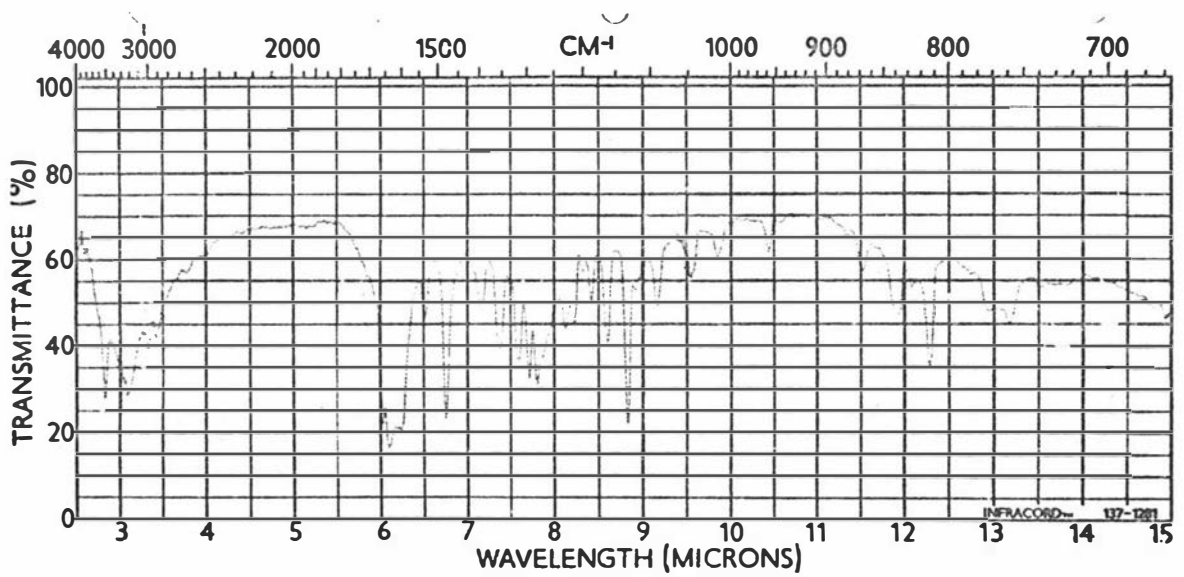


Figure 42

I.r. spectra (KBr disc) of $\not{\alpha}Y_2a$ and $\not{\alpha}Y_2b$ forms of $\not{\alpha}Y_2$



$\not{\alpha}Y_2a$ form



$\not{\alpha}Y_2b$ form

Figure 43

I.r. spectrum (KBr disc) of OC

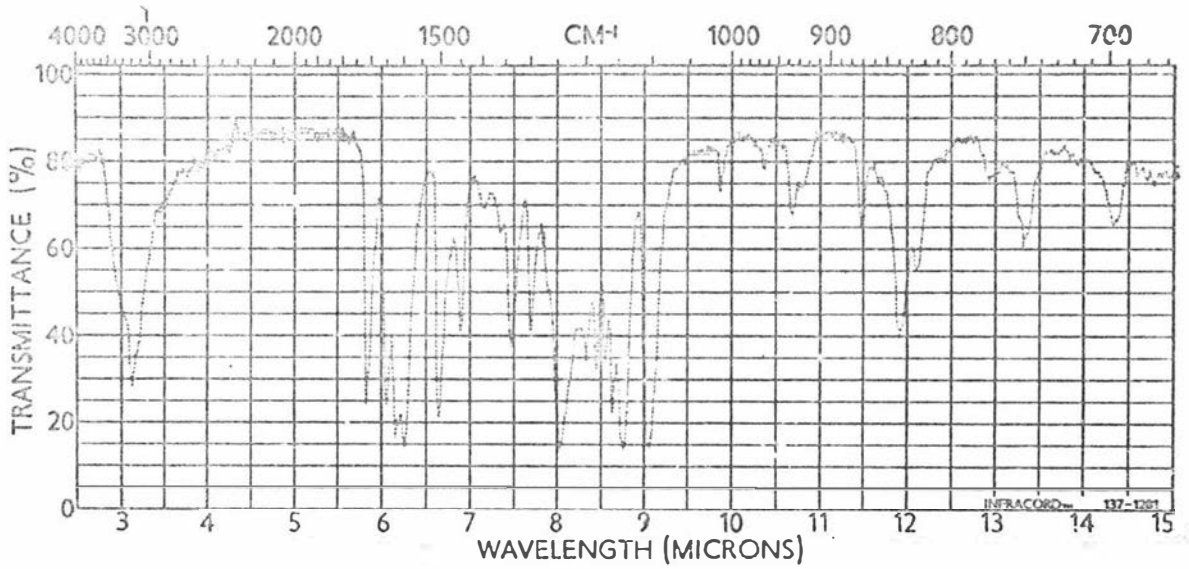


Figure 44

I.r. spectrum (KBr disc) of OC-methyl ether

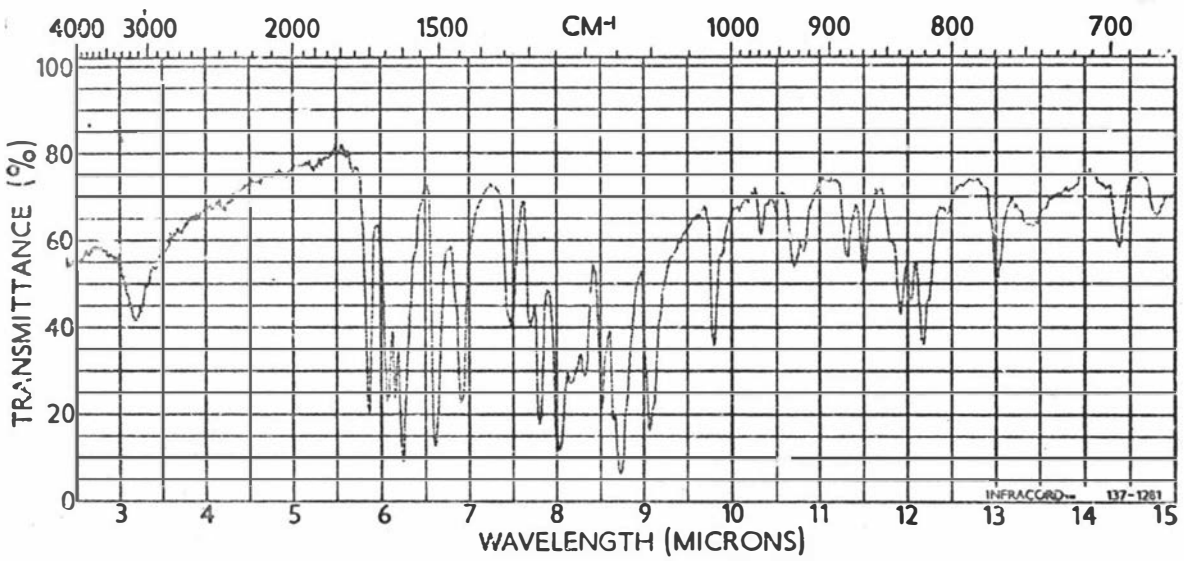


Figure 45

I.r. spectrum (KBr disc) of ØOC

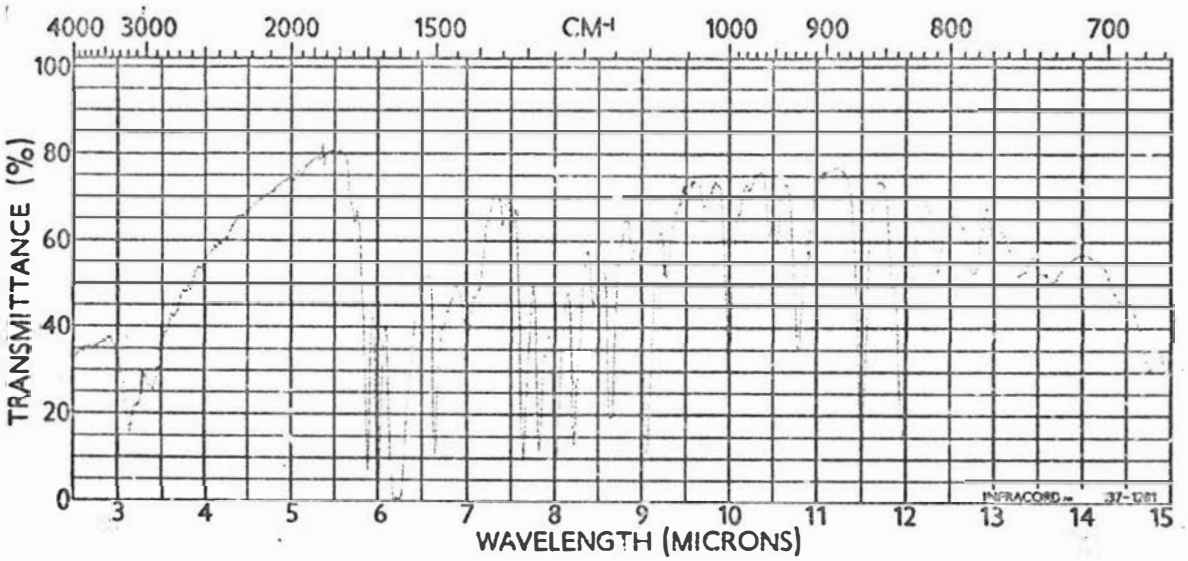


Figure 46

Mass spectrum of UC

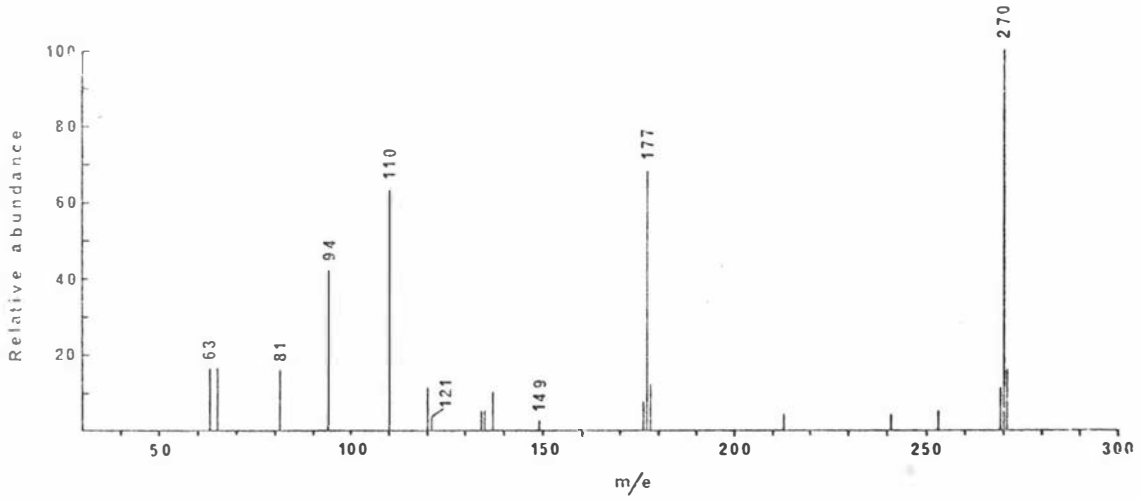


Figure 47

Mass spectrum of OC-methyl ether

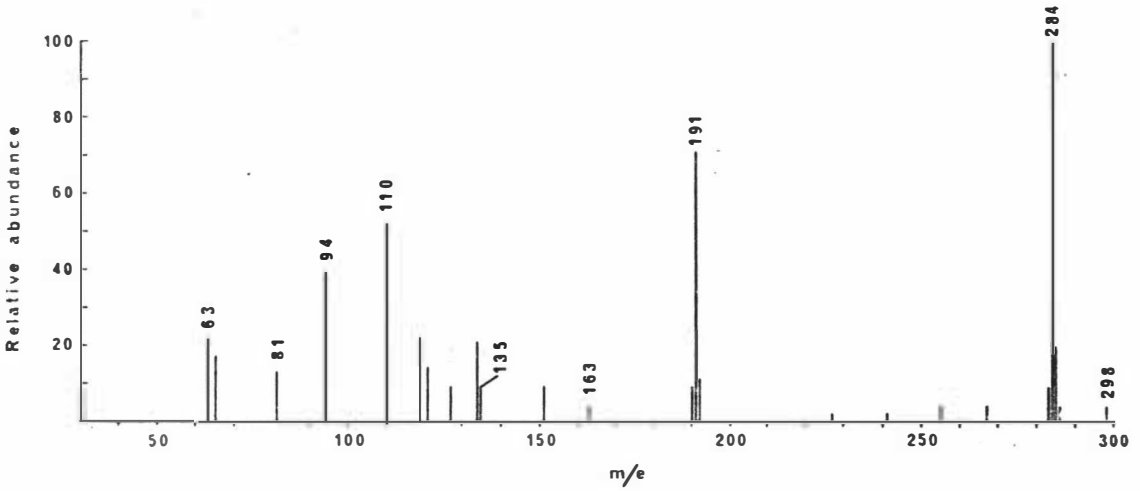
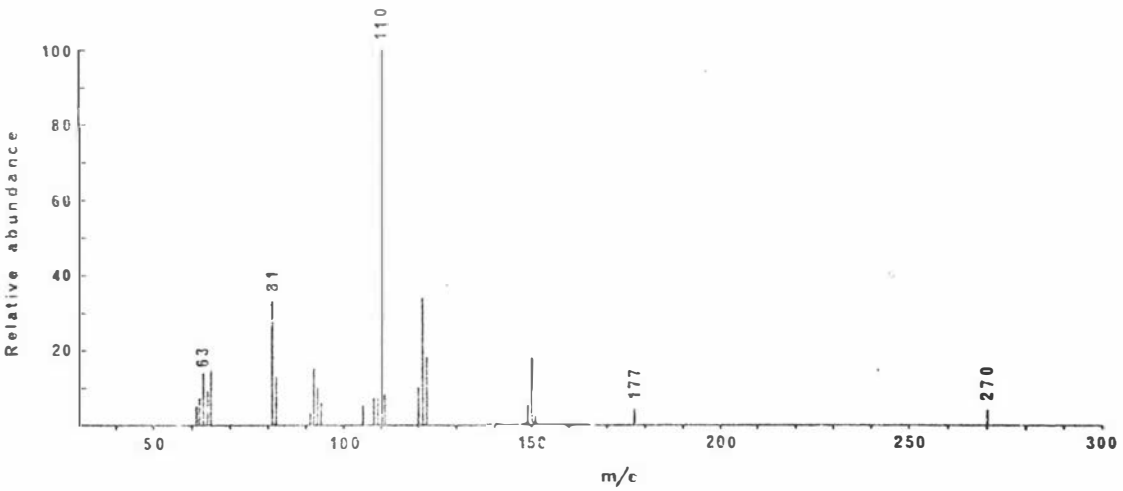
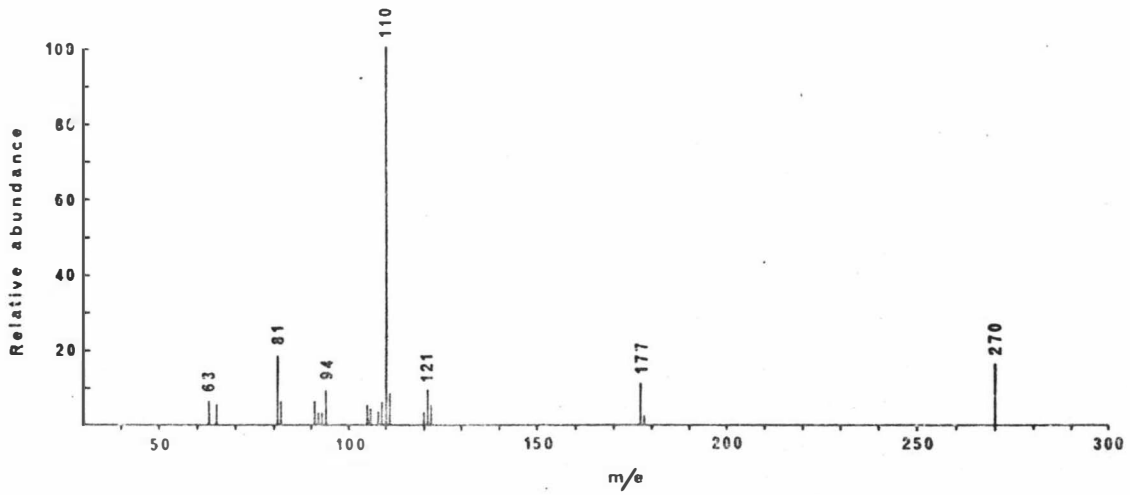


Figure 48

Mass spectra for MOC



Initial spectrum



Final spectrum

Figure 49

Mass spectrum of TMS-ether product from $\not{\alpha}Y_1$

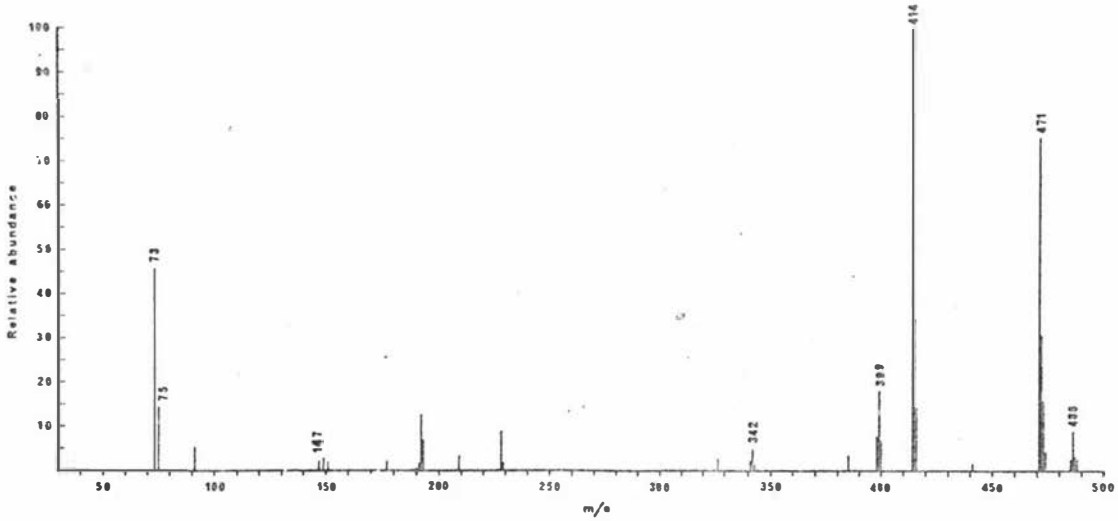


Figure 50

Mass spectrum of TMS-ether product from $\not{\alpha}Y_2$

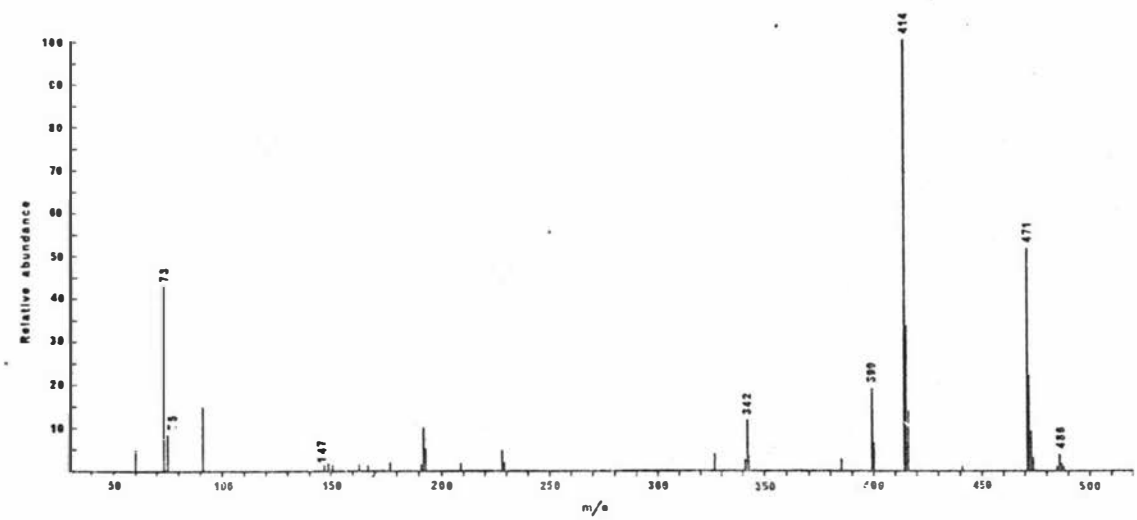


Figure 51

Mass spectrum of TMS-ether of flavonol

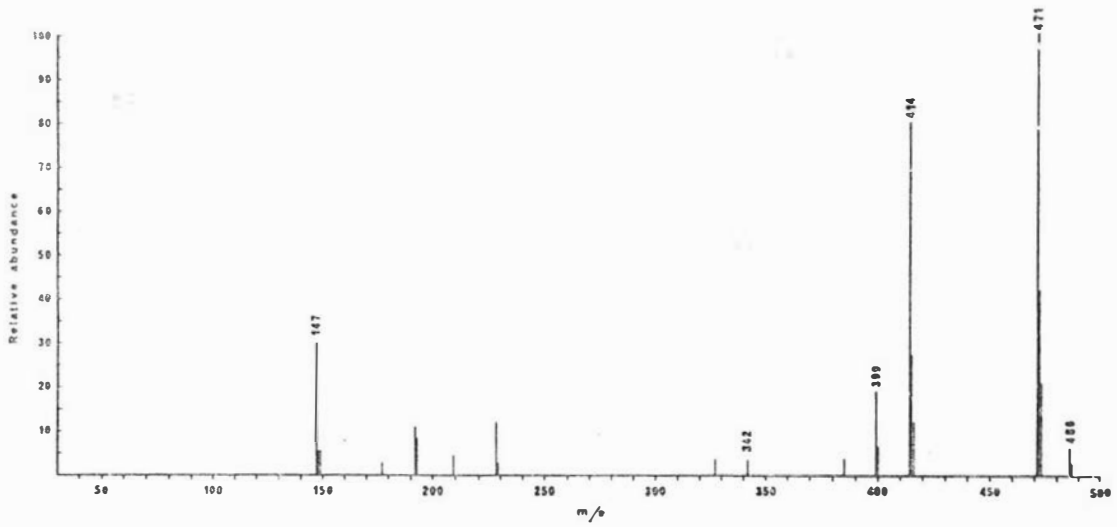


Figure 52

Mass spectrum of TMS-ether of OC

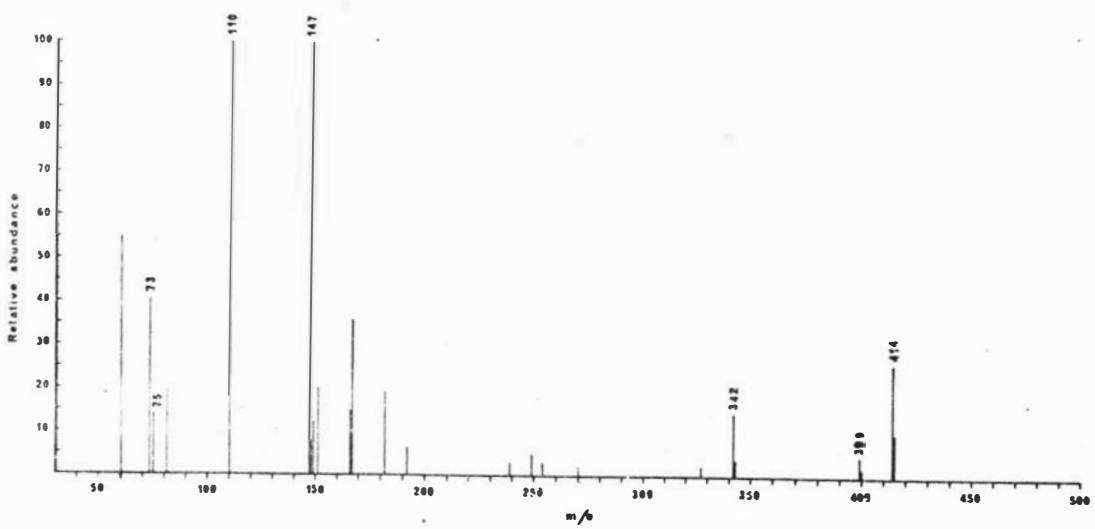
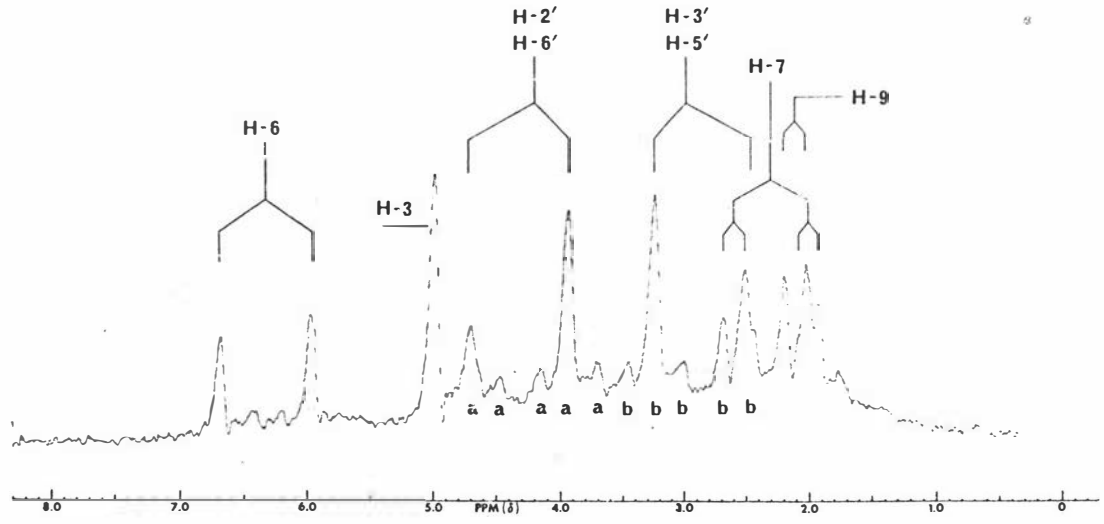


Figure 53

NMR spectrum of OC in acetone-d_6, sweep 390-490 Hz downfield from TMS internal standard, 12 sweeps on CAT (computer of average transients).



Locations of absorptions of the 2', 6' and 3', 5' protons are indicated by the letters 'a' and 'b' respectively.

Figure 54

NMR spectrum of OC in acetone-d_6 containing D_2O, sweep 390-490 Hz downfield from TMS. See Figure 53 for analysis of the spectrum.

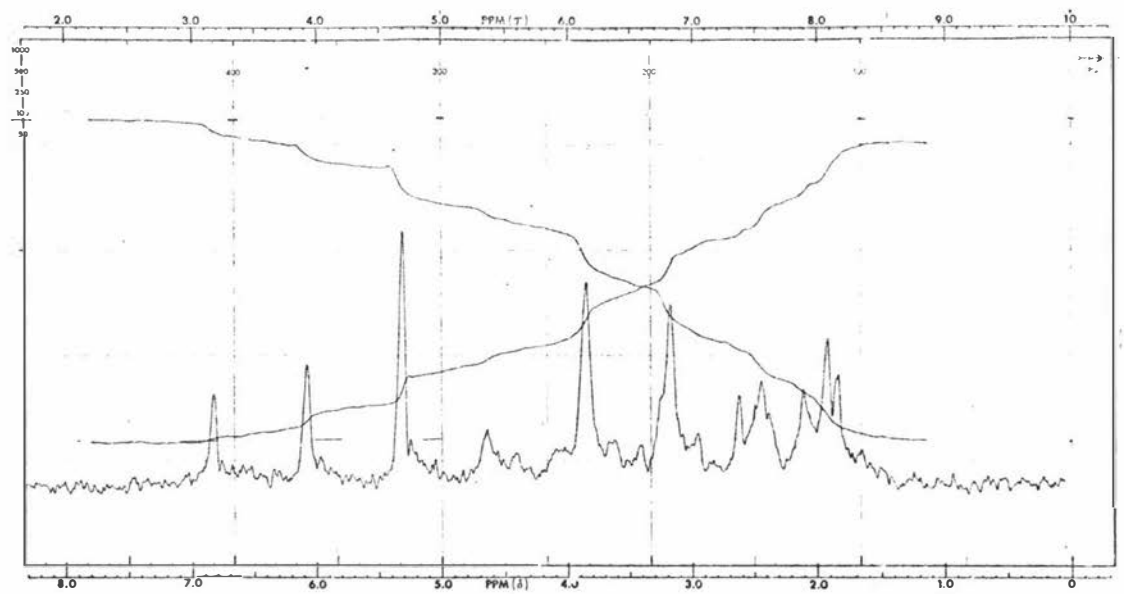


Figure 55

NMR spectrum of OC-methyl ether in acetone-d_6, sweep 390-490 Hz downfield from TMS, 16 sweeps on CAT. Refer to Figure 53 for details of the analysis of the spectrum.

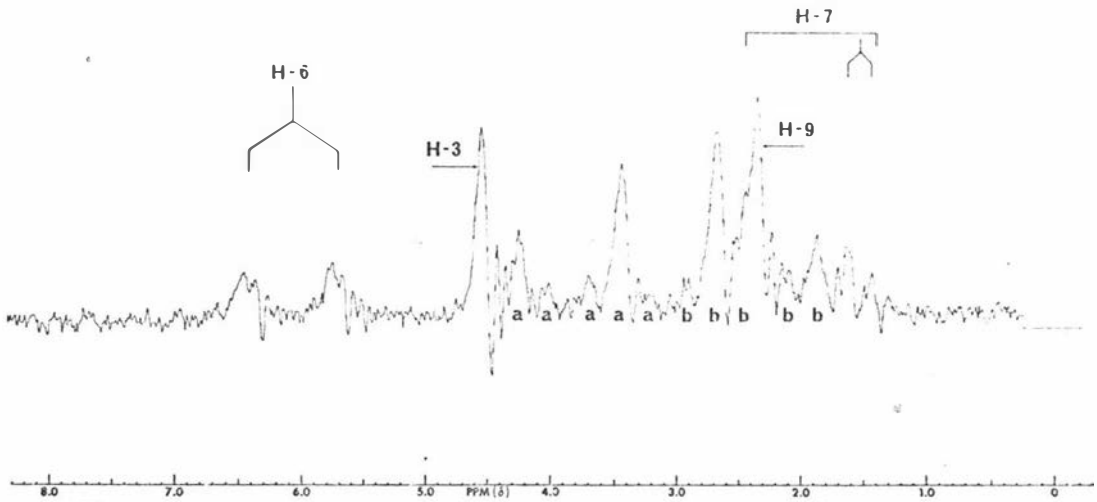


Figure 56

NMR spectrum of $\not{\alpha}Y_1$ in acetone-d_6, sweep 250-500 Hz downfield from TMS.

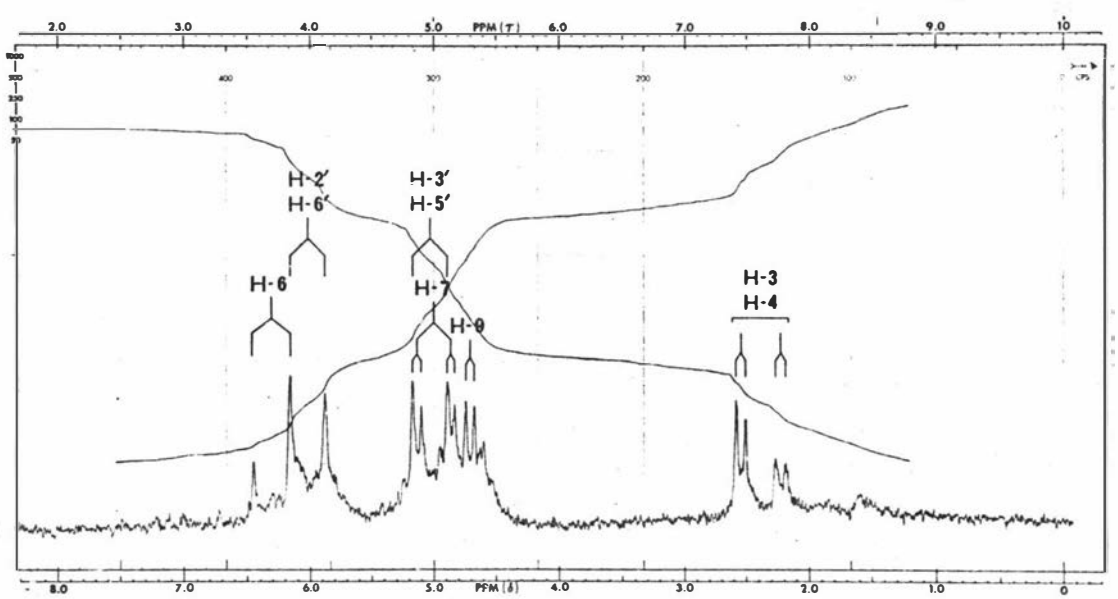


Figure 57

NMR spectrum of $\not{\alpha}Y_2$ ($\not{\alpha}Y_2$a form) in acetone-d_6, sweep 250-500 Hz downfield from TMS.

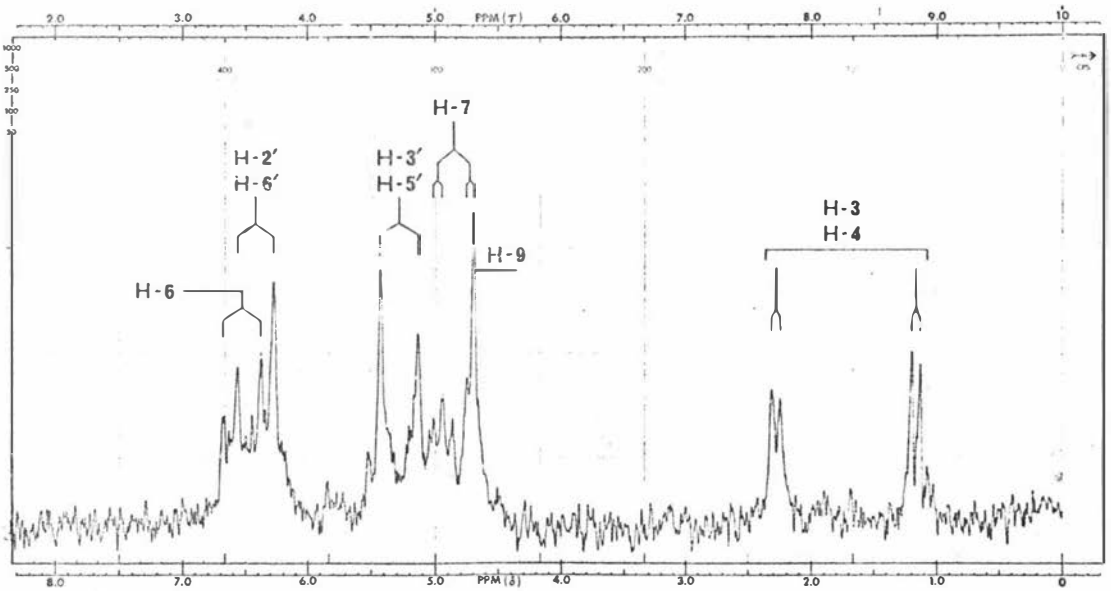


Figure 58

NMR spectrum of $\not{\alpha}Y_2$ ($\not{\alpha}Y_2$a form) in acetone-d_6, sweep 370-470 Hz downfield from TMS; 10 sweeps on CAT.

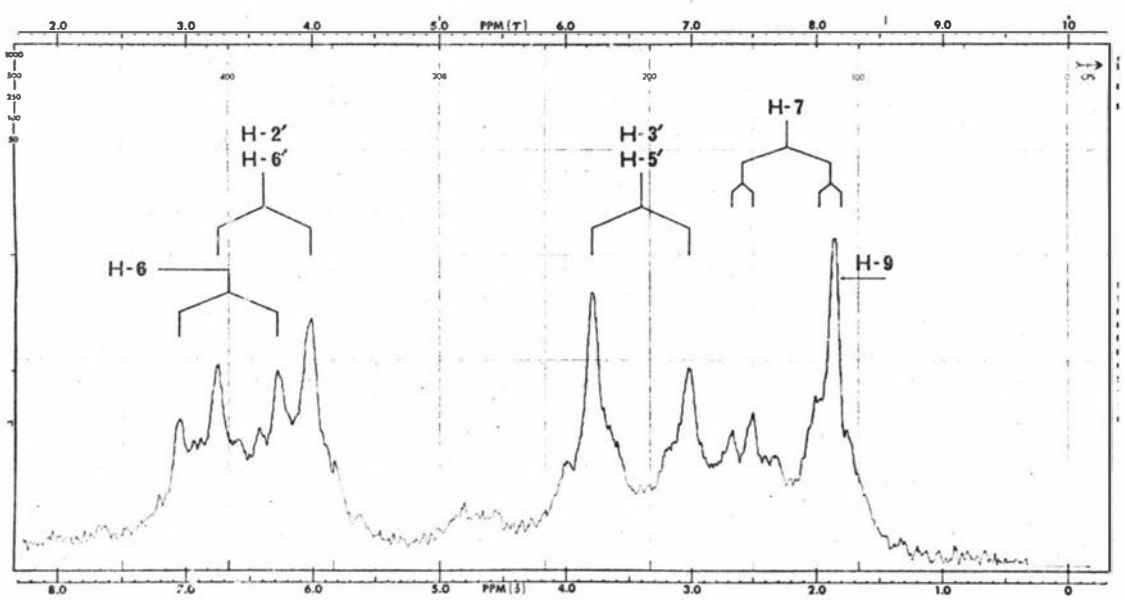


Figure 59

NMR spectrum of $\not{\alpha}Y_2$ ($\not{\alpha}Y_2$b form) in acetone-d_6, sweep 250-500 Hz downfield from TMS. Assignments are as in Figure 57.

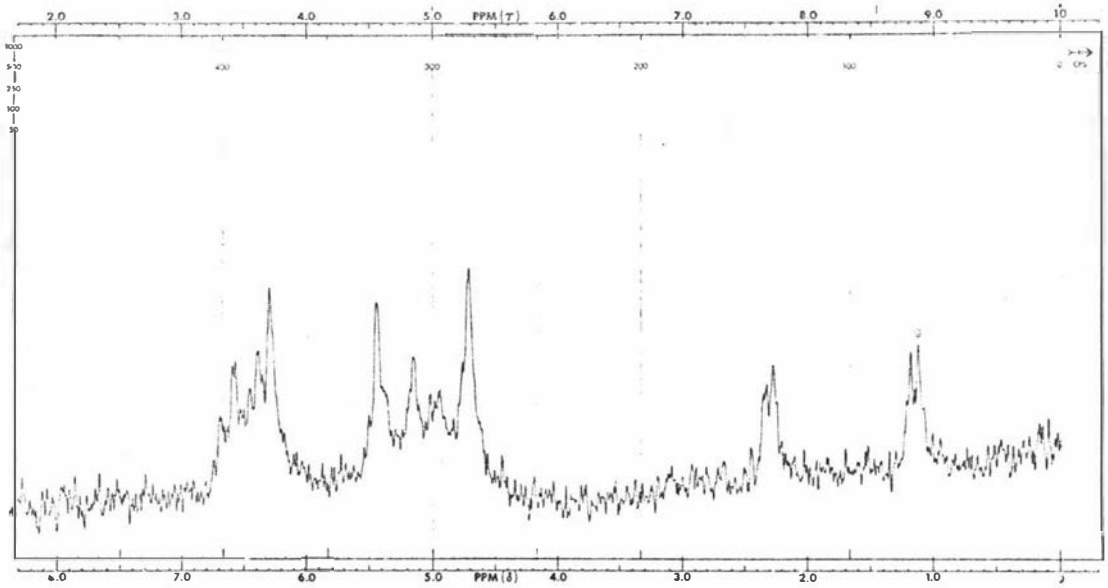


Figure 60

NMR spectrum of $\not{\alpha}Y_2$ ($\not{\alpha}Y_2$b form) in acetone-d_6, sweep 370-470 Hz downfield from TMS; 10 sweeps on CAT. Assignments are as in Figure 58.

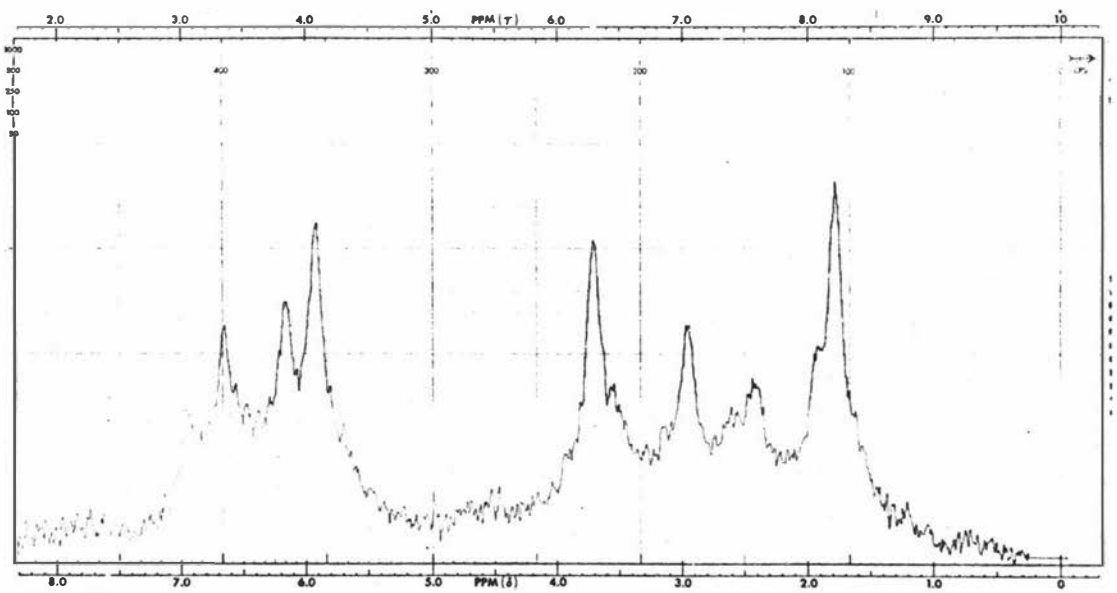
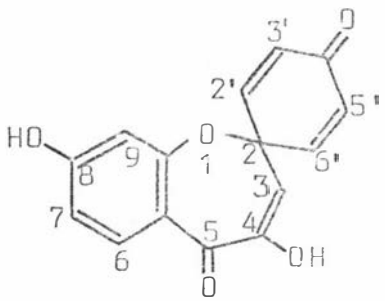
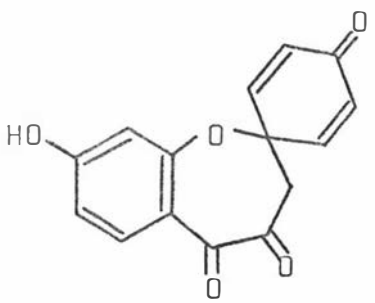


Figure 61

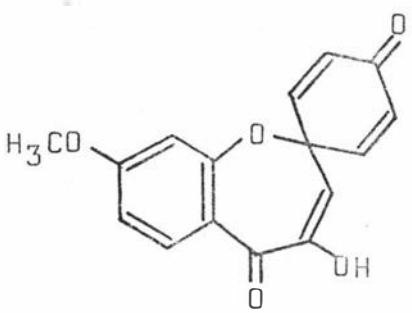
Structure of OC, ØOC, OC-methyl ether and ØY Compounds



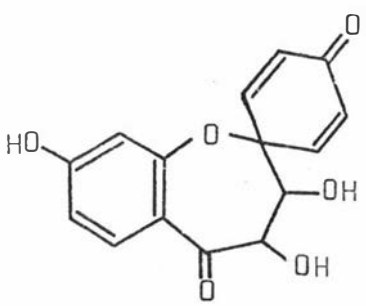
OC



ØOC



OC-methyl ether



$ØY_1$ and $ØY_2$ ($ØY_2$a and $ØY_2$b)

2E THE CHEMICAL OXIDATION OF CHALCONE WITH HYDROGEN PEROXIDE

In the course of biochemical studies recorded earlier (see section 2B-4), a chemical reaction was observed on addition of hydrogen peroxide to chalcone in tris buffer pH 8. The rate of disappearance of chalcone in this chemical reaction was slow, being about 1 - 4% of the rate observed when low amounts of enzyme were added to the same reaction mixture. The apparent products of this limited chemical reaction, detected chromatographically in very low amount, were OC and flavonol. Further studies of the chemical reaction were subsequently undertaken to define its nature and to permit comparison with the enzymic reaction.

2E-1 Products of reaction at pH 6 - 11.

In these studies, the chemical reaction was initially investigated in the pH range 6.2 - 11 using a much higher concentration of hydrogen peroxide than in the previous reactions, which had been strictly controls for the biochemical reaction. Once again, when products of the reaction were ether extracted at pH 7, 2-D chromatography confirmed the isolation of OC and flavonol as the only quantitatively significant products. The quantities of these compounds recovered are presented in Table 12, together with details of reaction mixtures and conditions investigated. Thus, the rate of reaction as judged by the quantity of products isolated was apparently insignificant at acid pH and increased in rate with pH in the alkaline range. At pH 8 and above, where the more significant rates of reaction apparently occurred, OC was the major product isolated.

Table 12

Amounts of OC and flavonol isolated chromatographically following the chemical reaction of chalcone and hydrogen peroxide in buffers pH 6.2-11

Reaction buffer		Product isolated (µg)	
pH	Type	OC	Flavonol
6.2	phosphate	0	0.05
7.0	phosphate	0.8	0.34
8.0	phosphate	6.0	0.48
7.0	tris	2.2	0.96
8.0	tris	6.5	0.37
9.0	tris	14.8	0.14
11	tris base	17.9	0.16

The reaction was conducted by the sequential addition to 4 ml of 0.05 M buffer (see Table) of chalcone (0.73 µmole) and 30% H_2O_2 (87 µmoles). Incubation was for 20 minutes at room temperature, after which the reaction mixture was adjusted to pH 7 and the product immediately extracted into ether (2 x 5 ml). On removal of the ether, the product was separated by 2-D chromatography and the OC and flavonol spots present were excised. OC was eluted in 85% EtOH and estimated by u.v. absorbance at 325 nm, while flavonol was eluted in an EtOH-phosphate buffer mixture and estimated fluorimetrically (see Experimental).

However, the small amounts of flavonol invariably present were noticeable on account of the intense fluorescence of the compound on chromatograms viewed in u.v. light.

In addition to OC and flavonol products, the 2-D chromatograms also showed clearly that very small amounts of compounds were present in the Y_1' and Y_2' spot positions, especially from the reaction in tris buffer pH 7 but also from phosphate buffer at pH 7 and 8. At higher reaction pH, no Y'-type spots were present. This constituted the first evidence of the possible existence of the ⌀Y compounds in the product of the chemical reaction and raised the possibility that these compounds were the precursors of the OC and flavonol isolated, as in the case of the enzymic reaction. From the known behaviour of the ⌀Y compounds, transformation of the small quantities required to account for the observed OC and flavonol products would have been reasonably expected under the conditions of reaction and extraction. Further, the presence of traces of these precursors at lower but not at higher reaction pH would be consistent with previous experience.

Evidence of aurone formation in these chemical reactions was revealed initially only at higher reaction pH by the occurrence of the compound as a spot beside chalcone. However, later inspection revealed the existence of Y_1 and Y_2 spots too on these chromatograms and also in those of products formed at lower reaction pH. These results were entirely compatible with aurone formation occurring only through Y_1 and Y_2 precursors, again in parallel to the findings for the aerobic biochemical reaction.

The origin of the routinely isolated products, OC and

flavonol, in the chemical reaction was then investigated following the circumstantial evidence which accrued from the previous series of chemical reactions that ØY compounds may be common precursors as in the enzymic reaction. A reaction was therefore carried out with conditions specially chosen to minimise the destruction of any ØY compounds which may be formed. Thus hydrogen peroxide concentration was increased to favour a faster reaction, and the reaction pH was selected as a compromise between the conflicting effects at higher pH of faster reaction but greater instability of any ØY compounds formed. A short reaction time was selected to virtually eliminate any loss of ØY compounds by conversion to CC, flavonol and other unidentified products whose formation was known to occur at slightly alkaline pH.

The reaction mixture contained chalcone (3.7 µmoles) and hydrogen peroxide (8.7 mmoles) in 40 ml 0.05 M tris buffer pH 7.5. After 2 minutes reaction at room temperature, the solution was adjusted to pH 2 - 3 and the ether-extracted (2 x 40 ml) product was chromatographed in 2-D on two papers with 20 minutes drying time between solvents. On the partially dry final chromatograms, characteristic Y_1' and Y_2' spots were detected in u.v. light and immediately eluted as the two separate types in 85% ethanol. The absorption spectra of the two eluates were typically those of ØY compounds and were of about equal intensity. The absorbance indicated the quantity of compound in each would have been, as ØY compound, equivalent to about 25 µg of CC. Each eluate was then transferred to aqueous solution and treated cautiously with the minimum amount of alkali required to raise

the pH above 9 (determined empirically on account of acetic acid present from chromatographic solvent) in order to convert any ØY compounds largely to OC but also to a detectable amount of flavonol. The alkali-treated product was recovered and analysed by 2-D chromatography in the usual manner. It was found to contain in both cases OC and flavonol in low amounts, but as the only significant products. Therefore, both $ØY_1$ and $ØY_2$ had been formed in the chemical reaction. The chromatograms of reaction product also indicated that the ØY compounds were the source of the OC and flavonol previously isolated as products of the chemical reaction since none of the latter compounds appeared in this case where conditions had been chosen to limit the transformation of any ØY compounds formed, and no precursors other than the ØY compounds were detected. The final chromatograms of the alkali-treated eluates showed after a period of storage that small amounts of the aurone precursors, Y_1 and Y_2 had also been formed in the reaction and recovered in the eluates of the respective Y' spots, but not transformed extensively in the alkali treatment step on account of the limited rise in pH.

When investigations were also carried out at higher reaction pH than the previous upper limit of pH 11 (see Table 12), the chromatographically apparent product pattern remained as OC and flavonol. In contrast to the previous experience (Table 12) the quantity of products on chromatograms no longer increased with reaction pH. The contents of the reaction mixture were as given in Table 12, but buffer was replaced by NaOH, either 0.01 or 0.1 N and reaction time was restricted to 5 minutes before ether extraction at pH 7

and chromatography of products. The lack of additional quantity of products from reaction at pH 13 compared with pH 12 was rationalised in terms of the product being formed as ØY compounds. Hence these compounds would have been transformed rapidly at either reaction pH to OC and flavonol, but the proportion of the latter would have increased considerably at the higher reaction pH (see Figure 31). However, since flavonol is degraded in alkali the losses of this product, and of total product therefore, would have been increased at higher reaction pH. Thus, the situation could have arisen where the expected additional rate of product (ØY compounds) formation at higher pH was offset by the added rate of destruction of the major derived form (flavonol) of the product at that pH. Under the conditions of reaction at pH 7 - 11 and extraction at pH 7 (see Table 12), transformation of ØY compounds would yield predominantly OC and hence the general effect of an acceleration of reaction rate with increasing pH would be linked with an increase in OC formation and recovery. The decreased amount of flavonol recovered from reaction at pH 9 and 11, compared with lower pH (Table 12) may well have been significant and a reflection of increased rates of destruction outweighing the trend at higher pH towards proportionately more flavonol formation from ØY compounds. The destruction of flavonol was found, for example, to be 7.3 µg when 25 µg of the compound was incubated in a 4 ml volume of 0.05 M tris base pH 11, at room temperature for 60 minutes. At 60°, however, the same amount of flavonol in the same incubation mixture was destroyed in about 5 minutes. Thus, from the foregoing survey of the amounts and balance of flavonol and OC

recovered from the chemical reaction, the hypothesis that ØY compounds are initially formed in this reaction under all pH conditions, besides at pH 7.5 as directly demonstrated, is proposed to uniquely accommodate the observed results. Therefore in terms of detailed product pattern the chemical reaction is considered to parallel the enzymic reaction.

2E-2 Products of reaction under A.F.O. conditions

An important extension of the study of the chemical reaction was made to include the general conditions established in practice for the synthesis of many flavonols from the corresponding chalcones. This general reaction has become known as the Algar-Flynn-Oyamada (A.F.O.) reaction. Typically, the A.F.O. reaction is conducted at high pH and at low temperature, about 0^{o}. Thus in the present investigation, chalcone (180 μmoles) was dissolved in 2.5 ml of 1.25 N sodium hydroxide and the solution cooled in ice to 0^{o} when hydrogen peroxide (960 μmoles in 0.11 ml) was added and the mixture held at 0 - 4^{o} for several days. Samples (100 μl) of the reaction mixture were withdrawn at intervals over the next 145 hours and were acidified immediately with HCl in a 4 ml volume of water and ether extracted. An aliquot of the evaporated ether extract was chromatographed in 2-D to reveal the product pattern. The only significant product quantitatively was flavonol. After 20 hours reaction, the yield was approximately 20% of the chalcone added; after 145 hours very little chalcone remained and the ether-extracted product was largely flavonol, as shown immediately by the u.v. absorption spectrum and subsequently by chromatography. At no time was any chromatographic evidence of OC formation

detected, even after limited reaction times of from 2 - 20 minutes or from 1 - 4 hours. Traces of the dihydroflavonol, garbanzol, were apparent in the product for reaction times of between 20 minutes and 20 hours only, with a maximum at about 2 hours. Very small amounts of Y_1 and Y_2 were detected (after aurone artifact formation) for reaction times of between 2 minutes and 2 hours only; the amounts decreased progressively with reaction time.

When considered in conjunction with the previous findings for the chemical reaction at lower pH and at room temperature it was considered (see Discussion) that flavonol formation in this A.F.O. reaction also occurred by way of ØY compounds as intermediates. The effect of the high reaction pH would have been to strongly direct transformation of these compounds to flavonol. Further, the low reaction temperature was expected to have contributed much to the stability of flavonol (compare rates of destruction given above at about 20^{o} and 60^{o}).

Chapter 3

DISCUSSION

In this Chapter, the results leading to the proposed structures for the new products isolated, namely OC, $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ are examined. Following this, the chemical and biochemical oxidations of chalcone are considered and plausible mechanistic schemes advanced for these reaction systems. It will be shown that the novel peroxidase-catalysed reaction encountered in this work can be rationalised in terms of a proposed sequence of previously known redox transformations of the enzyme.

3A STRUCTURE OF OC, $\not{\alpha}Y_1$ AND $\not{\alpha}Y_2$

A close structural relationship is indicated for OC, $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ from a number of results. Thus, the facile transformation of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to OC indicated a minimal structural difference. Further, the identity or close similarity in the spectral data points to a very limited difference between $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. This conclusion is reinforced by the common property of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ in giving rise to either OC or flavonol under alkaline conditions. As will become apparent in the following sections, various structural interpretations are automatically strengthened by virtue of the knowledge that close structural relationships exist among all the new compounds.

3A-1 Evidence from spectral data

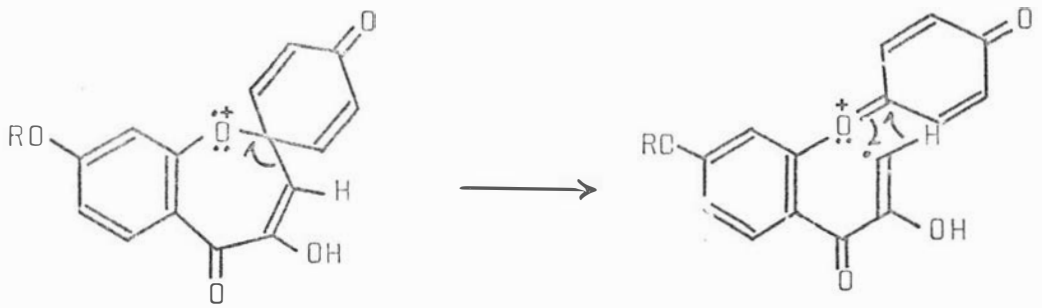
(a) Interpretation of the mass spectra of OC and OC-methyl ether. The molecular formulae of OC, $\not{\alpha}$OC

(both $C_{15}H_{10}O_5$) and OC-methyl ether ($C_{16}H_{12}O_5$), determined by mass measurement of the respective molecular ions, established OC and ØOC as structural isomers and OC-methyl ether as a mono-methyl ether of OC. The molecular formula therefore showed that in the formation of OC, a four-equivalent oxidation of chalcone had taken place resulting in the net removal of two hydrogen atoms and the addition of one oxygen atom.

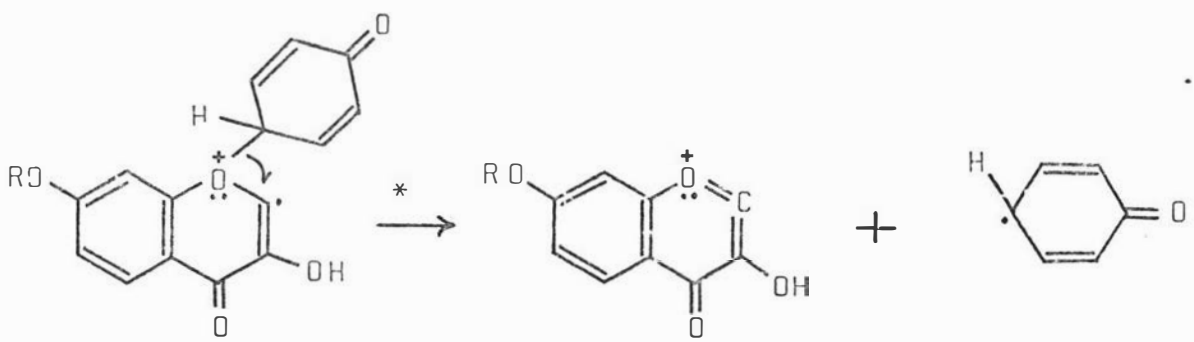
The mass spectra of OC and OC-methyl ether were very similar (Figures 46 and 47), the molecular ion being the base peak in each case and the major fragmentation paths closely similar for both compounds. A scheme for interpreting the paths of fragmentation is presented (Scheme 1). The structures presented in this Scheme for specific ions are considered to be plausible representations of the compositions established for the ions by mass measurement (Table 11) and serve to rationalise the fragmentation observed in terms of the proposed structure of the intact molecule. Thus, cleavage of the molecular ion in Scheme 1 is represented as an α cleavage of the ether linkage (41) at the spiro centre followed by hydrogen atom transfer and elimination of the fragment. Given the restrictions imposed by the original chalcone structure, this elimination of a fragment of mass 93 can only be reasonably formulated as a loss of the atoms originally of ring B of the chalcone. Furthermore, elimination of the same fragment from both OC and OC-methyl ether molecular ions indicated the oxygen atom present in the fragment could not be phenolic in the OC structure, otherwise methylation with diazomethane would have been expected. Successive eliminations

Scheme 1

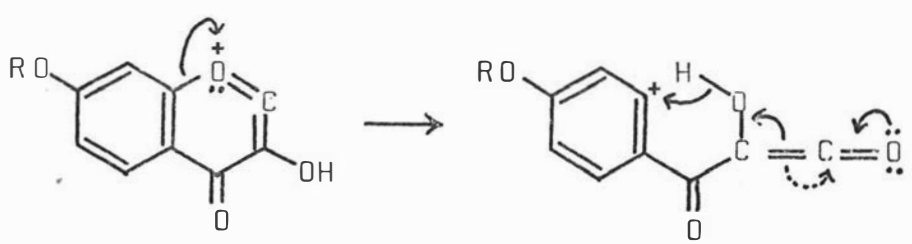
Scheme for the fragmentation of OC and OC-methyl ether upon electron impact



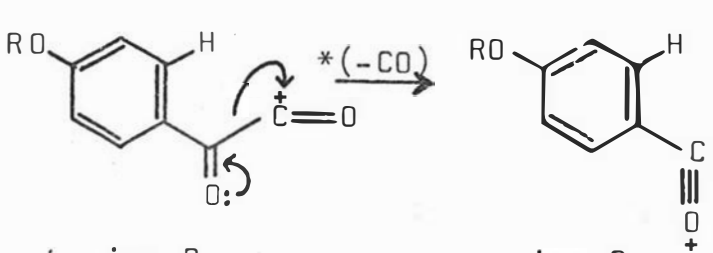
OC: R = H m/e 270 (100%)
OC-OMe: R = CH_3 m/e 284 (100%)



ion A
R = H m/e 177 (68%)
R = CH_3 m/e 191 (71%)



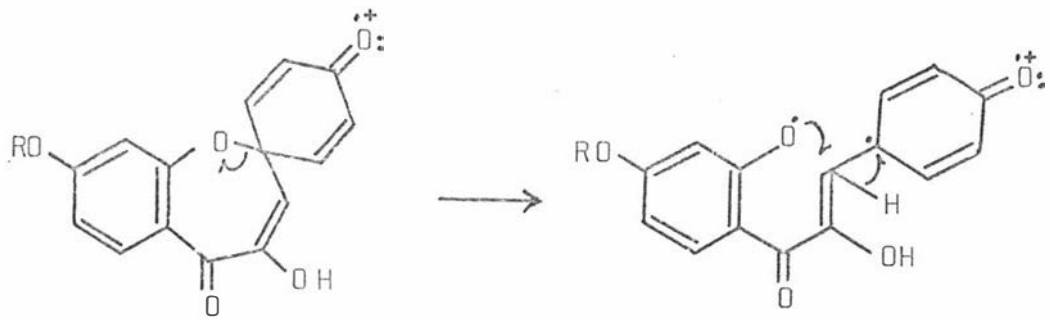
ion A



ion B
R = H m/e 149 (1.6%)
R = CH_3 m/e 163 (3.5%)

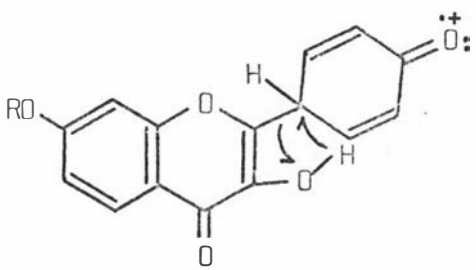
ion C
R = H m/e 121 (2.6%)
R = CH_3 m/e 135 (9.4%)

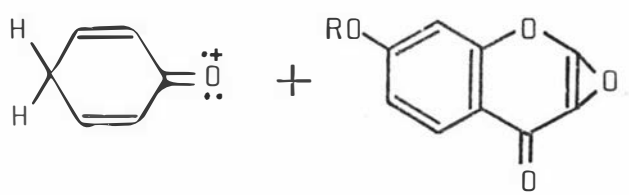
Scheme 1 Continued



R = H m/e 270 (100%)

R = CH_3 m/e 284 (100%)





ion D

m/e/ 94 (42% from OC)

(39.4% from OC-OMe)

* signifies presence of appropriate metastable peak

of two molecules of carbon monoxide from ion A to give ion C occurred to a small extent and could be rationalised in terms of the heterolytic cleavage sequences in the Scheme.

Ion D (m/e 94) was prominent and represented fragmentation of the molecular ion with retention of charge in the original chalcone B ring moiety, in contrast to the formation of the previously encountered fragment, ion A. Formation of ion D, however, requires a double hydrogen transfer. Homolytic cleavage of the ether linkage in the molecular ion gives the diradical, stabilised by mesomerism involving the rings adjacent to each electron, which can then rearrange with transfer of one hydrogen atom to the flavonol-like intermediate. This intermediate then cleaves (42) with another hydrogen transfer reaction to form ion radical D.

Fragmentation of deuterium-labelled OC, prepared by introducing OC and deuterium oxide in to the source (43) was consistent with the above Scheme. The molecular ion incorporated a maximum of two deuterium atoms, as would be expected, through exchange with hydrogen at the enolic (C-4) and phenolic (C-8) functions. Both deuteriums were retained in fragment ions A and B, while one was present in ion D.

The peak at m/e 110 $(C_6H_6O_2)^{\bullet+}$ in the spectra of both OC and OC-methyl ether was considered to be an artifact and to represent the molecular ion of hydroquinone which had been formed by partial pyrolysis of the sample introduced into the mass spectrometer. This diphenol was formed by spontaneous degradation of OC (section 2D-3(d)) and by hydrogenolysis of both OC and OC-methyl ether

(section 2D-3(c)). The main evidence pointing to this origin of ion m/e 110 was the wide variation in the intensity of the ion in relation to the other established ions (Scheme 1) in the spectra run under different inlet temperature regimes. The spectrum for the deuterated OC sample (see above) showed that two hydrogens were exchangeable with deuterium in this ion (m/e 110 —→ m/e 112) as expected for its origin as the proposed second molecular ion but not if it were formed from OC or OC-methyl ether molecular ions by fragmentation involving a double hydrogen transfer (compare deuterium exchange for ion D m/e 94). Peaks at m/e 81 and 63 in the spectra are typically fragment ions in the spectrum of hydroquinone (44).

The mass spectrum of ØOC was of little direct value beyond the establishment of the compound as a structural isomer of OC. A very prominent peak at m/e 110 in both the first and final spectra recorded for the ØOC sample was again attributed to hydroquinone formed by partial pyrolysis, which may have been more extensive with ØOC than with OC and OC-methyl ether. As the inlet temperature increased to about 200°, the spectrum resembled increasingly that of OC. It was considered likely that the diketone, ØOC, isomerised to the more stable enol, OC, in the mass spectrometer.

(b) Interpretation of the mass spectra of TMS derivatives from $ØY_1$, $ØY_2$, OC and flavonol. Silylation of $ØY_1$ and $ØY_2$ was undertaken in an effort to stabilise these highly reactive compounds sufficiently to permit determination of molecular weight at least, by mass spectrometry. If successful, this technique would have revealed

the number of hydroxyl groups in the molecule and, therefore, the validity of the diol structure proposed (Figure 61) would have been tested directly, a feat virtually unmanageable by other procedures on account of the instability of the compounds. Attempted mass spectrometry of the unprotected compounds was fruitless.

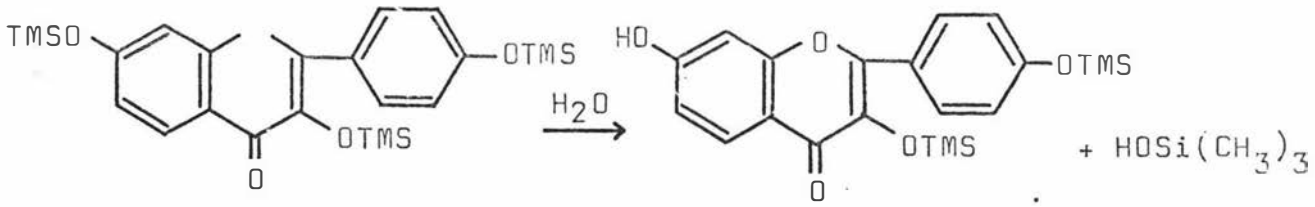
Mass spectrometry of the GLC-purified, major, TMS derivative of both $\emptyset Y_1$ and $\emptyset Y_2$ indicated that an identical product had been obtained from the two compounds (Figure 49 and 50). Further, direct comparison with the spectrum of an authentic trimethylsilylated flavonol sample (Figure 51), similarly isolated by GLC, provided strong support (see below) for the identification of the TMS derivatives of $\emptyset Y_1$ and $\emptyset Y_2$ as the TMS-flavonol product. Consequently, all evidence was found to be consistent with the conclusion that a transformation of both $\emptyset Y_1$ and $\emptyset Y_2$ to flavonol in the course of silylation with the BSTFA reagent had occurred. Analytical GLC (Figure 34) indicated that any TMS-OC product also formed was in relatively minor proportion to the TMS-flavonol since these two compounds were separately shown to be resolved on the column under the conditions employed. Therefore, the transformation of $\emptyset Y_1$ and $\emptyset Y_2$ in the silylation step had strongly favoured rearrangement to the flavonol nucleus (compare transformation in aqueous alkali- Figures 31 and 32). This catalysis in the silylation procedure with the BSTFA reagent was unexpected.

The mass spectrum of the TMS derivative from $\emptyset Y_1$ and $\emptyset Y_2$ displayed a weak molecular ion peak at m/e 486 in each case. A similar weak peak at m/e 486 was observed for the molecular ion of authentic TMS-flavonol, consistent with the

expected molecular ion of the fully trimethylsilylated, trihydroxy compound (270 plus 3 x 72). In contrast, the tri-TMS derivative of the intact diol structure proposed for $\emptyset Y_1$ and $\emptyset Y_2$ would have had a molecular weight of 504. The discrepancy of 18 mass units (504 minus 486) corresponds to the loss of the elements of water which would occur in the rearrangement of the diol structure ($\emptyset Y$) to flavonol. Using a $\emptyset Y_2$-derived sample of the TMS derivative, a check was made to ensure that the peaks at m/e 486 and 471 in the spectra of the $\emptyset Y$-derived samples did not represent, instead of the molecular ion and M - 15 fragments respectively, fragment ions derived from a higher mass molecular ion, in particular at m/e 504. Thus, no metastable peak could be detected for any transition yielding either m/e 486 or 471 fragments, apart from the transition m/e 486 $\longrightarrow$ 471, and no evidence whatsoever of the existence of a m/e 504 ion could be detected. Hence, the existence of a common molecular ion peak at m/e 486 leaves no other conclusion than that the $\emptyset Y$-derived samples are either identical to or isomeric with TMS-flavonol. When all other data are taken into account, the only tenable conclusion is that the product from these three sources is identical.

The spectra of the $\emptyset Y$-derived samples were complicated apparently by the occurrence of partial hydrolysis of the TMS derivative to produce a mixture of compounds, presumably while the sample tube was open to the air in the final stages of introduction into the mass spectrometer. Some TMS ethers are known to be very sensitive to moisture (45). As a consequence, the peak at m/e 414 was indicated, on the basis of relative peak intensities as inlet temperature

increased and on metastable peak intensities, to represent largely a molecular ion and only to a minor extent the transition m/e 486 ⟶ 414. At higher temperatures, the relative intensity of m/e 414 increased considerably in relation to the peak at m/e 471, which was derived from m/e 486 by loss of methyl (metastable peak detected). This relative increase in peak intensity was consistent with the presence of a separate compound of lower volatility, giving rise to much of the m/e 414 peak as a molecular ion. A compound of mass 414 could readily arise from the TMS-flavonol structure upon hydrolysis of one TMS ether linkage (equation 2), a reaction which would be expected to be more facile for phenolic rather than alcoholic, TMS-linked oxygen functions (45).



T_3MS-flavonol

mass 486

T_2MS-flavonol

mass 414

Equation 2

While the peak at m/e 414 was the base peak in the spectrum of each of the ΔY-derived samples, it was relatively less intense, but nonetheless highly significant, in the spectrum of TMS-flavonol. Although its origin in the latter sample was not specifically investigated, it is entirely reasonable to assume it again represented largely the molecular ion species arising by hydrolysis as in equation 2.

With the m/e 414 peak largely removed from the true mass spectra for the ØY-derived and TMS-flavonol samples, it thus emerges that the base peak in each case is at m/e 471. This is readily accepted as a fragmentation of the molecular ion by loss of a methyl radical from a TMS group, a favoured process in many TMS derivatives (46). The peak at m/e 399 in the spectrum of the $ØY_2$-derived sample was shown, by metastable peak scanning, to arise by the transitions m/e 414 ⟶ 399 and also m/e 471 ⟶ 399 (loss of methyl and $CH_2Si(CH_3)_2$ respectively)- the latter is equivalent to the minor contribution to the m/e 414 peak of the transition m/e 486 ⟶ 414 also observed in this sample.

The peak at m/e 147, prominent in the spectrum of TMS-flavonol and present also in the spectra of the ØY-derived samples, probably represents a well-established artifact in the spectra of TMS derivatives (46). This artifact arises by loss of a methyl radical from the condensation product (hexamethyldisiloxane) of trimethylsilanol, itself formed upon spontaneous hydrolysis of the TMS ether (as in equation 2 above).

$$2\ (CH_3)_3SiOH \longrightarrow \underset{\text{Hexamethyldisiloxane}}{(CH_3)_3-Si-O-Si-(CH_3)_3} + H_2O$$

$$[(CH_3)_3-Si-O-Si-(CH_3)_3]^{\cdot +} \xrightarrow{CH_3^{\cdot}} \underset{\text{m/e 147}}{(CH_3)_2-Si=\overset{+}{O}-Si-(CH_3)_3}$$

Another artifact peak at m/e 75 is also characteristically formed directly from this trimethylsilanol hydrolysis product by loss of methyl radical (46).

$$[(CH_3)_3SiOH]^{\cdot +} \xrightarrow{CH_3^{\cdot}} \underset{\text{m/e 75}}{\overset{+}{H}O=Si(CH_3)_2}$$

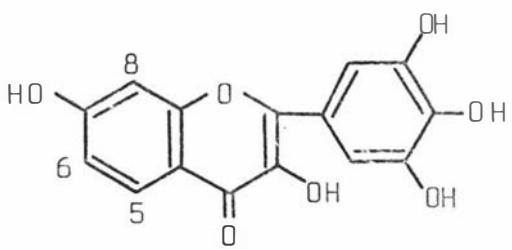
A strong peak at m/e 75 is present in the spectra of the ØY-derived samples; in the case of TMS-flavonol, however, the spectrum was not recorded below m/e 100. Thus the presence of peaks at both m/e 147 and 75 is indicative of the occurrence of trimethylsilanol, consistent with the contention that partial hydrolysis of the TMS-ether occurred prior to mass spectrometry in all three samples. The peak at m/e 73 is typically that of the trimethylsilyl cation (46).

To complete the study of the TMS derivatives, that of OC was also prepared by GLC and subjected to mass spectrometry. Of particular interest was the fact that this derivative was expected, and found (Figure 52), to display a molecular ion peak at m/e 414. Moreover, the spectrum of TMS-OC displayed a number of unique peaks which served to distinguish it from the other mass spectra considered. Thus the peak at m/e 110, typical of unprotected OC and already considered to be an artifact arising by partial sample pyrolysis, was unique to the TMS-OC sample, together with other less significant peaks. Hydrolysis of the TMS-OC sample would also have been expected, and was in fact indicated by the occurrence of typically-artifact peaks at m/e 147 and 75. Fragmentation of the TMS-OC molecular ion and a probable additional molecular ion at m/e 342 was overall similar to TMS-flavonol in that loss of methyl (M - 15) and $CH_2Si(CH_3)_2$ (M - 72) was observed.

General support for the OC structure was also forthcoming from this TMS-OC spectrum. The presence of the two hydroxyl groups in the molecule was indicated by the presence of two TMS groups in the derivative, while the absence of

the ion at m/e 94, prominent in the OC spectrum and requiring a double hydrogen transfer in its genesis (Scheme 1), was entirely predictable in that one of the required hydrogens was no longer available, having been displaced by the TMS group.

(c) Interpretation of the NMR spectra of OC, OC-methyl ether $\emptyset Y_1$ and $\emptyset Y_2$. In the NMR spectrum of OC (Figure 53 and Table 13) the absorptions assigned to the three aromatic ring protons H-6, H-7 and H-9 were similar in pattern to those of the A ring protons, H-5, H-6 and H-8, of the 5-deoxyflavonoid, robinetin (47), which serves as a suitable model compound.

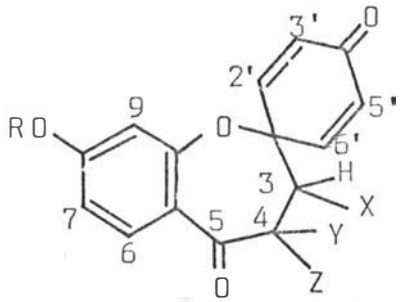


Robinetin

In OC, the presence of the carbonyl function at C-5 is evidenced in the low-field absorption of H-6, due to the deshielding effect of this group (47, 48). Coupling of the H-6 and H-7 protons ($J_{6,7}$=8.6 Hz) is within the range (6-10 Hz) for ortho coupling in aromatic rings (49). A spin decoupling experiment confirmed the low field doublet (H-6) arose by coupling with a proton (H-7) in the upfield

Table 13

NMR Data[x] for OC, OC-methyl ether, ØOC, $ØY_1$ and $ØY_2$



General formula

Compd	Chemical Shifts (ppm δ scale)					
	H-3	H-6	H-7	H-9	H-2',6'	H-3',5'
OC	7.33	7.60d	6.82dd	6.77d	7.21d	6.93d
OC-OMe	7.24	7.55d	6.76dd	6.81	7.11d	6.80d
ØOC	5.05d (H-3a)					
	3.89d (H-3b)					
	(H-3, H-4)					
$ØY_1$	5.32d 5.15d	7.16d	6.52dd	6.37d	7.02d	6.53d
$ØY_2$a	5.19d 4.65d	7.27d	6.43dd	6.37	7.22d	6.66d
$ØY_2$b	5.20d 4.65d	7.27d	6.43dd	6.38	7.22d	6.66d

x Chemical shifts are given in ppm (δ scale) relative to a TMS internal standard. Multiplicities are noted for signals other than singlets as d (doublet) and dd (double doublet). No separate assignment of chemical shift to H-3 and H-4 protons in $ØY_1$ and $ØY_2$ is made. Analyses of all spin systems (see text) were made by first order approximations.

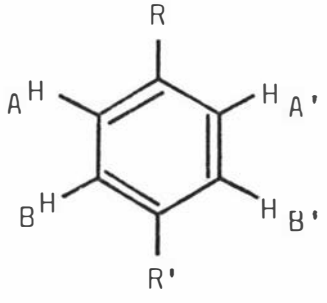
Table 13 Continued

Compd.	Coupling Constants (Hz)				
	H-3a,3b	H-3,4	H-6,7	H-7,9	H-2',3' H-5',6'
OC			8.6	2.1	9
OC-OMe			8.2	2.4	10
ØOC	6.0				
$ØY_1$		2.3	8.8	2.0	8.4
$ØY_2a$		2.0	9.3	2.0	9.0
$ØY_2b$		2.0	9.3	2.0	9.0

multiplet (see Figure 53) in keeping with the above assignments. Further splitting of the H-7 resonance into a double doublet, by meta coupling (49) with H-9 ($J_{7,9} \approx 2$ Hz) also occurred as shown in Figure 53. Partial overlap with some of the upfield signals of the dienone ring protons added to the complexity of the spectrum in this region.

A singlet at δ7.33, downfield of all but the H-6 resonance, was assigned to the C-3 proton, in keeping with its olefinic nature aided probably by its conjugation with the β-carbonyl group (50).

The four remaining protons in the spectrum of the OC molecule absorbed as an AA'BB' system (51), symmetrical about its mean chemical shift (δ7.07), which could be assigned satisfactorily to the protons of the p-cyclohexadienone ring in OC, derived from the aromatic B ring of the original chalcone. This type of spectrum is also shown typically by p-disubstituted benzenes (51,52) in which the various couplings are of the order shown below.



$J_{AB} \approx 7 - 10$ Hz

$J_{AA'} \approx J_{BB'} \approx 2 - 3$ Hz

$J_{AB'} \approx 0 - 1$ Hz

This system is nearly one in which the AB and A'B'proton pairs form two independent systems of coincident chemical shift which would then resonate as an AB-type quartet. The cross-ring coupling is responsible for the extra lines observed, which have not obscured, however, in the OC system,

the basic resemblance to the AB quartet. The simple AB coupling in p-cyclohexadienones, with the protons β to the carbonyl group the further downfield, can also be complicated by cross-ring coupling as in the aromatic system above (53). In the first-order analysis of the OC spectrum to provide the data given in Table 13, the AA'BB' system has been treated as a simple AB one with some error therefore in the values obtained for chemical shift and coupling constant. The approximate coupling constant obtained ($J_{2,3'} = J_{5',6'} = 9$ Hz) agrees with values found (9 - 10.5 Hz) for p-cyclohexadienones (53). While the assignment in OC of the AA'BB' system is made in respect of the p-cyclohexadienone system, rather than the para-disubstituted aromatic ring as in the original chalcone, it should be noted that on NMR evidence alone, either possibility may hold. However, other data necessitated assignment of the dienone ring system in OC.

Signals for both the enolic and phenolic exchangeable protons were not detectable for OC in the acetone solvent. All the signals discussed above persisted on addition of deuterium oxide.

Integration of the OC spectrum, after deuterium oxide had been added (Figure 54), provided support for the interpretation which has been discussed. Based on the integral for one proton, for example H-6 doublet or H-3 singlet, the total proton count for the spectrum was 8.5 (8 protons required in absence of enolic and phenolic signals), while for the AA'BB' system, the lower field half (AA') integrated for two protons. Further, all the signals upfield from the mean chemical shift of this system integrated for 4 protons,

namely the BB', H-7 and H-9 protons.

Thus, the NMR spectrum of OC indicated that all protons originally present in the chalcone A and B rings had remained in the rings of the new structure. This finding therefore excluded either ring as the site of the additional oxygenation detected in the molecule by mass spectrometry and consequently implicated oxygenation at either the carbon originally α or β to the carbonyl in the chalcone. The one-proton singlet observed in the OC spectrum showed an absence of protons on the neighbouring two carbons in keeping with the presence of the extra oxygen in the heterocyclic ring as a hydroxyl function bearing an exchangeable proton.

The spectrum of OC-methyl ether confirmed on integration that the compound was a mono-methyl ether; the methoxyl protons absorbed as a 3-proton singlet at δ4.02, a value typical of a phenolic methyl ether (50). A noticeable effect of this methylation was the relative downfield shift of the H-9 resonance. The spectrum for the H-6, H-7 and H-9 protons remained of the ABX type as in the case of OC.

In the spectrum of βOC, the structurally significant feature was the AX pair of doublets (δ5.16, δ3.96, J_{AX}=6.3 Hz) upfield of the main spectrum in the aromatic region. These doublets were assigned to the C-3 methylene group in the heterocyclic ring of this α-diketone tautomer of OC. Such protons would be expected to resonate well upfield of aromatic protons (48) and the difference in chemical shift indicated considerable contrast in the orientation of the individual protons in relation to the

diamagnetically anisotropic carbonyl function, at C-4. A Dreiding model of the proposed structure shows that, in all likely conformations of the molecule, one particular C-3 proton is approximately coplanar with the C-4 carbonyl group and would consequently be deshielded more effectively (48, 54, 55) than the other methylene proton.

The spectra of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ (Figures 56 and 57) also revealed in common a pair of doublets, upfield of the aromatic region, as required by the presence of vicinal protons in the heterocyclic ring of the proposed isomeric diol structure. Spectral identity of the $\not{\alpha}Y_2a$ and $\not{\alpha}Y_2b$ preparations of $\not{\alpha}Y_2$ (Figures 58 and 60) confirmed the conclusion from optical rotation measurement and i.r. spectroscopy that these two forms of $\not{\alpha}Y_2$ were enantiomeric. Differences between the $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ spectra, particularly in the chemical shifts of the C-H protons of the vicinal diol system, would be expected in such diastereoisomers (50, 54).

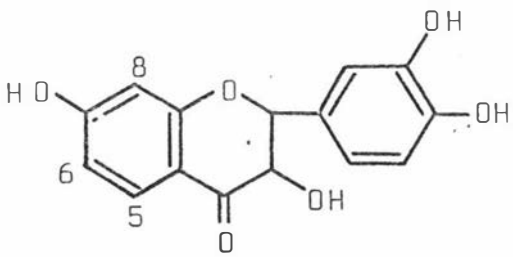
The assignment of the upfield pair of doublets in these spectra to the vicinal C-H protons of the isomeric diols is in accord with observed chemical shifts (50). Thus, each vicinal proton is α to an hydroxyl substituent in a 7-membered ring system and one of each pair is also α to a carbonyl group. No immediate separate assignment of chemical shift has been made for these protons (Table 13) in view of the uncertain conformational effects in the molecules which would influence the shielding of each proton by neighbouring groups. Since chemical shift data may make a contribution to the elucidation of the relative stereochemistry of the molecule (56), this question of assignment of shifts for $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ will be further considered later

(section 3A-2(b)) when additional evidence can be brought to bear.

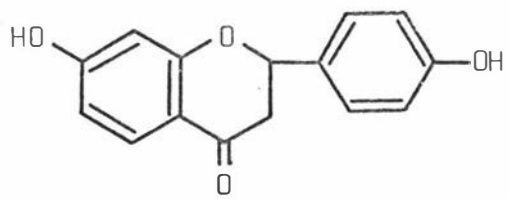
The coupling of these signals assigned to the H-3 and H-4 protons was similar ($J \approx 2$ Hz) in both $\not\!\!Y_1$ and $\not\!\!Y_2$ spectra. For vicinal protons the coupling constant may be used to define the relative stereochemistry according to the Karplus relation (56,55,57). However, this relationship of coupling constant to dihedral angle for vicinal protons is not necessarily valid for every system, for other factors such as the nature of neighbouring substituents and bond (H-C-C) angles can also influence the value of J (56,57). Hence, in the case of the proposed $\not\!\!Y_1$ and $\not\!\!Y_2$ structures, the deduction of conformational and stereochemical data directly from J values is uncertain, in the absence of data for closely related model compounds (compare the application of the Karplus relationship to deduction of the relative stereochemistry and conformation of flavan derivatives (55, 56)). In both $\not\!\!Y_1$ and $\not\!\!Y_2$, the mobile oxepin ring may permit conformational inversion so that the value of the coupling observed could possibly reflect a time average over the populations of a number of conformers. Therefore, while the isomeric $\not\!\!Y_1$ and $\not\!\!Y_2$ compounds may, on first analysis, be expected to show a distinct difference in vicinal coupling constant, the observed absence of any significant difference cannot be taken directly as evidence against the proposed structures. Put another way, no unequivocal interpretation can readily be taken from the observed coupling data.

The signals in the aromatic region of the spectrum of $\not\!\!Y_1$ (Figure 56) bear considerable similarity in pattern

to those of suitable model flavonoid compounds((47), see spectra numbers 34 and 36). The signals assigned to the aromatic ring protons H-6, H-7 and H-9 in $\not{\alpha}Y_1$ display relatively similar chemical shifts and couplings to equivalent protons (H-5, H-6 and H-8) in the model flavonoid, dihydrofisetin.



Dihydrofisetin



Liquiritigenin

The deshielding of the H-6 proton in $\not{\alpha}Y_1$ indicated the presence of the carbonyl function at C-5. As illustrated in Figure 56, half of the H-6 doublet is coincident with a line of the AB quartet absorption pattern of the dienone ring protons (see below); a separation of these coincident absorptions was clearly seen on addition of deuterium oxide. Similarly, the H-7 proton absorption is partially hidden in the upfield doublet of the dienone ring absorption. For the four dienone ring protons, a deceptively simple AB quartet is observed. This pattern is also typically observed for the protons of p-substituted flavonoid B rings, for example in liquiritigenin, where the difference in chemical shift of A and B protons is also about 0.5 ppm (47, 55, 56). The spin system again approximates to an AA'BB' case (56); the difference in comparison with the OC spectrum arises through the reduced chemical shift difference in the A and

B classes of protons in the latter case. Integrals recorded were in agreement with the upfield pair of doublets (diol) representing two protons and the aromatic signals a further 8 protons, as required by the analysis presented above. Again, hydroxylic proton signals were not observed.

Assignment of the $\not{O}Y_2$ spectra (Figures 57-60) in the aromatic region is generally the same as for the $\not{O}Y_1$ spectrum. The pattern of signals observed for the H-6, H-7 and H-9 protons is slightly different to that in $\not{O}Y_1$ and now corresponds more closely to that of the A ring protons in the model compounds dihydrofisetin and liquiritigenin. A pattern for the dienone ring protons, similar to that in $\not{O}Y_1$, persists in $\not{O}Y_2$, in agreement with the comparable chemical shift difference in A and B classes of protons in the two compounds.

Thus, the NMR spectra strongly support a very close relationship between the compounds $\not{O}Y_1$ and $\not{O}Y_2$ which is required by the facts of their formation from chalcone and their common transformations to OC and flavonol. Further, the essential difference between the OC and $\not{O}Y_1$ and $\not{O}Y_2$ structures has been identified as the presence of a pair of vicinal protons in the heterocyclic ring in the latter compounds in place of a single olefinic proton in the former. This is precisely what is required in going to the proposed diol structures. These structures will be found to receive further support from the observed chemical transformations (see section 3A-2).

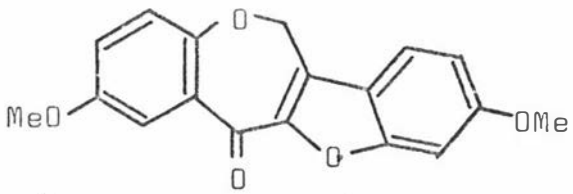
(d) Structural correlations from i.r. spectra. The i.r. spectra of OC and OC-methyl ether (Figures 43 and 44) show that methylation reduced in intensity, but did not

eliminate, the hydroxyl stretching vibration, 3200 cm^{-1} (OC) and 3150 cm^{-1} (OC-methyl ether), in agreement with the formation of a mono-methyl ether of OC, as confirmed by NMR and mass spectrometry. Evidence for this methylation, through the resultant alkyl-aryl ether linkage, was also found in the appearance of the band at 1280 cm^{-1} (C-O stretching) in the spectrum of OC-methyl ether (58).

The p-cyclohexa-2,5-dienone ring system typically gives rise to three bands in the i.r. in the 1590-1680 cm^{-1} region. A dienone doublet, apparently due to a splitting of the carbonyl absorption, accounts for the two of these bands which are separated by 20-40 cm^{-1} with the higher frequency member usually in the range 1660-1670 cm^{-1}, while the third dienone band, at lowest frequency of the trio and normally 20-25 cm^{-1} below that of the lower band of the dienone doublet, represents typically the C=C stretching vibration (53). Equivalent bands, which are attributed to the dienone carbonyl group vibration, are present in the spectra of OC and OC-methyl ether at 1650 and 1625 cm^{-1} in both cases. The third dienone band, however, overlaps the highest frequency aromatic C=C stretching vibration (about 1600 cm^{-1}) (59).

For the C-5 carbonyl group, the prominent band is located at 1710 and 1705 cm^{-1} for OC and OC-methyl ether respectively. While this frequency may be higher than expected for the stretching vibration of this carbonyl group in these compounds, the important influence which the state (solid, neat liquid, solution etc.) of the sample (KBr disc here) can have on the observed frequency must be kept in mind as a possible contributing factor (60).

Interestingly, a similar value (1710 cm^{-1}) has recently been recorded for the carbonyl absorption in the oxepin system below (61).



Factors which would be expected to lower the frequency of the C-5 carbonyl vibration of OC include conjugation with the aromatic ring and with the C3-C4 double bond. Additional contributions may come through hydrogen bonding with the C-4 hydroxyl and by the p-hydroxyl substituent at C-8 intensifying the effect of conjugation of the carbonyl group with the ring. To offset these factors, any movement of the carbonyl group out of a coplanar orientation with the aromatic ring would normally increase the frequency of vibration as would any additional strain effects arising from unsaturation and fusion in the oxepin ring. However, the u.v. spectrum of OC indicated the presence of an extended conjugated system of π bonds which would depend on coplanarity of the carbonly group with the aromatic ring (see below). A Dreiding model shows that this orientation of the carbonyl group would also favour hydrogen bonding with the adjacent enolic hydroxyl group. In a coplanar relationship to the carbonyl, however, this hydroxyl group could also exert a maximal field effect, as an α oxygen substituent, a factor which would operate to increase the

frequency of the carbonyl absorption (60, 62). Thus, more readily-identifiable effects which normally decrease rather than increase the carbonyl frequency are apparent and the observed frequency may then reflect, aside from the possibility of a sample state effect, some unidentified or anomalous effects in the carbonyl environment. As an illustration of such unexplained effects, Bellamy (60) details some anomalies in the frequency of the 4-carbonyl vibration encountered in certain flavones according to the hydroxylation pattern at the neighbouring 3- and 5- positions.

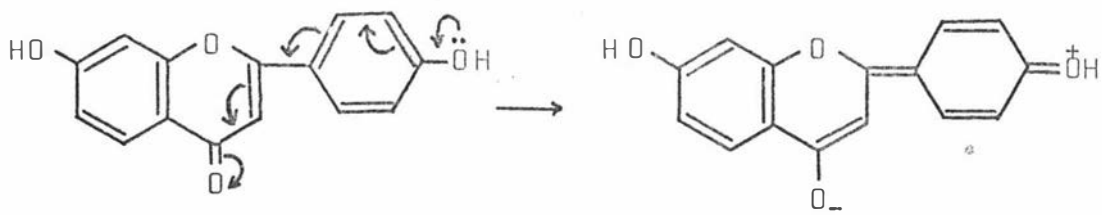
To enable direct comparison with the value observed for OC and to illustrate the extent of the frequency difference, carbonyl (C-4) frequencies (KBr disc) are given for a selection of 5-deoxyflavonoid compounds, all of which have the same A ring substitution pattern as OC ((18) and spectra specially recorded). Thus the values observed were; chalcone (isoliquiritigenin) 1620 cm^{-1}, γ_1 1660 cm^{-1}, flavonol (4', 7-dihydroxy) 1625 cm^{-1}, flavanone (liquiritigenin) 1650 cm^{-1}, flavone (4', 7-dihydroxy) 1635 cm^{-1} and aurone (hispidol) 1650 cm^{-1}.

In the case of ØOC, the i.r. spectrum (Figure 45) does not clearly show the extra carbonyl absorption which might be expected of this α-dicarbonyl structure. The frequencies observed for α-diketones will, however, depend upon the relative orientations of the carbonyl groups (60,62). If coplanar, cis-carbonyls may interact and show coupling in their vibrations. Alternatively, when the groups are twisted out of a coplanar orientation, coupling can disappear and then independent bands will occur for each carbonyl. This latter orientation of carbonyls is likely to occur in

ØOC due to the dipole-dipole repulsion of the adjacent cis-carbonyl groups (63). Thus, the diketone carbonyl absorptions in ØOC may be present as independent bands at 1700 and 1665 cm^{-1}, the latter band could overlap the highest of the dienone ring frequencies (another carbonyl band), the rest of which are not clearly resolved from the aromatic C=C absorption. Comparisons of the intensities of carbonyl bands in OC and ØOC may not necessarily be made in a straightforward manner since the type of carbonyl (for example, conjugated or unconjugated) affects the intensity by an amount which can reach, for example, a factor of four in ketosteroids (60). This intensity effect also raises the possibility that in ØOC both carbonyls vibrate at the same frequency and, in the absence of interaction to cause splitting, one band at 1700 cm^{-1} is observed. A similar result is found for o-quinones where two bands would be expected but only one is evident (60, 62).

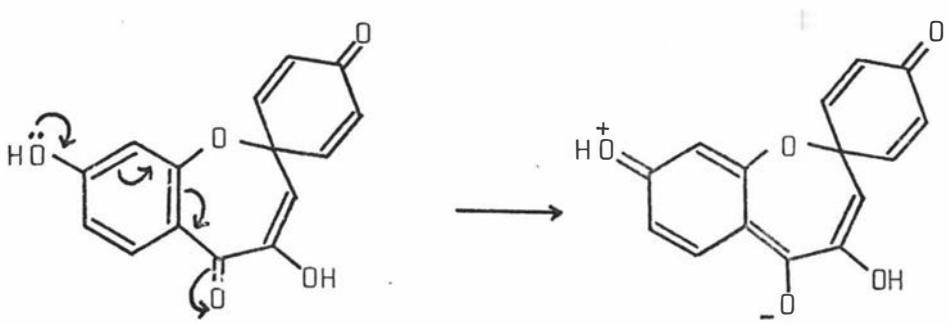
The major contribution of the i.r. spectra of $ØY_1$ and $ØY_2$ to the structural argument is the demonstration of identity of spectra of $ØY_2a$ and $ØY_2b$ forms of $ØY_2$. Such identity of spectra for samples with opposite optical rotations is conclusive evidence for the resolution of enantiomers.

(e) U.v.spectral data. The absorption spectrum of OC in ethanol is strikingly similar to that of 4',7-dihydroxyflavone in the same solvent. An electronic transition involving the cinnamoyl moiety is considered chiefly responsible for the 325 nm (Band I) absorption in the flavone system (64).



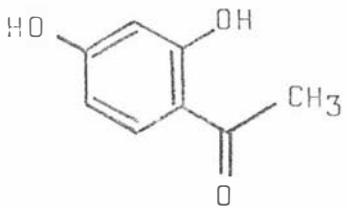
4', 7-dihydroxyflavone

In OC, the presence in the aromatic ring of para-orientated electron donating (C-8 hydroxyl) and electron-withdrawing (carbonyl) substituents would facilitate the predominant generation of the resonance form shown below upon electron transfer (65).



OC

This resonance of a benzoyl chromophore, however, gives rise typically to resacetophenone-like absorption in which the main band is at 275 nm and a weaker band at 315 nm approximately (65)



λ max 277 nm
and 314 nm

Resacetophenone

Thus, the resacetophenone-type spectrum of 5-deoxyflavonoids such as garbanzol, liquiritigenin and Y_1 and Y_2 reflects the predominant contribution of the benzoyl chromophore. Hence in OC, additional factors, presumably associated with the enolic system, must operate to reduce the transition energy to that of the flavone system and cause the absorption band to shift to 325 nm. This situation finds some parallel in the case of the aurone structure which absorbs at a higher wavelength than similarly oxidised flavonoids (example, flavones), a phenomenon for which several explanations have been proposed(64). Whatever the effect which operates in the OC molecule, the high wavelength of the absorption maximum suggests that a near planar system of π bonds is likely to occur in the molecule since deviations from planarity in conjugated systems are associated with distinct reductions in the wavelengths of absorption maxima (65). Examination of a Dreiding model indicates that the C3-C4 double bond is optimally aligned and nearly coplanar with the aromatic ring when the carbonyl group and aromatic ring are also coplanar. Hydrogen bonding with the enolic hydroxyl, then also at maximum effectiveness, may stabilise

the molecule in this conformation.

The absorption of the dienone ring system is chiefly in the 230-250 nm region (53) and may be largely responsible for the band in this part of the OC spectrum.

No bathochromic shift in OC absorption occurred on the addition of aluminium chloride to a solution of the compound in methanol (66) in contrast to the effects noted with 3- and 5-hydroxy flavones in which chelation gives rise to new chromophores and consequent bathochromic shifts.

Ionisation of the major chromophore in OC is almost complete upon saturation of the solution with sodium acetate, which is in agreement with similar ease of ionisation of a phenolic group para to the carbonyl as, for example, in flavonoid compounds (64). The slight further change on the addition of alkali probably reflects the limited effect of ionisation of the enolic hydroxyl.

Spectral data for OC-methyl ether, in ethanol alone and with sodium acetate added, are as expected for the compound with the readily-ionised hydroxyl function at C-8 methylated. Methylation in flavones of 7- and 4' hydroxyl functions produces only a small effect on the relevant absorption bands (64), the p electrons of the oxygen atom being still available for conjugation with the ring system. The absence of effect of sodium acetate on the OC-methyl ether spectrum is in agreement with the interpretation that the spectral change found for OC with this reagent is entirely due to ionisation of the hydroxyl group now methylated. The effect of a free C-8 hydroxyl group on the alkali stability of the molecule was evident in the rapid degradation of OC-methyl ether upon addition of alkali.

Being the diketo form, ØOC showed a spectrum in ethanol which approached that of an expected resacetophenone-type absorption to a considerable extent, in contrast to OC. This clearly illustrates the extent of change wrought in the chromophoric system by enolisation. Addition of alkali catalysed tautomerisation of ØOC to the more stable enolic system.

Both $ØY_1$ and $ØY_2$ exhibited spectra in keeping with the diol structures for which the chromophore is typified by those 5-deoxyflavonoids such as garbanzol and liquiritigenin in which the carbonyl is conjugated only with the A ring. Again, sodium acetate was sufficiently basic to fully ionise the activated hydroxyl group at C-8. Irreversible transformation to OC took place in stronger base and occurred rapidly on addition of alkali.

3A-2 Chemical evidence for the diol structure of $ØY_1$ and $ØY_2$.

The diol structure proposed for $ØY_1$ and $ØY_2$ has to accommodate not only the spectral data presented in the previous section but also the various facile chemical transformations of these two compounds which have been observed. As a consequence of these transformations, interrelationships between compounds have been established which collectively provide much support for the individual structures assigned. Structural interrelationships concerning $ØY_1$ and $ØY_2$ are discussed in this section.

(a) Rearrangement of $ØY_1$ and $ØY_2$ to ØOC The observed conversion of $ØY_1$ and $ØY_2$ to ØOC is readily explicable in terms of dehydration of a vic-diol structure in a carbonyl-forming elimination reaction, namely a pinacol-pinacolone

type rearrangement (Scheme 2). This reaction, which occurs under acidic conditions, may be considered to proceed by the formation of a carbonium ion intermediate (67). The relative ease of removal of the hydroxyl groups in the diol and hence the direction of the rearrangement (68) will depend on the relative stability of the alternative carbonium ions. Since carbonium ion character is unlikely to develop α to the carbonyl in the ØY compounds, the hydroxyl β to the carbonyl will be lost, resulting in an α-diketone as the rearrangement product. Thus, on mechanistic grounds, the assignment of the α- rather than the β-diketone structure for ØOC is indicated. Formation of ØOC from both $ØY_1$ and $ØY_2$ is evidence for the diol structure in both compounds.

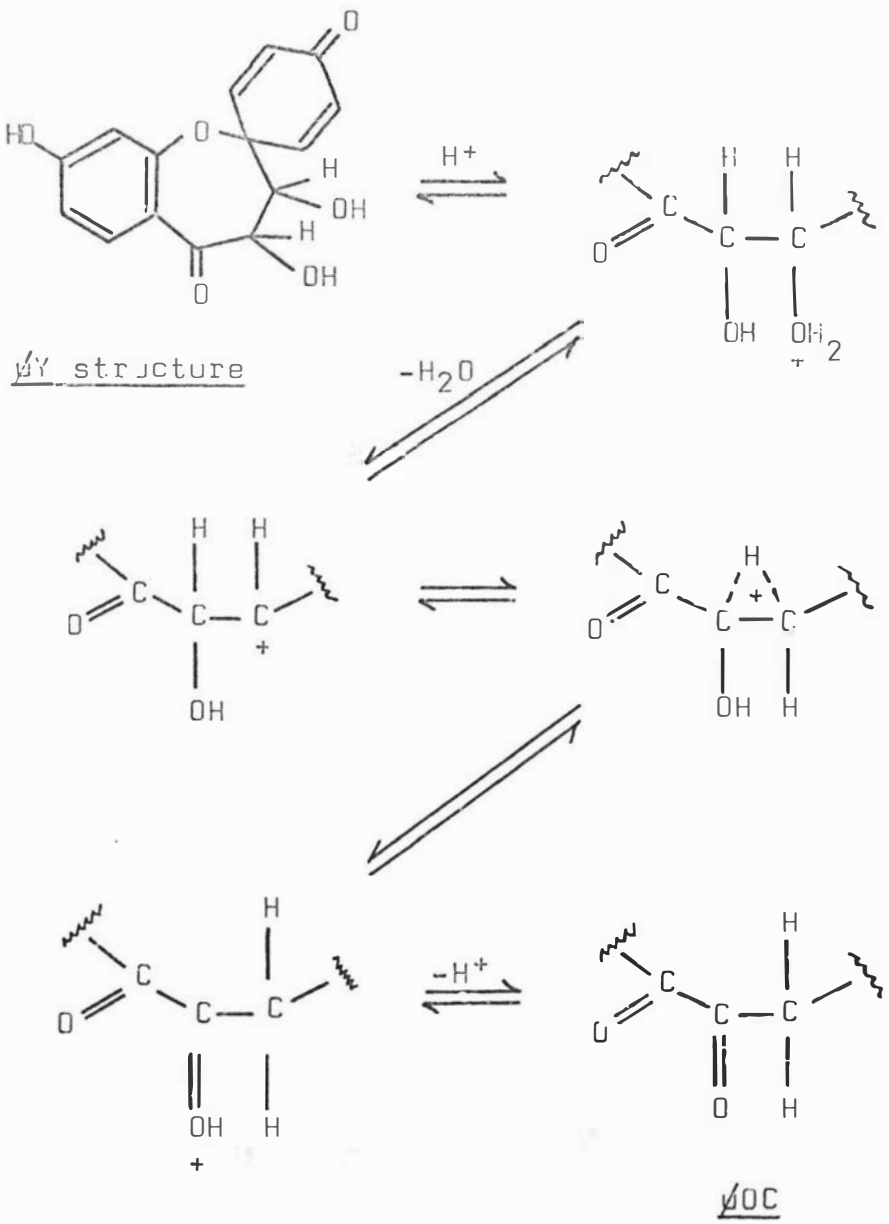
It is recalled that experimental conditions which favoured ØOC formation were always definitely acidic, if only slightly so. This acidic condition was ensured by ether extraction of ØY compounds from aqueous buffer acidified to pH 2 - 3, following enzymic reaction, and concentrating the wet ether extract, as required for the stabilisation of the ØY compounds. After removal of the ether, subsequent manipulations of the ØY sample which resulted in ØOC formation (short term storage of moist samples at room temperature or solution in acetone) would have preserved this acidic environment.

(b) Base-catalysed conversion of $ØY_1$ and $ØY_2$ to OC.

The ready conversion of $ØY_1$ and $ØY_2$ to OC under basic conditions is consistent with the α,β-dihydroxyketone structure proposed for the ØY compounds. This conversion is clearly an olefin-forming elimination (dehydration) reaction (69).

Scheme 2

The pinacolic rearrangement of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to $\not{\alpha}OC$



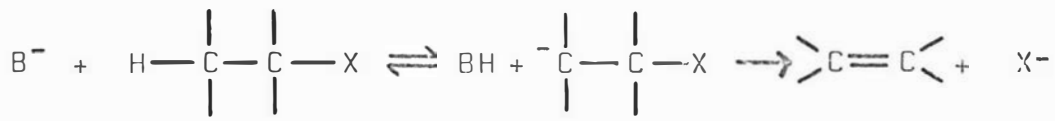
$\not{\alpha}Y$ structure

$\not{\alpha}OC$

The most widespread mechanism of base-catalysed elimination is the bimolecular (E2) mechanism (69) which operates by the concerted process summarised in equation 3.

Equation 3

In addition, eliminations involving ionic intermediates in non-concerted mechanisms are recognised (69, 70). Such a mechanism involving an anionic intermediate (the E1cB mechanism) is depicted in equation 4 (70, 71).

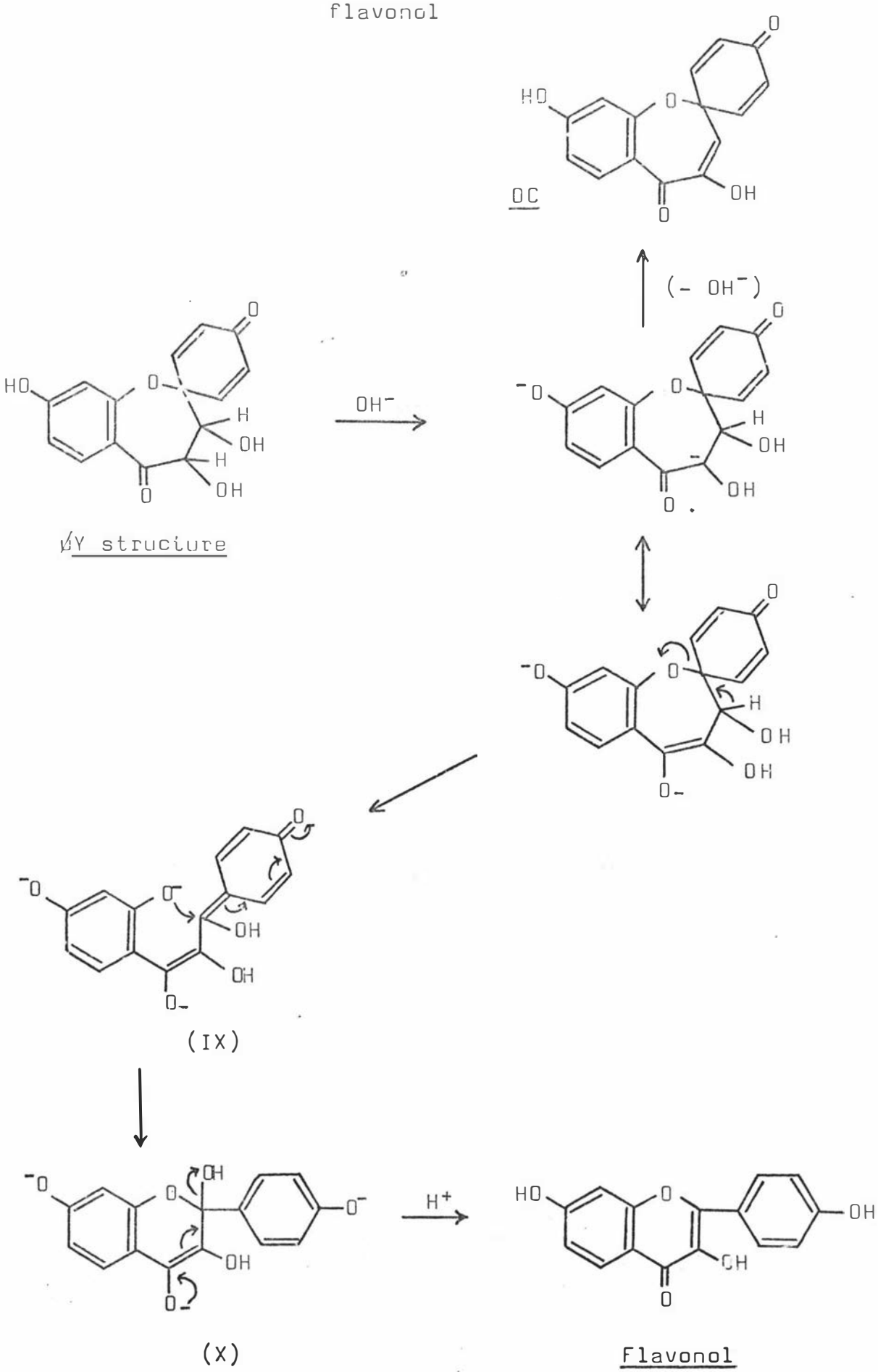


Equation 4

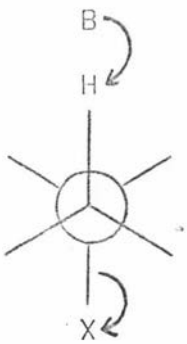
Recently, Bordwell et al (72) have suggested that most eliminations in systems where the proton lost is activated by an adjacent electron-withdrawing group proceed by a carbanion mechanism. Thus in ϕY_1 and ϕY_2, the presence of the carbonyl group will increase the acidity of the hydrogen atom attached to the α-carbon (C-4) and may be expected to promote elimination from these compounds by an E1cB mechanism. The subsequent elimination of a hydroxyl ion from the β-carbon (C-3) will result in the enolic form of an α-diketone, which is OC (Scheme 3).

Scheme 3

Base-catalysed transformations of ϕY_1 and ϕY_2 to OC and flavonol

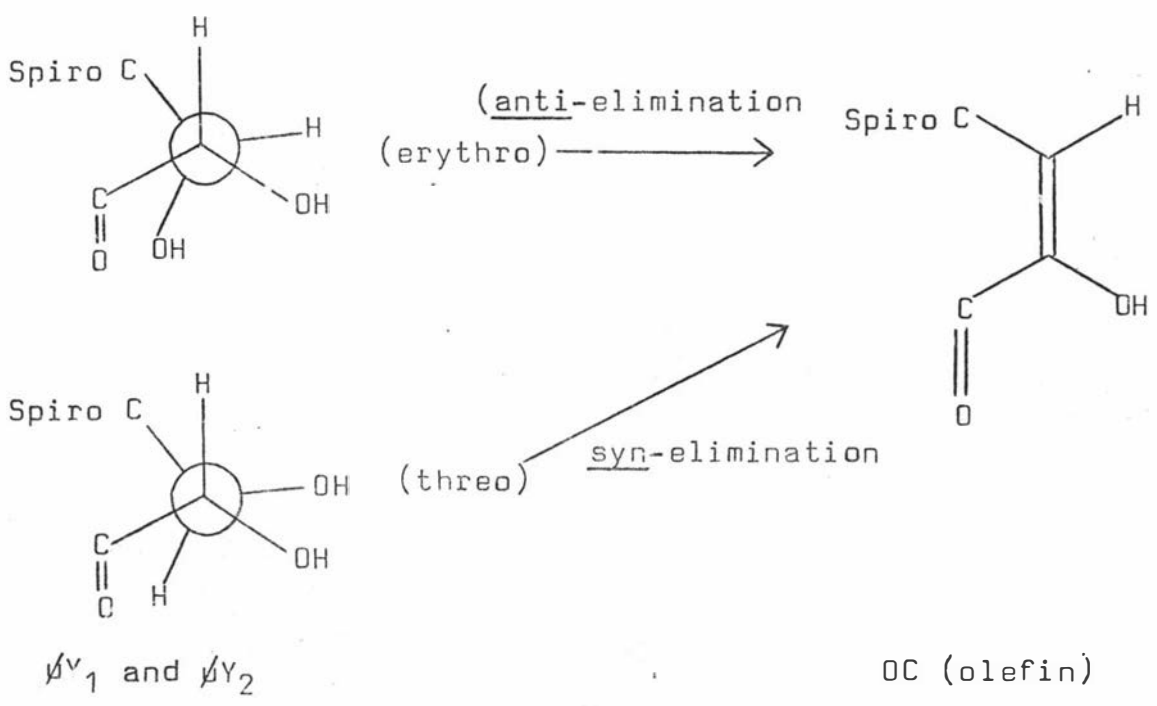


The preferred stereochemical course of E2 reactions has been noted to be one of anti elimination (68,70,73) in which the four atoms involved in the elimination reaction are coplanar in the transition state.



When base-catalysed eliminations have not been anti-stereospecific (for example, syn elimination), the carbanion mechanism (E1cB) has been frequently invoked to explain the different stereochemistry of elimination.

The E1cB mechanism accommodates conveniently a number of features of the reaction of ϕY_1 and ϕY_2 in the presence of base. It permits, in particular, rationalisation of the observed similarity in the rates of elimination of the diastereoisomeric erythro and threo forms, both of which, furthermore, give the same olefinic product, OC.



This is an example of a stereoconvergent elimination (that is, where two diastereoisomers give the same olefin, one necessarily by a syn elimination) which is an expected feature of the unimolecular mechanism.

As a consequence of the limited difference in rates of elimination from $\not\beta Y_1$ and $\not\beta Y_2$, no indication is available from the data as to the assignment of erythro or threo form to $\not\beta Y_1$ or $\not\beta Y_2$. However, a tentative assignment may be made on further analysis of the chemical shifts of the C-3 and C-4 protons in the NMR spectra of $\not\beta Y_1$ and $\not\beta Y_2$, given certain assumptions as to conformation. Thus it is assumed that both compounds exist largely in the conformation with the C-5 carbonyl and C-4 hydroxyl groups coplanar with the aromatic ring, for which the above Newman projections are relevant, as this would favour strongest hydrogen bonding. In this assumed conformation, the resonance of the C-4 proton would be expected downfield of the C-3 proton by virtue of the carbonyl α to the former (50). In the erythro and threo forms, shielding of H-4 by the carbonyl is common but shielding of this proton by the C-3 hydroxyl (C-O bond) is likely to be greater in the threo form (compare C-C bond shielding of axial and equatorial protons (54)). Thus the H-4 signal is predicted to be relatively upfield in the spectrum of the threo form.

Concerning the shielding of the C-3 proton, the effect of the dienone ring and bonds therefrom will be equal in the threo and erythro forms since the proton is merely switched between equivalent orientations in these forms. Similarly, there is little differential effect expected of the C-4 hydroxyl group. However, between forms there is an

appreciable difference in orientation with respect to the C-5 carbonyl group; in the threo form the C-3 proton lies much closer to the shielding cone of this group (54). Thus this effect would cause a relative upfield shift of the H-3 signal in the spectrum of the threo form. In addition, shielding by the C-C bonds of the oxepin ring would tend to reinforce this effect. Hence for the threo form, both the H-3 and H-4 signals are predicted to be upfield of those for the erythro form. Chemical shifts observed for $\not{\alpha}Y_1$ (δ5.32, 5.15) and $\not{\alpha}Y_2$ (δ5.19, 4.65), assigned in relation to the above argument, thus suggest that $\not{\alpha}Y_1$ is the erythro and $\not{\alpha}Y_2$ the threo compound.

(c) Base-catalysed rearrangement of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to flavonol. The base-catalysed conversion of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to flavonol was clearly shown to be a competing process with the elimination of water to form OC (Figures 31 and 32); the formation of flavonol being promoted by higher concentrations of base. The carbanion mechanism of elimination in OC formation, discussed atove (Scheme 3), conveniently accommodates this competing rearrangement to flavonol. Thus, the E1cB mechanism generates a carbanion intermediate which may be envisaged as a common intermediate in the subsequently divergent pathways of OC and flavonol formation (Scheme 3). In competition with the second and final step of OC formation (elimination of hydroxyl ion) the base-catalysed removal of a further proton from the intermediate is envisaged as the initial step of the flavonol pathway. Subsequent rearrangement by heterocyclic ring opening would lead to the quinone methide structure (IX). Cyclisation by internal addition of the phenoxide anion to the reactive

quinone methide structure would re-establish the aromaticity of the B ring system and concurrently generate the flavonoid skeleton in the intermediate form (X). This intermediate would then readily eliminate hydroxyl ion as shown to form flavonol.

Again, the similarity of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ is demonstrated in that both undergo competitive elimination and rearrangement reactions to yield OC and flavonol. Just as $\not{\alpha}Y_1$ eliminates water to form OC somewhat more readily than $\not{\alpha}Y_2$, a more competitive rearrangement to flavonol at the lower concentrations of base is also observed with $\not{\alpha}Y_1$ (Figure 32).

The presence of trace precursors of flavonol, detected chromatographically (section 2C-3(d)), would indicate the presence of intermediates in the rearrangement of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ to flavonol (assuming that there is no alternative pathway to this compound in the system). Isolation of the quinone methide intermediate (IX), postulated in Scheme 3, would not be expected, but an addition product with another nucleophile such as water may be isolated and may subsequently rearrange to flavonol. It is distinctly possible that the hydrated flavonol, as intermediate (X), may be isolated upon acidification if its rate of elimination to give flavonol is slow in relation to its rate of formation.

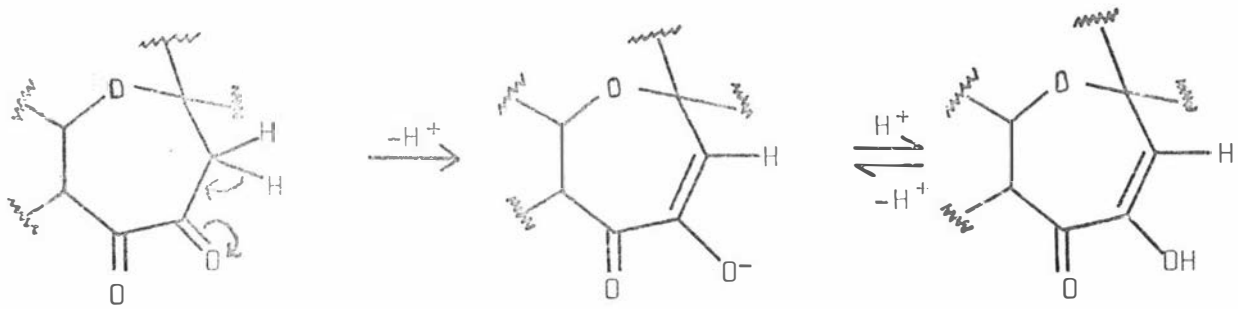
(d) Reaction of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ with cupric ion. The destruction of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ in pH 8 tris buffer upon addition of cupric ion is characterised by the appearance of fragment molecules, including p-hydroxybenzaldehyde. This ready degradation may be attributed to an oxidative glycol cleavage reaction (74) catalysed by copper and as such would provide added support for the proposed vic-diol grouping in $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$.

Circumstantial evidence for glycol intermediates in the base-promoted formation of flavonol from ØY compounds may be gleaned from the relatively greater suppression of flavonol than of OC formation which occurred in the presence of copper (section 2C-3(b)). Although a factor in this greater reduction in flavonol formation has earlier been rightly identified as the reduction in base concentration because of the presence of copper, the relative extent of flavonol suppression, however, was such that a further unique means of restricting flavonol formation may well have operated. An additional specific means of control, affecting the flavonol-forming pathway only, is now suggested to have operated by virtue of the existence of a new flavonol-specific diol precursor, such as intermediate (X) of Scheme 3, which may be degraded also in a copper-catalysed reaction.

3A-3 Reactions of OC and ØOC

The α-diketone structure for ØOC follows from the pinacolic rearrangement of the diols $ØY_1$ and $ØY_2$ just as the base-catalysed elimination of water from these compounds predictably leads to the same oxygenation pattern in the enolic form, OC. Greater stability of OC is predictable on the grounds of dipole-dipole repulsion of the carbonyl groups in the cyclic α-diketone ØOC(63). Enolisation of ØOC occurs readily, even on solution and heating in an organic solvent, as well as on alkali treatment.

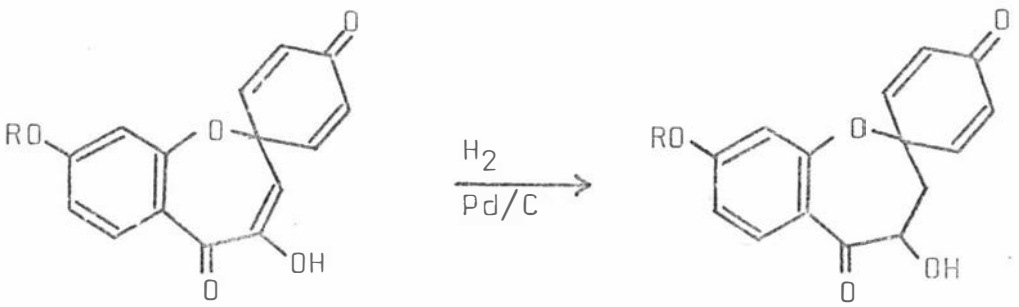
The enolisation reaction can be envisaged as follows (63).



The formation of hydroquinone upon hydrogenation of OC and OC-methyl ether and to a much lesser degree upon certain manipulations of OC under mild conditions (for example, polyamide column chromatography with aqueous ethanol, section 2D-3(d)) is conceivable from a spiro structure such as OC where the cyclohexadienone ring is para-substituted and the tendency for reversion to the aromatic system is likely to be a significant driving force in its reactions (53). Hydrogenation of OC or OC-methyl ether over palladium should lead to ready reduction of the C3-C4 double bond conjugated with the carbonyl group (75). The elimination of hydroquinone from this reduced form may then occur, possibly by the type of sequence shown in Scheme 4. Hydrolytic cleavage of the spiro ether bond in dihydro-OC is envisaged, by analogy with the relative ease of cleavage of tertiary or allylic ether structures (76). Elimination of hydroquinone may then occur in conjunction with the attainment of aromaticity. The hydrolytic cleavage to liberate hydroquinone leaves a second molecule from which 7-methoxychroman-4-one, tentatively identified as a product from the hydrogenation of OC-methyl ether, may be derived. Scheme 4 accounts for the formation of this compound via

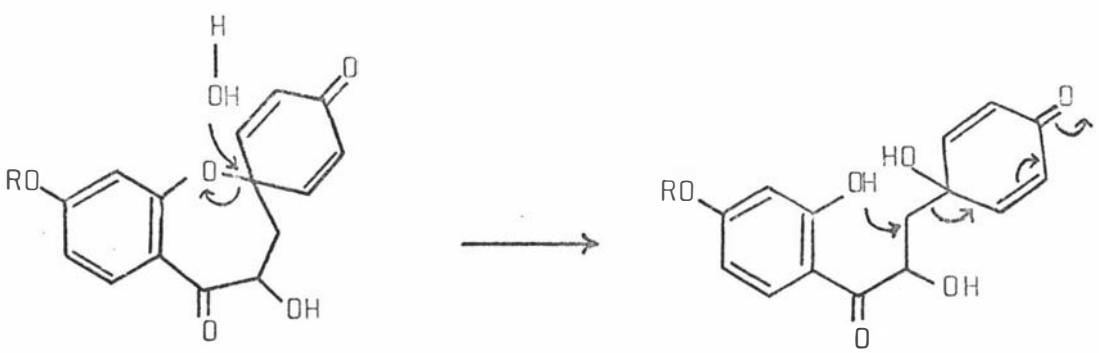
Scheme 4

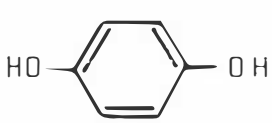
Proposed route for hydroquinone and chroman-4-one formation upon catalytic hydrogenation of OC and OC-methyl ether



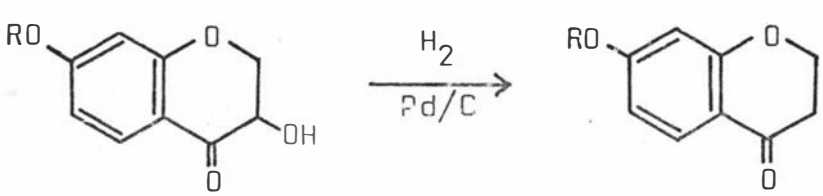
OC: R = H

OC-methyl ether: R = CH_3





hydroquinone

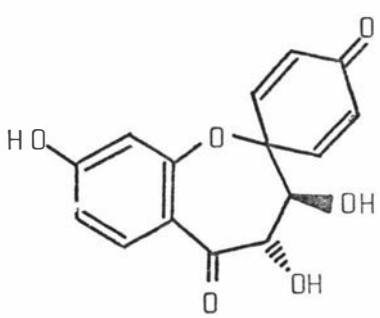


R = CH_3: 7-methoxy-chroman-4-one

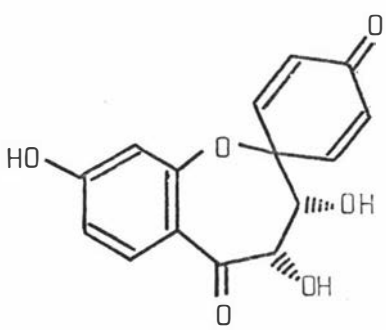
its 3-hydroxy derivative which would be expected to undergo ready hydrogenolysis as shown (77).

3A-4 Postulated epoxide precursors of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$.

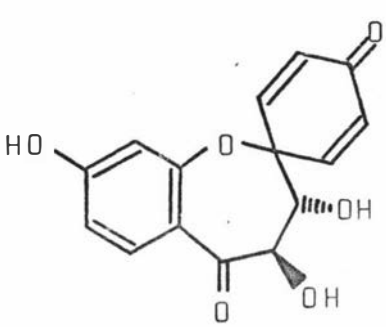
The diols $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ are the first products which can be isolated from the enzymic oxidation of chalcone and there is no evidence that the spectrophotometrically observed initial product is not also essentially a mixture of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. Thus, the enzymic reaction apparently gives rise to comparable amounts of both $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ (Table 7). Furthermore, $\not{\alpha}Y_2$ consists of two enantiomers and this also presumably holds, by analogy, for $\not{\alpha}Y_1$. Hence four stereoisomers of the diol structure are formed. Their configurations are reproduced in (XI) - (XIV)



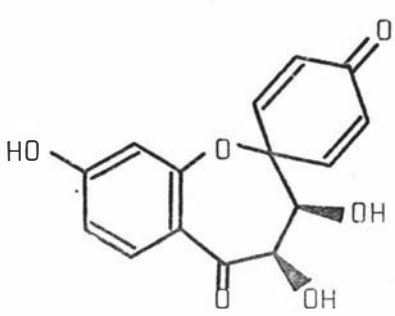
(XI)



(XII)

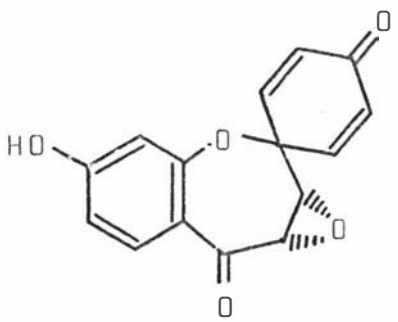


(XIII)

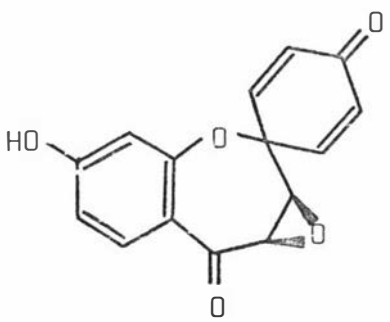


(XIV)

Since no interconversion between isomers under conditions of their formation or separation could be detected, it remains that their formation must be explicable in terms of the product of oxidation of the chalcone substrate. This complex situation can be best interpreted, in the light of the restrictions imposed by biochemical data and in mechanistic terms, by postulating the formation of two enantiomeric epoxides, (XV) and (XVI), as the true initial product of the enzymic reaction and consequently as the immediate chemical precursors of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ diols.



(XV)



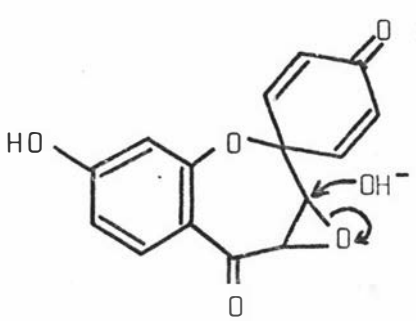
(XVI)

Spontaneous hydration of these reactive epoxides could then chemically generate all four isomers (XI) - (XIV) of the $\not{\alpha}Y$ diol structure. This requires each epoxide to give rise to two diastereoisomeric diols, one a $\not{\alpha}Y_1$ and the other a $\not{\alpha}Y_2$ isomer. Thus, (XV) is considered to be the progenitor of (XI) and (XII) and (XVI) the corresponding progenitor of (XIII) and (XIV). The implications of this proposal will now be considered more closely in relation to the current understanding of epoxide ring-opening mechanisms and stereochemistry (78, 79).

Epoxide ring opening reactions under basic or neutral conditions involve attack by a nucleophilic species (78) which is a water molecule or hydroxyl ion in the case of diol formation (equation 5).

Equation 5

Attack at a particular one of the two carbon atoms of the epoxide will be influenced by a combination of steric and electronic (inductive and conjugative) effects. The presence of an electron-withdrawing carbonyl group adjacent to the epoxide ring results in breaking of the C-O epoxide bond at the carbon β rather than α to the carbonyl on account of electron release from the former centre being favoured (78). In the particular examples of epoxides (XV) and (XVI), the carbonyl group would thus be expected to direct attack to the C-3 (β) carbon with opening of the epoxide ring in the direction indicated below.

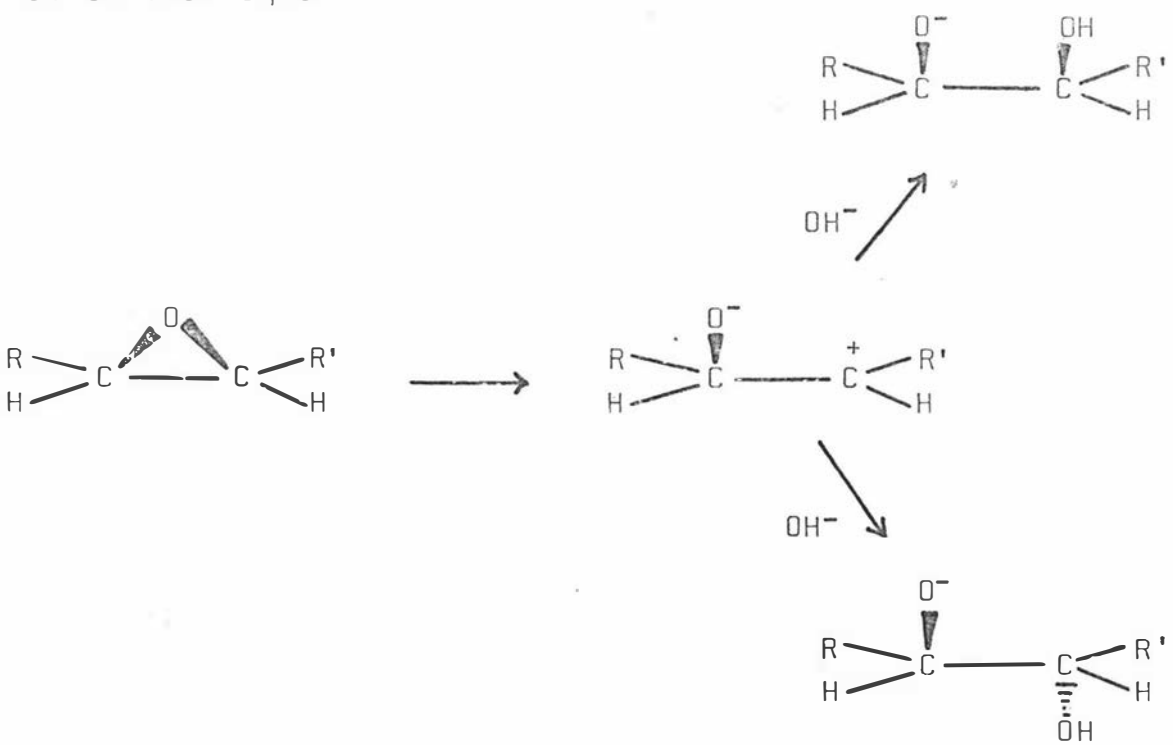


Epoxide ring opening reactions occur usually by an S_N2 mechanism (78). This mechanism has the stereochemical consequence that inversion of configuration invariably results at the carbon atom attacked, as illustrated below, since in the attack, a back-side approach of the nucleophile occurs.

This S_N2 mechanism, applied to the epoxides (XV) and (XVI) would lead only to the formation of the enantiomeric forms of the trans-diol, (XI) and (XIII). Thus, to account for the formation of the missing pair of cis isomers, (XII) and (XIV), a mechanism of epoxide opening at C-3 which would also permit retention of configuration, must be implicated. Inversion of configuration would lead to the formation of either a $\not{\alpha}Y_1$ isomer and retention of configuration to a $\not{\alpha}Y_2$ isomer or vice versa, depending on which compound is the threo (trans-diol) and which is the erythro (cis-diol) form. Since neither $\not{\alpha}Y_1$ nor $\not{\alpha}Y_2$ isomers predominate in the product mixture to any great degree (Table 7), retention and inversion of configuration at C-3 in the opening of the epoxides would need to be adequately competitive to account for the $\not{\alpha}Y_1$:$\not{\alpha}Y_2$ ratios observed in the product.

One such mechanism of epoxide opening which is applicable to this situation is the S_N1 mechanism in which an

intermediate carbonium ion is formed (78). With a coplanar, trigonal carbon atom, this intermediate permits bi-directional approach of the nucleophile giving rise to both cis and trans products. Normally the facility of approach would differ between directions because of the disposition of substituents in the molecule and hence the proportions of the two diastereoisomeric products would tend to be unequal rather than equal.



Ingold (80) discussed a pH-independent hydrolysis of the epoxide ring which may occur by a combination of separate S_N2 and S_N1 mechanisms and which is typically encountered under neutral aqueous conditions. In terms of the S_N1 contribution, the production of two stereoisomers would be expected, one of which would be identical with the exclusive product of the S_N2 mechanism. Thus the observed ratio of diastereoisomers in the product would be controlled in an even more complex manner than would be encountered in the S_N1 mechanism above.

The observed variation in the ratios of $\not{\alpha}Y_1$:$\not{\alpha}Y_2$ and in $\not{\alpha}Y_2$a:$\not{\alpha}Y_2$b isomers (Table 7) can now be considered against this background of the postulated origin of these diols and of epoxide ring opening mechanisms. Firstly, the ratios of enantiomers, $\not{\alpha}Y_2$a:$\not{\alpha}Y_2$b, should reflect directly the proportions of epoxides (XV) and (XVI) in the enzyme product, irrespective of the details of the mechanisms responsible for the postulated retention and inversion of configuration in diol formation, since the mirror image epoxides would undergo identical reactions in the common conditions of a given reaction medium. Thus a consistent, definite, trend in the balance between epoxides (XV) and (XVI) is apparent from the ratio of $\not{\alpha}Y_2$ enantiomers observed as the reaction pH drops from 8 to 7. This clearly reproducible trend contrasts with the absence of a unifying pattern in the balance between $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ diastereoisomers. Since this ratio would represent, in the above hypothesis of diol formation, the net balance of <u>cis</u> versus <u>trans</u> attack in opening of the epoxide ring, it is reasonable to accept the occurrence of much more random variation in this balance because of the plurality of the factors which determine it and the likelihood of subtle influences affecting their competitiveness. The definite pattern in the ratios of $\not{\alpha}Y_2$ enantiomers, on the other hand, indicates a degree of control in epoxide formation which is readily accepted for an enzyme-catalysed reaction.

In summary therefore, it is held that the data in Table 7 show that significant amounts of each of the four isomeric diols are invariably present in the product obtained under the enzymic reaction conditions. Formation of these four isomeric diols from the two enantiomeric epoxide

precursors, the true initial enzymic product. requires both *cis* and *trans* attack in the spontaneous hydration of the epoxides, with *cis* attack giving the erythro and *trans* attack the threo diols. Hence the S_N2 mechanism of ring opening leads to the threo form. Characteristically, the proportion of $\not{\alpha}Y_2$ exceeds that of $\not{\alpha}Y_1$ in the product, (this measured difference (Table 7) is held to be real if greater than about 5-10% of the absolute value, on account of the carefully controlled procedures employed to obtain the data limiting any tendency for the somewhat greater lability of $\not{\alpha}Y_1$ to lead to losses greater than for $\not{\alpha}Y_2$). Thus, if the reasonable assumption is made that in the combined S_N1 and S_N2 mechanisms the product of the normally-favoured S_N2 mechanism should predominate, $\not{\alpha}Y_2$, the major component, would be equated with the threo form. This deduction is at least consistent in outcome with the one made on the basis of NMR chemical shift data (see section 3A-2(b)).

This concept of the epoxides as the true initial reaction product is of merit to rationalise not only the concurrent formation of four stereoisomeric diols but also the biochemical and chemical oxidations of chalcone. Consideration of the chemical reaction of chalcone with hydrogen peroxide at this juncture will facilitate discussion of the biochemical reaction to follow.

3B CHEMICAL OXIDATION OF CHALCONE WITH HYDROGEN PEROXIDE

In aqueous buffered media at pH 7-11, the oxidation reaction at room temperature yielded small amounts of OC and flavonol as the main products isolated. This

result strongly suggested that both products may have originated from $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ since, in the enzymic reaction, the same two products were also encountered in the absence of any precautions in the extraction step to stabilise $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. A direct demonstration of this origin of OC and flavonol in the chemical reaction was achieved by the isolation of product as $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ only, when suitable precautions were taken to virtually halt the transformation to OC and flavonol.

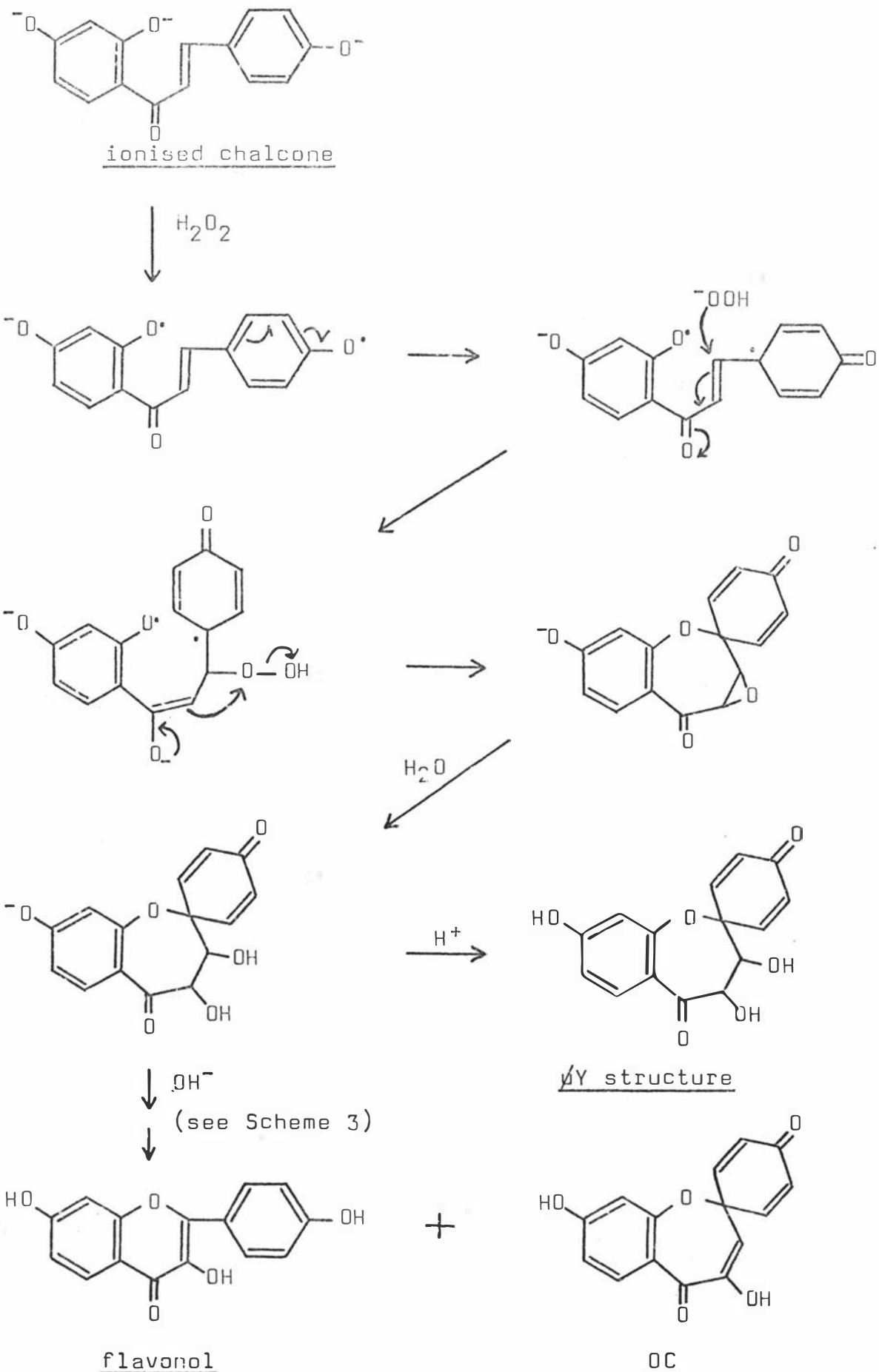
3B-1 Postulated scheme for the chemical reaction.

The chemical reaction of chalcone with hydrogen peroxide in aqueous buffered media pH7-11 at room temperature has been formulated (Scheme 5) as a $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$-forming reaction, through the intermediate isomeric epoxides as discussed previously. Hence, the chemical reaction is suggested to parallel the enzyme-catalysed reaction in terms of the true product in each case being the enantiomeric epoxides which then undergo further chemical reactions to form the range of extracted products, $\not{\alpha}Y_1$, $\not{\alpha}Y_2$, OC and flavonol.

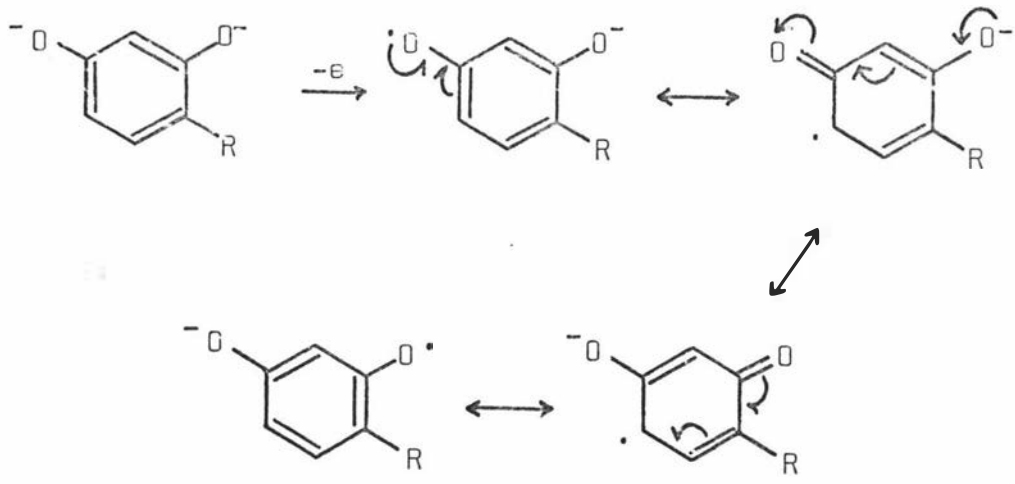
In Scheme 5 the fully-ionised chalcone molecule is represented, in keeping with the degree of ionisation apparent spectrophotometrically in the higher pH media where the rate of reaction is considerably greater. In the formation of the epoxide product, two separate oxidative processes are involved; firstly, intramolecular oxidative coupling and secondly, the introduction of an atom of oxygen, as the epoxide oxygen. The intramolecular coupling is most conveniently written as a free radical dimerisation; hydrogen peroxide being the acceptor of the two electrons removed in

Scheme 5

Postulated mechanism for oxidation of chalcone by hydrogen peroxide



discrete steps from separate A and B ring phenoxide oxygens of the ionised chalcone. The generation of phenoxy radical by one-electron oxidation of the phenoxide anion is the initial step envisaged in the oxidative coupling reactions of phenols (81). Resonance forms (81) of the chalcone diradical would be expected through interaction of each unpaired electron with the electrons of the respective aromatic A or B ring. Thus, the generation of the A ring radical could be accomplished in principle by electron extraction from either the 2' or 4' oxygen function of the chalcone.



Chalcone; R= p-coumaroyl

The oxygenation step is envisaged as a base-catalysed epoxidation of an α ,β -unsaturated ketone, a reaction which has been found in general to proceed by the nucleophilic addition of hydroperoxide anion to the β-carbon atom of the unsaturated ketone (82). Epoxide formation is completed by an intramolecular displacement of hydroxyl ion from the hydroperoxide intermediate. An important feature of this addition

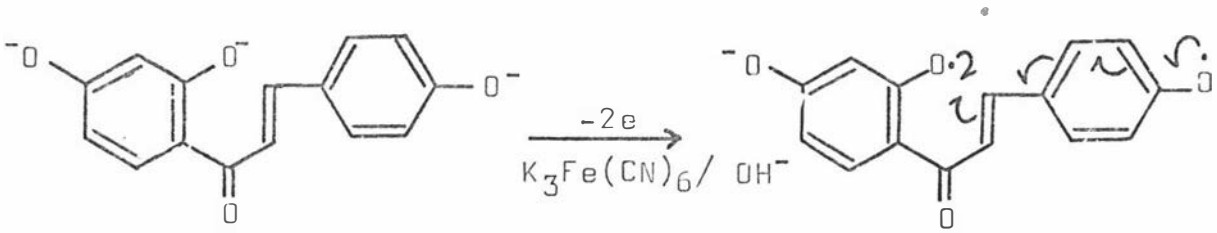
of hydroperoxide anion to chalcone is the conformational mobility achieved in the absence of the α,β -double bond, for this mobility is essential for making intramolecular coupling of the envisaged intermediate diradical a sterically feasible process. This dimerisation is impossible in the trans-chalcone system which exists originally on account of the distance between these radical centres (see (83) for examples of the existence of chalcones exclusively as trans isomers). The formation of isomeric epoxides is readily explained on the basis of competitive addition of hydroperoxide anion to either side of the double bond, since specific steric effects are likely to be absent in this system. Formation of the diradical may be expected to facilitate the epoxidation step since internal electronic inactivation (see example below) of the system towards attack by hydroperoxide anion (84), which can stem from any of the anionic oxygen functions in this particular chalcone, is correspondingly reduced.

The intramolecular coupling of chalcone to generate the oxepin ring system involves the formation of a linkage para to the oxygen of the dienone system which is also commonly found in cyclohexadienones formed by radical coupling (53). In phenoxy radicals, this para carbon atom is, next to the oxygen itself, the most favoured locus of the odd electron in the resonance forms(81).

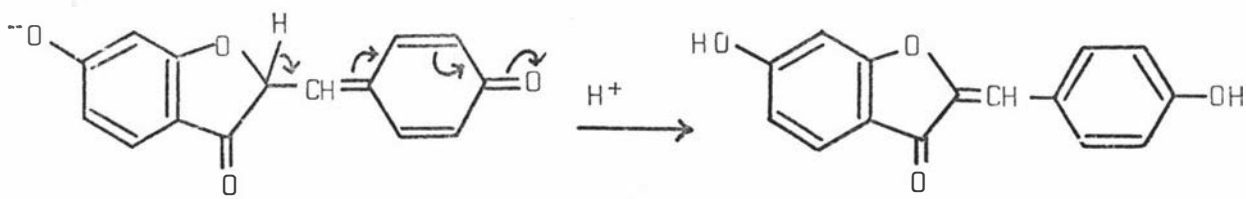
From the foregoing considerations it is clear that the oxygenation step is vital to permit the intramolecular coupling required for oxepin ring formation; in other words, for steric reasons, addition of hydroperoxide anion must precede heterocyclic ring formation. Epoxide ring formation

may well be completed as a rapid process which accompanies the radical coupling, since in the simple epoxidation of α ,β -unsaturated ketones, addition of hydroperoxide anion is the rate determining step in the reaction (82).

It is interesting to compare this oxidation by hydrogen peroxide in alkali with alkaline ferricyanide oxidation of the same chalcone, in which case the product (17) is the corresponding aurone, hispidol, formed in the following radical coupling process (84).



Chalcone (anion)



aurone (hispidol)

Here, in the absence of oxygenation of the exocyclic double bond, the conjugated system permits the radical centre to move freely to the carbon α to the carbonyl group whence immediate radical coupling is sterically possible and the heterocyclic 5-membered coumaranone ring system is formed, in contrast to the 7-membered oxepin ring. This contrast in product formation serves to highlight the well-established difference in the two oxidising agents compared; alkaline

ferricyanide being especially associated with dimerisation and intramolecular coupling in the products while hydrogen peroxide tends to effect hydroxylation more than coupling (81). Of course, only an oxidant with the dual properties (oxygenating and coupling) of hydrogen peroxide could effect the present oxidation of chalcone. The presence of suitably located free phenolic groups in the chalcone has the interesting effect of permitting oxidative coupling to accompany the postulated epoxidation reaction. Simple epoxidation of the unsubstituted chalcone structure has been shown to give only the trans-epoxychalcone, starting with either the cis- or trans-chalcone isomer (85). In the present reaction the epoxide formed is formally equivalent to a cis-epoxychalcone, this change from the normal epoxide geometry being ensured by the oxidative coupling step which generates the oxepin ring.

3B-2 A possible new insight into the mechanism of the A.F.O. reaction.

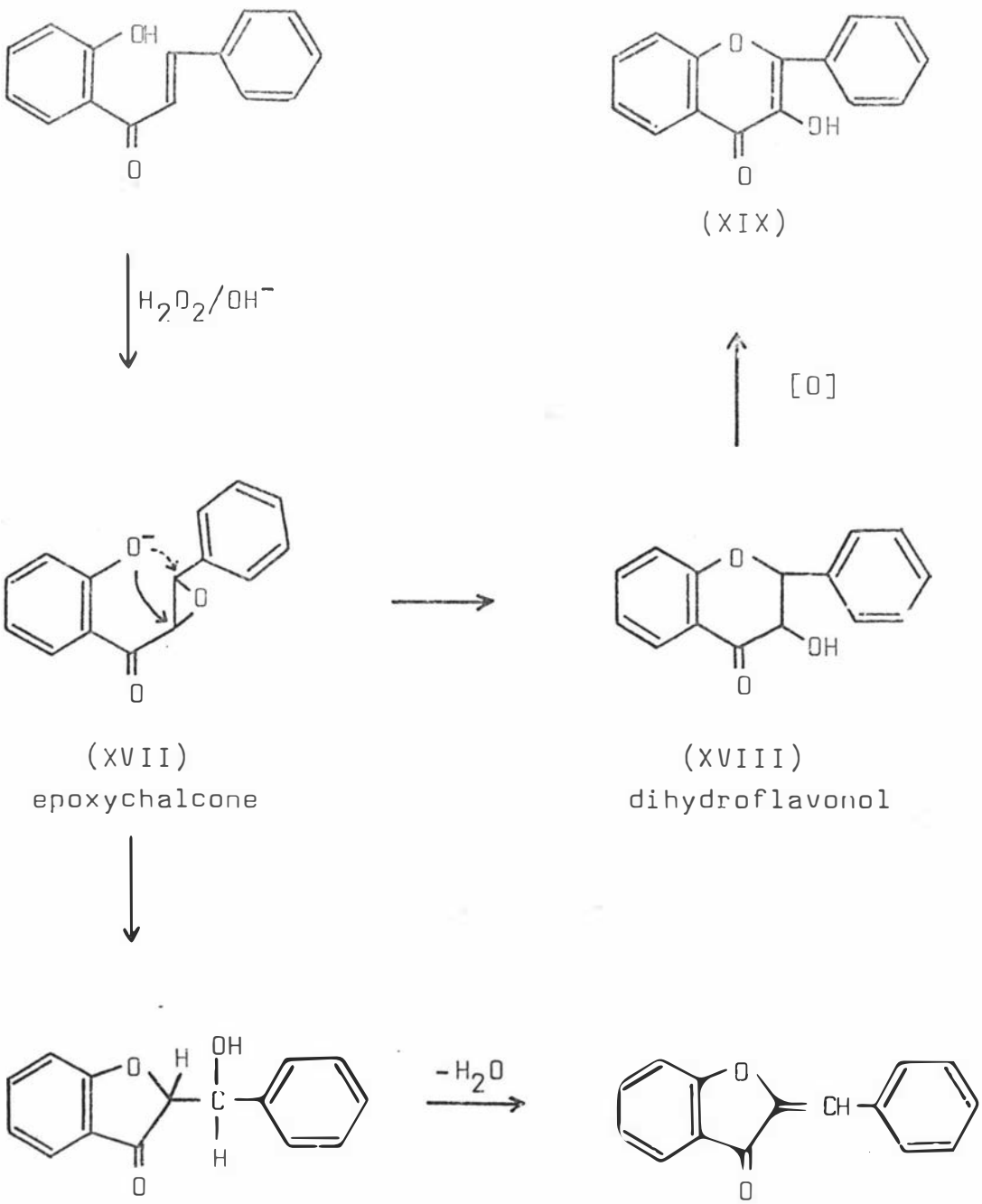
A plausible mechanism has been discussed in the preceding section for the chemical oxidation of chalcone by hydrogen peroxide in weakly alkaline media, pH 7-11 at room temperature. The trend of increased recovery of products as reaction pH increased, in keeping with increased rate of reaction, did not continue when still higher pH conditions were investigated. Rather, reduced amounts of the usual products, OC and flavonol, were recovered. This result probably reflected the extent of degradation of the main expected product, flavonol, rather than an inhibition of the reaction.

Of more direct interest however, under conditions of high pH, is the low temperature (about 0°) reaction of chalcone with hydrogen peroxide. This oxidation of chalcones by alkaline hydrogen peroxide constitutes an important general synthetic reaction affording chiefly the corresponding flavonols and is generally known as the Algar-Flynn-Oyamada (A.F.O.) reaction (84). In the light of the findings of the present study that precursors of flavonol are formed both in the chemical and enzymic oxidations of isocliquiritigenin in the presence of hydrogen peroxide, it is considered possible that the mechanisms and intermediates of the lower pH, room temperature, reaction system could be applicable also to the reaction under A.F.O. conditions, where such intermediates, if formed, would never be observed on account of their great lability. If correct, this would mean a considerable new insight is gained into the mechanism of the A.F.O. reaction which is still far from being settled in detail (84, 86, 87).

The situation prior to the key study of the A F.O. reaction by Dean and Podimuang (84) was that chalcone epoxides were favoured as intermediates in the formation of not only flavonols but also of aurones which were also often encountered as products and occasionally in major amounts. Thus, alkaline hydrogen peroxide was envisaged to convert the chalcone to the chalcone epoxide. Ring opening of this epoxide by an internal nucleophilic substitution would lead eventually to the flavonol or the aurone depending upon whether the attack was at the α- or β-carbon (see Scheme 6). This duality of the position of attack, and therefore of the direction of epoxide ring opening, is in

Scheme 6

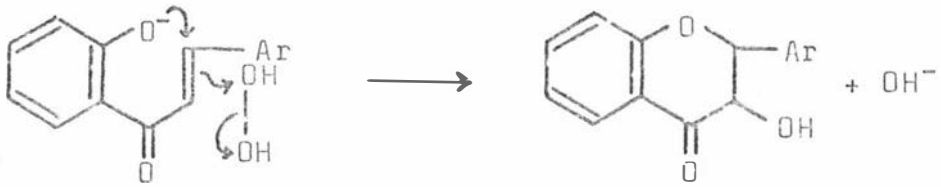
Postulated rearrangements of epoxychalcone to dihydroflavonol and hydrated aurone



agreement with the observed reactions of epoxides with an aryl group attached to one carbon atom and a carbonyl group to the other (78). The intermediate in flavonol formation by this sequence is the dihydroflavonol (XVIII) while the hydrated aurone (XX) is the intermediate in the aurone pathway.

While the postulated chalcone epoxide intermediate has never been isolated in the A.F.O. reaction, recent work has provided support for some of the postulated interconversions in Scheme 6. Thus the chalcone epoxide, specifically substituted as (XVII) (Scheme 6) has been synthesised and found to undergo epoxide ring opening with rearrangement to the corresponding dihydroflavonol (XVIII) upon treatment with alkali in deoxygenated solution (86). With oxygen present, further oxidation of (XVIII) to the flavonol (XIX) occurred. No aurone formation from the chalcone epoxide was, however, recorded.

Dean and Podimuang have recently examined mechanisms postulated in Scheme 6, however, and have concluded that a chalcone epoxide intermediate by no means accounts for the generation of products in many A.F.O. reactions (84). In particular, the evidence was found to be weighted against an epoxide intermediate in those reactions leading predominantly to the flavonol product. These authors have alternatively suggested the dihydroflavonol intermediate is formed in a concerted mechanism of cyclisation and hydroxylation as depicted below, rather than via the chalcone epoxide.



The case for the intermediacy of the dihydroflavonol in flavonol formation in the A.F.O. reaction apparently rests on work reported in the 1930's on this stepwise conversion (see (84)). The epoxide intermediate was however retained to explain aurone formation. This latter conclusion in turn has recently been questioned on stereochemical grounds (87).

The detailed 2-D chromatographic screening reported earlier in this thesis of the products of the A.F.O. reaction of the chalcone isoliquiritigenin during the course of the reaction (section 2E-2) showed clearly flavonol was always the main product. Minor amounts of two other products, garbanzol (dihydroflavonol) and Y_1 and Y_2 (hydrated aurone) were noted in the earlier hours of the reaction. The known course of flavonol formation operating both in the chemical reaction at lower pH and in the enzymic reaction does not implicate the dihydroflavonol as an intermediate.

It is proposed, therefore, that under A.F.O. conditions, flavonol is also formed by way of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ as outlined earlier in Scheme 5.

The effect of the low reaction temperature in the A.F.O. reaction is therefore seen to limit the destruction of flavonol predominantly. At room temperature, this

destruction would be very significant and would apparently approximate to the rate of flavonol formation. Transformation of $\emptyset Y_1$ and $\emptyset Y_2$ to the flavonol, to the exclusion of OC, may be reasonably accepted under the A.F.O. conditions of high pH, low temperature and slow rates of formation of these precursors. Certainly no OC exists in detectable amounts at any stage of the A.F.O. reaction.

The formation of small amounts of the dihydroflavonol, garbanzol, and hydrated aurone, Y_1 and Y_2, in the A.F.O. reaction of the chalcone isoliquiritigenin can best be rationalised in terms of Scheme 6 where the chalcone epoxide serves directly as the common intermediate. This in effect classifies all these compounds as by-products of the main flavonol-forming reaction which is now proposed to be dependent upon oxepin-type intermediates.

In general terms then, in the A.F.O. reaction, direct epoxidation of the chalcone with alkaline hydrogen peroxide to yield the epoxychalcone which could rearrange under basic conditions to form the dihydroflavonol or the hydrated aurone is viewed as a competing reaction, frequently minor, with that of oxepin formation. The effect of substitution pattern in the chalcone substrate on the proportions of products (flavonol and aurone) obtained (84) can be viewed as resting on two effects; firstly, on the balance between the formation of epoxyoxepin and epoxychalcone intermediates and secondly, on the balance in competing orientations in the ring opening of epoxychalcone to give 5- or 6- membered heterocyclic products. The competition in the formation of epoxyoxepin and epoxychalcone intermediates may be visualised as being dependent upon the relative

ease with which the chalcone substrate is 'dehydrogenated'. If this mode of oxidation is sufficiently rapid, the course of oxygenation will be diverted from simple epoxychalcone formation by the forces of the coupling reaction and epoxy-oxepin formation will occur. Thus, in cases where aurone is the major product of chalcone oxidation in the A.F.O. reaction, it is suggested that oxygenation dominates dehydrogenation and epoxychalcone formation as previously supported (84) is assured.

In conclusion, therefore, a definite mechanistic distinction is made between those reactions involving a four-equivalent oxidation (epoxyoxepin formation) and those involving a two-equivalent oxidation (epoxychalcone formation) by hydrogen peroxide. This distinction, in addition to providing a valuable insight into the chemical reaction, will be found in the following sections to be equally suited as a basis for rationalising product formation in the enzyme-catalysed reaction.

3C THE ENZYMIC REACTION

In this part, the results of biochemical studies of the reaction of chalcone catalysed by peroxidase are considered in relation to the recorded findings on peroxidase-catalysed reactions. Further, a scheme is advanced to explain the reaction of chalcone. Finally, some instances of the occurrence of certain features of the chalcone reaction in other areas of study are briefly noted.

3C-1 General discussion of the biochemical results

Comparative studies of the garbanzo and HRP enzymes

clearly demonstrated a uniformity of catalytic properties and behaviour which established the identity of the garbanzo preparation as a peroxidase. Thus, the products of chalcone oxidation were identical for the two enzymes, oxygen was obligatory for the reaction and the effects of various additives on the rate of reaction were similar. Some quantitative differences were, however, apparent between the two enzymes; for example, the activity toward chalcone in relation to the peroxidatic activity toward guaiacol was greater for the HRP enzyme by a factor of about 5 (see Figures 25 and 26). Further, the garbanzo enzyme exhibited maximal reaction rates at a concentration of hydrogen peroxide approximately half that similarly required by the HRP enzyme. The pH optimum for the garbanzo enzyme (pH 7.5) was also significantly lower than that for the HRP preparation (pH 7.9) (Figure 24). Both enzymes were shown by gel electrophoresis to be mixtures of isoenzymes. Consequently the observed detailed properties of the preparations would be expected to be the weighted average of those of the component isoenzymes. Examples of the variation in properties between peroxidase isoenzymes are known (88, 89, 90).

The absolute requirement for oxygen in the formation of $\not{A}Y_1$ and $\not{A}Y_2$, the major product, immediately placed the reaction in the peroxidase-oxidase category (25, 28). The absence of reaction under anaerobic conditions with the amounts of enzyme normally used in aerobic reactions indicated that chalcone is not a ready peroxidatic donor. When much higher concentrations of enzyme were subsequently used, some direct formation of aurone at pH 7 was noted.

This would be the expected product of peroxidative oxidation of chalcone judging by the result of ferricyanide oxidation (17). However, as previously mentioned, the formation of a similar quantity of aurone in the presence of boiled enzyme means that no definite claim can be made for the occurrence of slow enzymic peroxidation of chalcone under anaerobic conditions where the extremely rapid formation of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ is inhibited. Nevertheless, it is clear that the enzyme is a much more effective catalyst of the oxidatic than of the peroxidatic reaction of chalcone, a situation which is analogous to that noted by Klapper and Hackett (91) in comparing catalysis of the oxidatic and peroxidatic reactions of 2-methyl-1,4-naphthohydroquinone. The dihydroflavonol, garbanzol, was also found not to function as a hydrogen donor in the peroxidase reaction at pH 8. It is not known whether either chalcone or garbanzol would undergo peroxidation at acidic pH where many peroxidatic reactions take place.

In this study, reduction in the rate and also in the extent of reaction at higher hydrogen peroxide concentrations were noted. An inhibitory effect of hydrogen peroxide has also been observed in a number of other peroxidase catalysed reactions (90, 92, 93).

The redogenic donors, hydroquinone, catechol, pyrogallol and p-phenylenediamine were strong inhibitors of the chalcone oxidation reaction (Table 4). This is in accord with the effect of such peroxidase substrates on the peroxidase-oxidase reaction (25, 27); the redogenic donors apparently undergo preferential peroxidative reaction thus sparing the chalcone substrate. With the oxidogenic donors,

phenol, resorcinol and p-cresol, however, a stimulatory effect on the reaction, which may have been expected (25), was not observed, phenol being essentially without effect and the other two donors apparently inhibitory. It is not certain whether this inhibitory effect is an intrinsic one or merely a reflection of impurities which may have been present in the resorcinol and p-cresol. The inhibition observed in the presence of manganous ion is in contrast to the stimulatory effect of this ion in many cases on the peroxidase-oxidase reaction (25, 93, 94) but parallels the strongly inhibitory effect noted on the aromatic hydroxylation reaction (26). This similarity of effect of manganous ion in the chalcone and aromatic hydroxylation reactions will be discussed later in relation to the possible nature of the active oxygenating species in these peroxidase-catalysed reactions. Both mercaptoethanol and diethyldithiocarbamate are inhibitory in the chalcone reaction but further study is required to uncover the mode of action of these compounds in this system. The observed rate inhibition by added cupric ion is probably confounded by the additional effect of the additive to promote breakdown of the ϕY_1 and ϕY_2 product. Since evidence was obtained of the occurrence of product (ϕY_1 and ϕY_2) inhibition in the enzymic reaction, chemical degradation of ϕY compounds promoted by added cupric ion would therefore be expected to reduce this inhibition. The greater extent of the chalcone reaction observed in the presence of added cupric ion than in the control reaction would support such an additional effect of copper.

The heat-stable factor(s) in the crude garbanzo

enzyme which suppressed oxepin (OC) formation while promoting Y_1 and Y_2 formation is considered to function as a redogenic donor which consequently spares chalcone by restricting the key reaction, epoxyoxepin formation. However, it is proposed that Y_1 and Y_2 formation is still a side reaction, as in the normal oxepin-forming reaction of the purified system. This Y_1 and Y_2 formation somehow occurs more effectively during the oxidation of this unknown, chalcone-sparing donor than during the oxidation of chalcone itself. Thus one compound, the unknown donor, could cause the observed dual effect of inhibition of oxepin and promotion of Y_1 and Y_2 formation. A parallel is drawn between Y_1 and Y_2 formation in the presence of a reaction supported by this unknown donor and the hydroxylation of a substrate in the aerobic dihydroxyfumaric acid-peroxidase system (see Section 3C-3)

The qualitative results gained from the examination of the activity of a range of chalcones (Figure 30) as substrates in a peroxidase-catalysed reaction indicated that the 4-hydroxyl substituent in the B ring of isoliquiritigenin is required for the activity of this compound. This finding is readily explained on the basis of the expected ease of phenoxy radical formation and the existence, through appropriate conjugation, of the mesomeric form of the radical in which the free electron is in the position <u>para</u> to the oxygen as required by the formation of the p-cyclohexadienone ring system (53, 81, 95).

3C-2 A scheme for the peroxidase-catalysed oxidation of chalcone

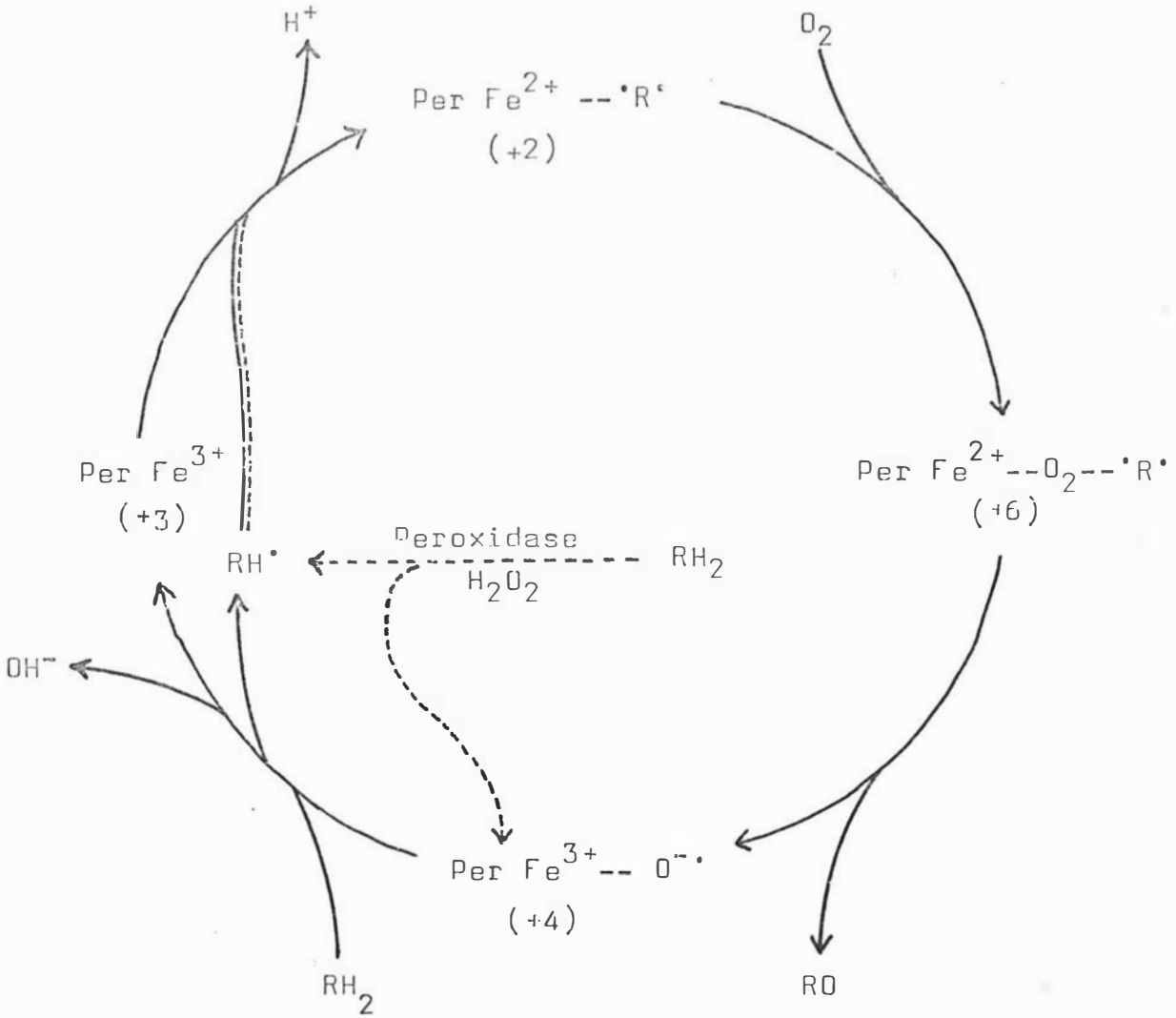
A scheme for the enzymic reaction has to accommodate

a number of salient features which were revealed in the biochemical studies. These features include the observed stoicheiometry of chalcone and oxygen consumption (1:1) and the absence of other than a net catalytic consumption of hydrogen peroxide, despite an effect of this compound on reaction rate suggestive of a co-substrate role. Further, the formation of one type of product, the ØY diol structure, has to be accounted for by the scheme. Finally, in the light of these requirements, a scheme has been sought which embodies only known redox states of peroxidase (see Introduction). Scheme 7 is proposed to explain the enzymic reaction.

An essential concept of Scheme 7 is that the true enzymic product is considered to be the epoxyoxepin structure rather than the ØY diol structure. This concept is strongly supported by the biochemical data as has been stated earlier in the discussion of the origin of $ØY_1$ and $ØY_2$ diols. The basis for this statement is that a chain reaction sequence which accommodates the biochemical data can only be constructed using the known redox states of peroxidase, if the product is assumed to be the epoxyoxepin or an isomeric structure (that is, chalcone minus two hydrogens and plus one oxygen). Acceptance of the ØY diol structure as the true initial enzymic product, in contrast, does not permit a peroxidase-mediated chain reaction to be similarly constructed. Besides being invaluable to explain the biochemical features of the reaction, the postulated epoxide structure has considerable merit from a chemical viewpoint. Thus, the origin of the stereoisomeric ØY diols, a most complex product mixture stereochemically, can be

Scheme 7

Proposed chain reaction for peroxidase-catalysed oxidation of chalcone (RH_2)



----- chain initiating reaction

——— chain carrying reaction

conveniently rationalised as already discussed (see section 3A-4). Further, from the concept of epoxide as true enzymic product, it follows that the ensuing chemical conversions of epoxide, by way of $\not{O}Y_1$ and $\not{O}Y_2$, to OC and flavonol, become non-redox reactions since all the structures involved are of the same oxidation level (four-equivalent higher with respect to chalcone). This is entirely consistent with the facile nature of these reactions.

Thus, the enzymic reaction, interpreted on the basis of the epoxyoxepin structure (RO) as the product, is a four-equivalent oxidation (dehydrogenation plus oxygenation)of the chalcone substrate (RH_2) - equation 6.

$$\underset{(RH_2)}{\text{chalcone}} + O_2 \xrightarrow{\text{enzyme}} \underset{(RO)}{\text{epoxyoxepin}} + H_2O$$

Equation 6

Scheme 7 simply embodies a self-sustaining (in theory) chain reaction which accounts for the reaction summarised in equation 6. Thus, one oxygen atom from molecular oxygen is combined in the oxidised chalcone product as the epoxy oxygen while electrons concurrently removed in the intramolecular oxidative coupling process in oxepin formation reduce the other atom of the oxygen molecule to water.

To start and to maintain the chain reaction in the face of any breakdowns, the simple peroxidatic reaction of chalcone to generate chalcone free radical is envisaged. In this peroxidatic step, hydrogen peroxide substrate will be required in stoicheiometric amounts.

$$\text{Per Fe}^{3+} + H_2O_2 \longrightarrow \text{Compd I} \xrightarrow{RH_2} RH^{\cdot} + \text{Compd II} + H_2O$$

Equation 7

The products of this peroxidatic reaction, chalcone free radical and Compound II are both intermediates in the chain reaction and thus the cyclic mechanism is established by this peroxidatic step. Further, the stoicheiometric requirement for hydrogen peroxide in this essential step is considered to be the reason for the observed rate of the reaction being sensitive to the hydrogen peroxide concentration, despite the minimal net consumption of this oxidant. Thus, if the chain reaction is always relatively fast, the peroxidatic reaction of chalcone would invariably be rate limiting overall when called into play to initiate the chain reaction at the outset and after any chain-breaking side reactions. Hence in this manner, the substrate-like effect of hydrogen peroxide concentration on reaction rate may be explained. The limited overall consumption of hydrogen peroxide is therefore seen as a measure of the efficiency of the chain reaction.

A central feature in the chain reaction sequence is the postulated participation of the ferrous enzyme, formed from the free ferric enzyme by addition of the active intermediate, the chalcone radical, as in equation 8.

$$\text{Per Fe}^{3+} + RH^{\cdot} \longrightarrow \text{Per Fe}^{2+}\text{---}{}^{\cdot}R^{\cdot} + H^{+}$$

Equation 8

In equation 8 the concept of a diradical product formed from chalcone at the active site of the enzyme is summarised

(compare Scheme 5 for the chemical reaction). This diradical is considered to be enzyme bound and is therefore not free to undergo intramolecular coupling, for example, to aurone. Effective reduction of the free ferric enzyme in this manner would demand a strong affinity between the enzyme and chalcone free radical. In the ferrous form, exclusively, the enzyme can be oxidised by a molecule of oxygen in the formation of an active oxidising complex equivalent in overall oxidation level, if not in electronic structure, to Compound III (see Introduction and Figure 5). Thus in Scheme 7, the complex equivalent to Compound III in oxidation level is generated following oxygen addition (equation 9) to the reduced enzyme, and within this final complex, in the presence of bound chalcone diradical and activated oxygen, the oxygenation step is accomplished in conjunction with radical coupling to generate (equation 10) the epoxyoxepin product (compare Scheme 5). In the process, the oxidation level of the active enzyme intermediate is reduced by two equivalents from that of Compound III (+6) to that of Compound II (+4). No definite electronic structures are intended to be assigned for the enzyme intermediates by the representations in equations 9 and 10.

$$\text{Per Fe}^{2+}\text{---}\cdot\text{R}\cdot + O_2 \longrightarrow \text{Per Fe}^{2+}\text{---O=O---}\cdot\text{R}\cdot$$

Equation 9

$$\text{Per Fe}^{2+}\text{---O=O---}\cdot\text{R}\cdot \longrightarrow \text{Per Fe}^{2+}\text{---}\cdot\text{O}\cdot + \text{RO}$$

Equation 10

Reaction of another chalcone molecule with the active enzyme compound, Per Fe^{2+}---·O·, equivalent to Compound II,

from equation 10 is responsible in a normal peroxidatic reaction step (equation 11) for the continuation of the self-sustaining chain reaction. Thus the products formed in this step, free enzyme and chalcone free radical, are species of the initial chain reaction step, equation 8.

$$\text{Per } Fe^{2+}\text{---}{}^{\cdot}O^{\cdot} \text{ (Compd II)} + RH_2 \longrightarrow \text{Per } Fe^{3+} + RH^{\cdot} + OH^{-}$$

Equation 11

Summation of equations 8 - 11 gives equation 6, the summary of the chain reaction.

In this chain reaction of enzyme-catalysed epoxy-oxepin formation, the essential sequence of reaction steps is one of dehydrogenation prior to oxygenation. This is the same sequence which was proposed for the chemical reaction. In the enzymic system it is not feasible to accept any alternative sequence, since the enzyme, to combine with and thereby activate molecular oxygen, must undergo a four-equivalent oxidation step and hence with the relative oxidation level of Compound III (+6) as the upper limit, a ferrous enzyme of relative oxidation level (+2) must be first generated by one-electron reduction of the free, ferric enzyme (see Figure 5). The free radical of the hydrogen donor is a logical source of this reducing power.

An essential tenet of the epoxyoxepin argument for $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ formation is the existence of isomeric epoxides. Consequently, in the enzymic reaction the epoxidation of the α,β double bond in the bound diradical derived from chalcone must have occurred from both sides of the bond, with the balance affected somewhat by reaction conditions, as expressed in the observed ratios of $\not{\alpha}Y_2$ enantiomers

previously discussed (section 3A-4).

The cyclic mechanism in Scheme 7 accounts for the major product of chalcone oxidation, namely the isomeric epoxide precursor of diols $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. In the reaction, however, definite amounts of Y_1 and Y_2, besides traces of garbanzol were also formed. These latter products are considered to represent, as in the chemical reaction, by-products of a competing, minor reaction. This is a chain-breaking reaction in the case of the enzyme-catalysed system. Again, this minor reaction is envisaged as basically an epoxidation of the unoxidised chalcone molecule, the product of which rearranges predominantly to the 5- rather than the 6-membered heterocyclic ring compound and consequently Y_1 and Y_2 accumulate more than garbanzol. A corollary to this proposed origin of Y_1 and Y_2 and garbanzol is that for chalcone epoxide formation to occur an epoxidising species must be proposed to be occasionally freed from the enzyme or, alternatively, made accessible on the enzyme to a chalcone molecule, rather than to the diradical derived from chalcone oxidation.

Following this explanation of the proposed course of the peroxidase-catalysed oxidation of chalcone it is of interest to consider the reaction in relation to other studies of well-established, peroxidase-catalysed reactions recorded in the literature.

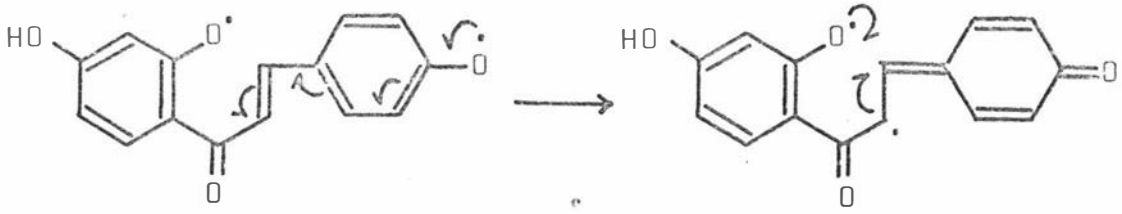
3C-3 Proposed chalcone reaction scheme in relation to previous work with peroxidase and other enzymes

The free radical mechanism proposed above (equations 7 and 8, section 3C-2) for the oxidation of the chalcone

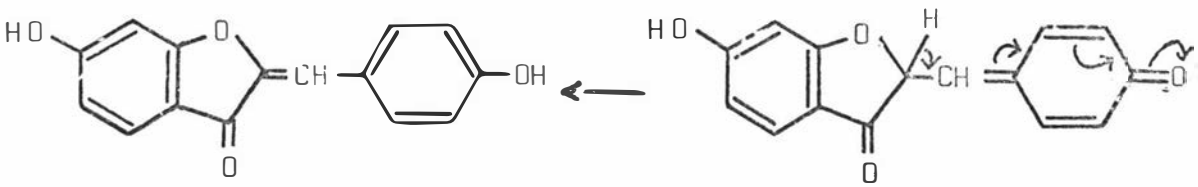
substrate by dehydrogenation (or electron removal from the anionic form) is to be expected in the oxidation of electron donors catalysed by peroxidase (25, 28, 30). Direct demonstration by E.S.R. spectroscopy of the one-electron oxidation of phenols to phenoxy radicals, as envisaged in chalcone oxidation and generally postulated in the past, has recently been reported (96) in a peroxidase-catalysed reaction. Reduction of the free ferric enzyme by chalcone free radical (equation 8) assumes not only the likelihood of such a reaction but also that the chalcone free radical is not preferentially consumed by further peroxidative reaction. There is support for both these assumptions in general terms in the literature; thus the reduction of ferri-peroxidase by the free radicals of the oxidatic reaction donors IAA and NADH is well established (28, 33) and the free radicals of donors are thought in general (28) and have been shown in particular cases (30) to be poor electron donors for peroxidase Compounds I and II.

While the oxygenation of ferrous peroxidase is known to be a rapid reaction (22, 28), the scheme postulated for chalcone oxidation requires that this oxygenation occurs while the dehydrogenated (two-equivalents) chalcone is held on the enzyme. Little is apparently known about the degree of binding of organic substrates in the course of reactions with peroxidase in which other than simple dehydrogenation is involved (93, 97, 98). In the chalcone reaction, the high yields of epoxyoxepin-derived products points to an efficient means of linking the dehydrogenation and oxygenation processes involved in product formation. Any significant release of dehydrogenated chalcone only into the

reaction medium would be expected to result in aurone formation.



dehydrogenated chalcone



aurone

This aurone product, under aerobic conditions, apparently was formed only through the hydrated precursors Y_1 and Y_2 which are considered to be products of a separate minor reaction pathway. Hence, in view of the experimental evidence in the case of chalcone, some highly efficient binding of the dehydrogenated intermediate must be entertained.

The oxygenation step, as in the chemical reaction sequence (Scheme 5), is of much significance in permitting the limitations imposed by the geometry of the trans-chalcone structure to be removed, thus allowing oxepin ring formation to occur. Again, this steric requirement for coupling would strongly suggest an oxygenation mechanism in which a carbon-oxygen bond is first formed at one carbon

only, either that α or β to the carbonyl, to permit conformational mobility in the system before the epoxide ring is generated by the formation of the second carbon-oxygen bond. This final bond formation to give the epoxide may be envisaged as the step which releases the product from the enzyme; the group which is eliminated in this cylisation being retained on the enzyme. According to Scheme 7, this group must contain one equivalent oxidising power and confer upon the enzyme the catalytic powers of Compound II. It is suggested that the reactions summarised in equations 8, 9 and 10 (section 3C-2), starting with the reduction of enzyme by chalcone free radical and proceeding by addition of oxygen through the formation of epoxide product and release of oxidised enzyme (Compound II), are rapid processes which under enzymic catalysis may exhibit a considerable measure of concerted character.

A biologically significant process which is occurring in this reaction sequence is the addition to the substrate of an atom of oxygen derived from molecular oxygen. In this respect, the reaction is analogous to the numerous mono-oxygenation reactions catalysed by mixed-function oxidase enzymes in which considerable interest is centred on the mechanism of oxygen activation and the nature of the 'active' oxygen species in particular (74, 99, 100, 101).

The peroxidase-catalysed oxidation of chalcone, however, is of the internal mono-oxygenase type, where the substrate being oxygenated provides the reducing power to combine with the second atom of the molecular oxygen (99, 101).

$$RH_2 + O_2 \xrightarrow{\text{enzyme}} RO + H_2O$$

This contrasts with the more common external mono-oxygenase type (mixed-function oxidase) where a second different molecule is required as a source of reducing power to combine with the second atom of molecular oxygen.

$$R + H_2X + O_2 \xrightarrow{\text{enzyme}} RO + X + H_2O$$

Atomic oxygen, or 'oxene', has been suggested as a likely form of active oxygen introduced into the substrate in reactions catalysed by mixed-function oxidases (74). The concept here is essentially of an enzyme-mediated oxygen transfer process. Details of the sequence of events on the enzyme leading from molecular oxygen to the generation and transfer of the 'oxene' species, in conjunction with the supply of reducing power, are not known but are likely to vary among the many mixed-function oxidases (74). Recently, Jerina et al, studying the hydroxylation of aromatic compounds by a rat liver microsomal mixed-function oxidase system, found that hydroxylation of naphthalene proceeded via the epoxide (102). This intermediate was held to be suggestive of the enzymic formation of 'oxene' as the reactive oxidant in accordance with earlier proposals.

This oxenoid mechanism, while acceptable in principle on stoicheiometric grounds for the peroxidase-catalysed oxidation of chalcone, is not considered likely in this reaction on closer examination. Thus, the problem is encountered of explaining in terms of known, well established, peroxidase compounds and oxidation states, the synchronous supply of reducing power (two electrons) while the 'oxene' is being generated from molecular oxygen. In Scheme 7, the first

electron removed from chalcone is lost in the reduction of (formally) hydroxyl radical to water before there is any interaction with molecular oxygen. That is, an essential feature which permits the chain mechanism to operate in the peroxidase-mediated reaction in terms of recognised intermediate enzyme compounds is the utilisation of an electron from each of two separate molecules of chalcone to reduce that atom of oxygen not incorporated from the molecule of oxygen consumed. This type of 'overlap' in the supply of reducing power is apparently not envisaged for the oxenoid mechanism. A further point against the operation of the direct oxenoid mechanism is that the addition of oxene to the double bond could be expected to give rise to the trans-epoxide by retention of the geometry of the chalcone system.

Rather than the oxenoid mechanism, an alternative one of oxygen activation is envisaged in the peroxidase system which is in keeping with the known occurrence and reactions of the peroxidase redox compounds (see Introduction and Figure 5). Thus, ferro-peroxidase is considered to react with molecular oxygen to form oxyperoxidase (probably identical to Compound III), an oxidising agent which does not dissociate molecular oxygen as would a simple oxygen carrier (22). Rather, it has been shown to contain activated oxygen (28). This peroxidase compound may also be considered as enzyme-bound superoxide anion (or perhydroxyl radical), see Figure 5 and equation 12 below.

$$\text{Per Fe}^{2+} + O_2 \longrightarrow \text{Per Fe}^{2+}\text{---}O_2 \longleftrightarrow \text{Per Fe}^{3+}\text{---}O_2^{-\cdot}$$

Equation 12

It is suggested that this oxygenated enzyme, which may be presently considered as enzyme-bound superoxide, is the oxygenating agent. Free superoxide has been shown (103) to be nucleophilic in its reactions in dipolar aprotic solvents and to generate the corresponding epoxide from cyclohexen-3-one, but not from cyclohexene itself in which the double bond is not activated towards nucleophilic attack. The enzyme-bound superoxide may even display similar nucleophilic properties and attack the chalcone-derived substrate at the carbon β to the carbonyl as in the case of hydroperoxide anion in the chemical reaction (Scheme 5). The epoxide-forming elimination step following intramolecular coupling would then lead to final product release and the appearance of oxidised enzyme (Compound II). If the enzyme-bound superoxide is not nucleophilic (see below), attack may occur at the α instead of the β carbon on the substrate but with the same general sequence leading to product. The net effect is simply transfer of an oxygen atom as in the oxenoid mechanism of mixed-function oxidation.

It is now of interest to compare oxygenation in the chalcone reaction with non-specific aromatic hydroxylation which occurs in the course of the peroxidase-catalysed aerobic oxidation of dihydroxyfumaric acid (DHF) and which has been studied by Mason's group (26). Both in chalcone oxygenation and in aromatic hydroxylation the reaction is essentially one of oxygen introduction. In addition to this superficial resemblance there are a number of further strong similarities in the two systems, including the absolute requirement for molecular oxygen and the inability of added hydrogen peroxide to substitute for the oxygen-derived

oxygenating species in the reaction. Further, manganous ion strongly inhibits both reactions. Hence a strong similarity in the nature of the oxygenating species and even in the mechanism of oxygenation is indicated by these common features of the two systems. Consequently it is now suggested, as in the chalcone system, that oxyperoxidase (Compound III) is the effective hydroxylating agent (oxygen donor) in this non-specific aromatic hydroxylation reaction. This proposal is in contrast to the final conclusion of Mason's group, following extensive studies (26), that the hydroxylating agent is perhydroxyl ($HO_2^{\cdot}$) or a related free radical species generated in the concurrent peroxidase-oxidase reaction of DHF. However, it is in agreement with Mason's original suggestion, following preliminary studies (104), that an oxygenated form of peroxidase equivalent to Compound III may be the hydroxylating species.

There are several attractive features of this proposed role of oxyperoxidase as the oxygenating species in the aromatic hydroxylation reaction. The first rests on the structure of oxyperoxidase (Compound III) which is likely to be a hybrid and not simply a ferroperoxidase-oxygen or ferriperoxidase-perhydroxyl complex (28, 105) - compare equation 12. Consequently, this situation with respect to structure and hence properties means that oxyperoxidase may quite conceivably be the hydroxylating agent, necessarily radical in nature on the findings of the final extensive studies (26). This interpretation of the properties of oxyperoxidase contrasts with that of Mason when he originally suggested the positively-charged

oxyperoxidase (Per Fe^{2+}-O_2) as the hydroxylating species, based on preliminary studies which indicated the oxygenating agent was electrophilic in nature (104). A further attractive feature of this proposed role of oxyperoxidase is that the formation of Compound III in the oxidation of DHF is well known (25, 34, 106) even if poorly understood. This Compound appears rapidly at the start of the oxidatic reaction of DHF but does not react with either DHF or DHF free radical (34). Thus, the DHF system used to support aromatic hydroxylation would be ideally suited to provide the proposed active oxygenating species. Further, in the presence of manganous ion the oxidatic reaction of DHF is accelerated (26, 34) but quite a different mechanism may then operate (34) since in particular Compound III is no longer present (106). Here, then, is a plausible explanation of the cause of the observed strong inhibition of oxygenation by manganous ion through its effect on reaction mechanism leading to the absence of the active oxygenating species. Previously (26), the effect of manganous ion was thought to operate apart from the enzyme by accelerating the reduction of perhydroxyl radical to peroxide (see Figure 7). This present concept of peroxidase-catalysed aerobic aromatic hydroxylation makes the reaction an enzyme-catalysed one rather than a chemical reaction associated with radicals liberated in the course of a supporting enzymic reaction. In the chalcone reaction, oxygenation is likewise held to be an example of enzyme-mediated oxygenation in contrast to the alternative of an extra-enzyme chemical reaction. The failure of both reactions to occur under anaerobic conditions

but with hydrogen peroxide supplied can only mean a failure to generate the active enzymic species; this conclusion indicates Compound III is probably not formed under the anaerobic conditions used with either DHF or chalcone.

Recent work by Jerina and Daly (40) on the absence of the NIH shift when non-phenolic aromatic compounds are hydroxylated in the above peroxidase-catalysed reaction in the presence of DHF supports the presence of a radical rather than an electrophilic oxygenating agent. This is because electrophilic oxygenating species are associated with the occurrence of the NIH shift in phenol formation. Consequently in terms of the enzymic hypothesis presented above, oxyperoxidase is further indicated to behave as a radical reagent. The enzyme-bound radical may be a more powerful oxidising agent than free perhydroxyl radical which has been held to be incapable of effecting hydroxylation of some substrates and therefore probably not to be the aromatic hydroxylating species (40). With oxyperoxidase proposed to display radical properties, the oxygenation step in chalcone oxidation may occur by attack at the α rather than the β carbon in relation to the carbonyl group.

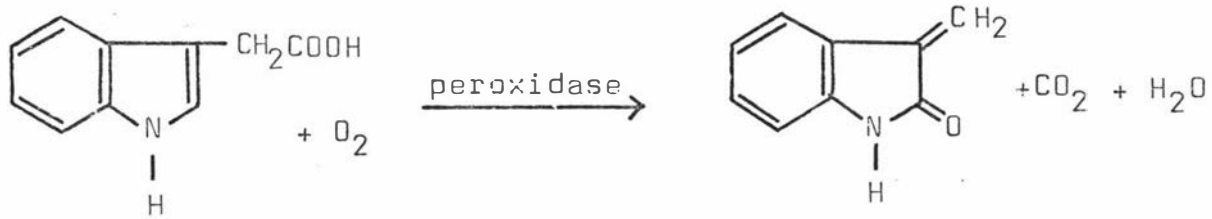
While an epoxide is the postulated product of the enzyme-catalysed addition of oxygen in the chalcone reaction, the nature of any intermediate in the aromatic hydroxylation system is not known. Arene oxide intermediates, however, have been implicated in hydroxylation of aromatic substrates catalysed by mixed-function oxidases and in similar chemical oxidations mediated by peroxyacids (102, 107, 108). In support of this proposal, direct evidence

has recently been obtained that 1,2-naphthalene oxide is the intermediate in enzymic formation of naphthol from naphthalene (102). A common feature of these mixed-function oxidase and peracid oxidations of aromatic substrates is the occurrence of the NIH shift (108). However, the NIH shift is not observed in peroxidase-mediated hydroxylation (40) or when a number of other model systems for chemical hydroxylation are employed (108). These model systems include Fenton's reagent in which hydroxyl radical is the active hydroxylating species (109). In view of this evidence with respect to the occurrence of the NIH shift, it is likely that aromatic hydroxylation catalysed by peroxidase occurs by a substitution reaction involving a radical species without the intervention of an epoxide intermediate. Thus the parallel between peroxidase-catalysed oxygenation in aromatic hydroxylation and chalcone oxidation apparently does not extend to the final generation of epoxide products. However, a common feature is indicated in the apparent difference of mechanism in the peroxidase-mediated oxygenation reactions and the mixed-function oxidase systems discussed.

From the above discussion it is clear that the peroxidase-catalysed oxidation of chalcone described in this thesis constitutes a reaction which combines the essential features of two separate, well-established reactions mediated by the enzyme. These are on the one hand, the oxidatic reaction in which dehydrogenation occurs and molecular oxygen functions as the electron acceptor and on the other, an aromatic hydroxylation reaction where the oxygen atom incorporated is derived from molecular oxygen. The chalcone-type of four-equivalent

oxidation of a substrate mediated by peroxidase is suggested to be dependent upon the formation of oxyperoxidase as the active oxygenating intermediate. It would be interesting to test for the presence of superoxide anion in this system using superoxide dismutase enzyme (110). This enzyme, by catalysing the dismutation of superoxide, would inhibit chalcone oxidation if the free superoxide is an intermediate but if essentially this enzyme-bound radical is involved, as predicted, the dismutase may be without effect (see for example (111)).

Several previous examples are known of the four-equivalent oxidation of substrates catalysed by peroxidase (24, 93, 97, 112). The two notable examples, the oxidation of indole-3-acetic acid (97) and the oxidative decarboxylation of methionine (93, 113), are shown formally below.



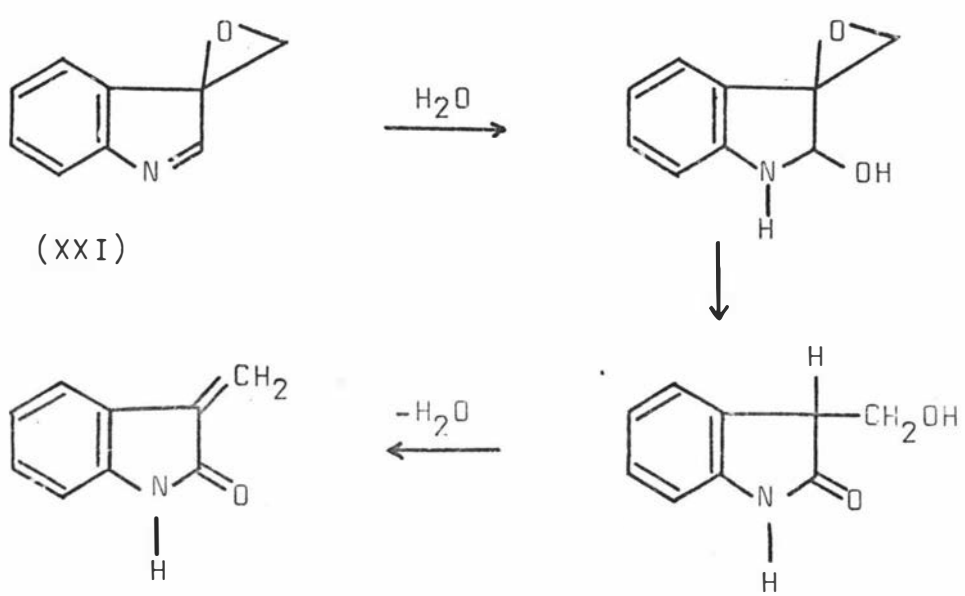
Indole-3-acetic acid (IAA) 3-methyleneoxindole

Methionine

In neither of these reactions was added hydrogen peroxide required;in fact it was inhibitory in the latter one. Additives were not required in the IAA oxidation but there was an

obligatory requirement for catalytic amounts of manganous ion and pyridoxal phosphate in the methionine oxidation. (Pyridoxal phosphate added was typically at one-quarter the concentration of the substrate amino acid).

While these reactions are both equivalent to the chalcone reaction in overall stoicheiometry, it is not suggested that they are equally comparable with the chalcone reaction. Rather, it is considered, for the reasons given below, that the IAA oxidation has most in common with the chalcone reaction. Thus, the enzymic oxidations of both IAA and chalcone require no external metal (manganous ion) or monophenol or other additives, although the reaction of IAA has been stimulated under certain conditions by the addition of manganous ion and of oxidogenic donors (114). It is important to note that the products of IAA oxidation have been characterised for the reaction in the absence of manganous ion and monophenol (97). Thus, the final main product, 3-methyleneoxindole, is considered (97) to be derived from an epoxide intermediate (XXI) which represents the true product of the enzymic reaction.



3-methyleneoxindole

The epoxide oxygen in (XXI) represents the atom derived from molecular oxygen. While Hinman and Lang (97) formulated the oxygenation step as a reaction of the IAA free radical with molecular oxygen, it is possible that an enzyme-bound oxygenating species is involved. This idea is a limited feature of the mechanism which has been proposed by Fox and Purves (98, 115).

Various known features of the IAA reaction are in agreement with a proposed mechanism similar to that presented for chalcone oxidation. Thus the IAA free radical is known to reduce the free enzyme and in addition Compound III reacts rapidly with IAA (32). As a result, Compound III is not detected in the course of IAA oxidation in contrast to the situation with NADH and DHF as substrates in the oxidatic reaction (34). With these latter two substrates, of course, no oxygenation reaction is envisaged, only dehydrogenation. It is possible that in the presence of manganous ion, the oxidation of IAA follows a different course, a possibility noted previously in the case of the reaction of DHF.

The mechanism of amino acid (methionine) oxidative decarboxylation, in being dependent upon pyridoxal phosphate and manganous ion, is likely to differ significantly from that of chalcone and IAA oxidation. In keeping with the requirement for pyridoxal phosphate, a mechanism involving electron transfer through covalently-bound Schiff base intermediates has been proposed (113) and an interesting feature is the suggested role of Compound III as the active form of the enzyme from which the oxygen in the amide product is derived. In terms of the somewhat earlier discussion,

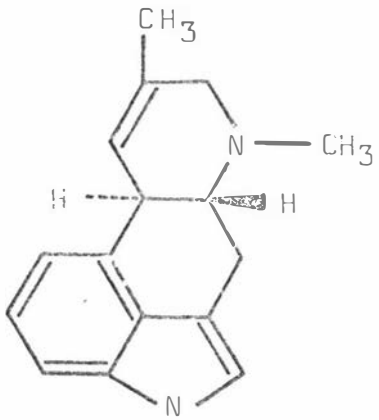
Compound III would not have been expected to be the oxygenating species in the presence of manganous ion. However, rather than being a strong argument against the proposed role of Compound III in oxygenations catalysed by peroxidase, the occurrence of oxygenation in the presence of manganous ion in this case may be considered as another expression of the operation of a mechanism, in this particular reaction, basically different to that in the IAA and chalcone systems. Bacterial flavoprotein enzymes are known which catalyse the oxidative decarboxylation of several amino acids according to the stoicheiometry of the peroxidase-catalysed reaction, but without any requirement for additives (101). The peroxidase-catalysed reaction may even be more complex than initially considered on account of the recent report (116) that pyridoxal phosphate is also a substrate for the enzyme.

3C-4 Results of present work in relation to other areas of study

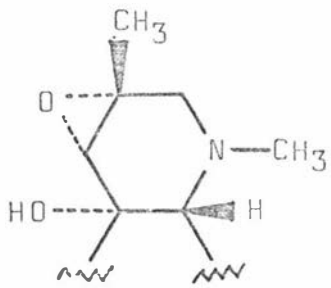
The peroxidase-catalysed chalcone oxidation to form the oxepin products involves, besides the oxygenation step, an intramolecular oxidative coupling reaction. Oxidative coupling is a well known general reaction of phenolic systems and is of considerable biosynthetic significance in plants and in micro-organisms (95, 117). Included among those compounds for which the known or proposed biosynthetic sequence incorporates an oxidative coupling step are various fungal metabolites (95, 118, 119) and alkaloids (95, 120, 121), the natural polymer, lignin, (122) and other polymeric phenols such as hydrolysable tannins (118) and proanthocyanidins (123).

The three main enzymes implicated in phenolic oxidation and coupling are peroxidase, o-diphenol oxidase (o-diphenol:O_2 oxidoreductase E.C. 1.10.3.1) and p-diphenol oxidase or laccase (p-diphenol:O_2 oxidoreductase, E.C. 1.10.3.2) (119). Many examples of oxidative coupling reactions of a wide variety of phenolic substrates have been observed in vitro in the presence of one or more of these enzymes (95, 119, 124-130). The difficulty in determining at times the type of enzyme responsible for certain of these oxidations, because of the use of crude enzyme preparations (124-126), has attracted comment (119) in terms of the limitations imposed on subsequent deductions concerning the properties of individual types of enzymes. However, in certain of the reports cited above (127-130), peroxidase enzyme was definitely the catalyst used.

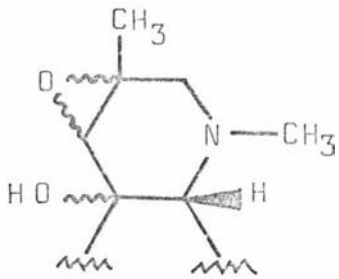
The formation of an epoxide product in a peroxidase-catalysed reaction as proposed in the case of chalcone oxidation, is not without precedent. A postulated intermediate with this structural feature (see(XXI) above) has already been noted in connection with the oxidation of IAA (97). Further, the isomeric epoxide products (XXIII) and (XXIV) have been obtained in the aerobic oxidation of agroclavine (XXII) catalysed by HRP in the presence of added hydrogen peroxide (131). There was an obligatory requirement for oxygen in the formation of epoxides (XXIII) and (XXIV) in this system which may indicate a similarity of the active oxygenating species with that of the chalcone reaction.



(XXII)



(XXIII)

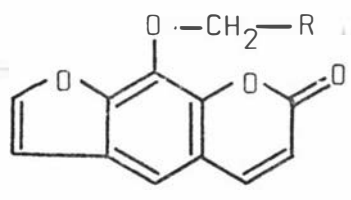


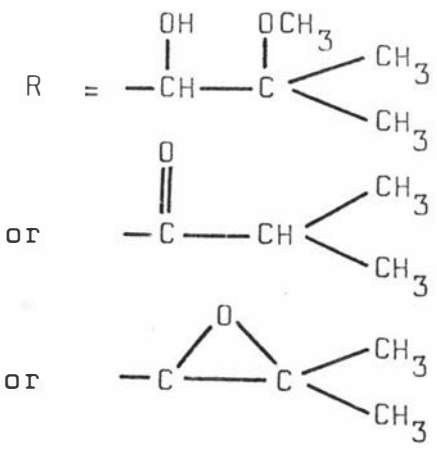
(XXIV)

The 1,2-epoxide structure itself is found in a wide variety of natural products representing many different classes of compounds, as reviewed by Cross (132), and numerous additional examples have accumulated in the meantime. Undoubtedly, a number of biosynthetic mechanisms for generating the epoxide ring exist. Formation through the incorporation of an atom of molecular oxygen by mixed-function oxidase enzymes has been noted in a number of cases recently. The example of microsomal hydroxylation of naphthalene proceeding by way of an epoxide intermediate has already been cited as a specific and possibly a more general example in microsomal aromatic hydroxylation (102, 107). Recently, epoxides have been implicated as intermediates in the hydroxylation of certain aliphatic olefins to form diols in a rat liver microsomal system (133). Microsomal mixed-function oxidase activity is also responsible for the epoxidation of cyclodiene insecticides such as aldrin and heptachlor (134). An epoxide intermediate of much significance is squalene epoxide, formed by the action of squalene epoxidase

(monooxygenase) on squalene and then cyclised enzymically, with concomitant epoxide ring opening, to the steroidal system (135). Epoxidation of the carotenoid in the formation of violaxanthin, on the other hand, is an example where epoxide oxygen is not derived from molecular oxygen but from water (136). Examples of the enzymatic hydration of epoxides to form diols are well established for arene oxides (102, 107), cyclodiene epoxides (134) and for fatty acid epoxides (137).

Certain substituted coumarins provide a particularly interesting example of the co-occurrence of related epoxide, glycol and keto forms of a parent compound (132, 138). Thus, from Heracleum candicans, the three coumarins (see below) with the indicated variation in the R group, have been isolated (138).





The similarity of the pattern in this range of products to that observed in the products of chalcone oxidation catalysed by peroxidase is striking.

3D CONCLUDING REMARKS

The study presented in this thesis reveals a clear example of a four-equivalent oxidation (dehydrogenation plus oxygenation) of an organic substrate, namely chalcone, catalysed by peroxidase. It has been possible to describe and explain this oxidation of chalcone in considerable detail from the combined results of biochemical and chemical studies.

The biochemical studies showed that an equivalent molar consumption of chalcone and molecular oxygen occurs in the enzymic reaction. Further, the oxygen requirement can not be replaced by added hydrogen peroxide, which is nonetheless required in catalytic amounts to obtain a rapid reaction. Thus, as a peroxidase-catalysed reaction, this one is of the oxidatic and not the peroxidatic category. However, the four-equivalent oxidation involved removes the reaction from the normal oxidatic category in which O_2 serves simply as an electron acceptor, to a little known category in which oxygen additionally adopts the role of an oxygenating agent. The reaction is seen to provide a particularly clear-cut example of this unusual and complex activity of the enzyme.

Two new compounds, $\emptyset Y_1$ and $\emptyset Y_2$, have been isolated as the major products of the reaction. These compounds are identified as diastereoisomeric diol derivatives of an oxpein structure. Evidence is presented that each diastereoisomer is a mixture of enantiomers. This therefore requires the formation of all four stereoisomeric diols from the chalcone. It is proposed that the diastereoisomers $\emptyset Y_1$ and $\emptyset Y_2$ are formed from enantiomeric epoxide precursors

which spontaneously hydrate to form the diols. Thus, these epoxides and not ϕY_1 and ϕY_2 are considered to be the true initial products of the enzymic reaction. Interpretation of the biochemical reaction in terms of the intermediacy of known redox states of the enzyme is made possible by this concept of the epoxides as initial product. The concept also provides an attractive chemical explanation for the occurrence of all four stereoisomers of the ϕY diol structure.

Identification of ϕY_1 and ϕY_2 is founded not only on direct physical studies on the compounds themselves but also upon their common chemical transformations to three other products, OC, flavonol and ϕOC. The base-promoted transformation leads to competitive formation of OC and flavonol, the former product being another new compound and the latter a known one. Characterisation of OC indicated a structure isomeric with flavonol but with retention of the oxepin heterocyclic ring of the ϕY structure in contrast to the pyran ring system of flavonol. Thus, the competing formation of these two products from the ϕY structure is formulated as an elimination of the elements of water with concomitant skeletal rearrangement in the case of flavonol. In addition, the ϕY diol structure accommodates the formation, under acidic conditions, of ϕOC, identified as the keto form of the enolic OC compound. A rearrangement of the pinacol-pinacolone type of the ϕY structure accounts for ϕOC formation. Thus the structural argument relating to all the new compounds, ϕY_1 ϕY_2, OC and ϕOC is given much strength in the collective analysis made possible by the facile chemical interconversions observed. These chemical transformations also established among the products a valuable

reference structure in the known compound flavonol.

Besides flavonol, other known flavonoids, namely the hydrated aurone isomers Y_1 and Y_2 and the dihydroflavonol, garbanzol, are formed in the reaction. However, these additional compounds occur in minor amounts only. It is interesting that the first evidence of the entire reaction was the observed formation of Y_1 and Y_2 from chalcone in the presence of crude peroxidase-containing enzyme preparations. Only on purification of the system did the true, complex nature of the reaction emerge. The minor formation of garbanzol in the reaction is considered to be competitive with Y_1 and Y_2 formation on the basis that all these products derive chemically by rearrangement of a common chalcone epoxide precursor. Significantly, this peroxidase-catalysed system leads to flavonol, ultimately, not by way of the dihydroflavonol intermediate (garbanzol), normally favoured in schemes of flavonoid biosynthesis, but by way of the newly-found oxepin precursors, $\emptyset Y_1$ and $\emptyset Y_2$.

The chemical reaction of chalcone with hydrogen peroxide, initially investigated as a control in the course of studies on the enzymic reaction, is also revealed on further study as being very similar to the enzymic reaction. Thus, $\emptyset Y_1$ and $\emptyset Y_2$ are the major initial products detectable in the chemical reaction under the conditions comparable with the enzymic reaction. It is furthermore considered that the chemical reaction under A.F.O. conditions, in which flavonol is the only major product detectable, also proceeds by way of the $\emptyset Y$ compounds as intermediates. This is in contrast to the currently accepted intermediacy of the dihydroflavonol in flavonol formation in the A.F.O. reaction.

These findings on the nature of the chemical reaction and proposals for the course of the A.F.O. reaction have relied heavily upon the enzymic reaction studies to enable structure and interrelationships of the products to be determined.

The biosynthetic significance of the observed transformations of chalcone initiated by the peroxidase-catalysed reaction is uncertain. To what extent the enzymic reaction is a model for the *in vivo* biosynthesis of flavonols, aurones and dihydroflavonols is a question of great interest and remains to be determined. Despite this uncertainty, the enzyme reaction is noteworthy as the first recorded *in vitro* biochemical system for the oxidation of chalcone to flavonol. The mild conditions and rapid rates of reaction associated with the use of the enzymic catalyst have enabled a most original and unexpected pathway to be elucidated for this oxidation.

A particularly lucid example of peroxidase catalysis of a four-equivalent oxidation involving linked dehydrogenation and oxygenation processes is provided by the reaction of chalcone recorded in this thesis. Consequently, the present study has also contributed significantly to knowledge of this complex and ill-defined activity of the enzyme in addition to the matter of flavonoid biosynthesis.

Chapter 4

EXPERIMENTAL

4-1 Garbanzo seedlings

Seeds of garbanzo bean (Cicer arietinum) were spread out on stainless steel mesh trays which were then suspended in a continuous mist of tap water at 15-20^{0} in the dark for 5-8 days. Seedlings were then harvested, rinsed in distilled water and used immediately in the preparation of garbanzo enzyme.

4-2 HRP enzyme

Horseradish peroxidase, RZ 1.73, was obtained from Worthington and the same batch was used throughout the present study. The activity of this enzyme, dissolved at a concentration of 1 mg/ml in 0.05 M tris buffer pH 7.5, was assayed by the guaiacol method (section 4-5) at 80 units/ug.

4-3 Spectra

Instruments used to obtain spectra were as follows; mass spectra (A.E.I. MS 9), NMR spectra (Varian HA 60), i.r. spectra (Perkin-Elmer 137 sodium chloride spectrophotometer) and u.v. spectra (Unicam SP 800 spectrophotometer).

4-4 Protein estimation

The biuret method (139) was used except in the case of the fractions obtained by chromatography on DEAE A-50 Sephadex where the protein concentration was very low. Protein in these fractions was estimated spectrophotometrically by the Δ (E215 - E225) method (140). The observed

optical density difference at these two wavelengths was converted to a relative protein concentration using the relationship applicable to serum albumin:

$$\blacktriangle E \times 144 = \mu g \text{ protein/ml}$$

4-5 Assay of peroxidase activity

The method based on the oxidation of guaiacol in the presence of hydrogen peroxide to coloured products which were monitored spectrophotometrically at 470 nm was used (141). The reaction volume totalled 2 ml in 0.05 M tris buffer pH 7.5 and contained guaiacol (25 µl of 26.7 mM solution in water), hydrogen peroxide (10 µl of 26.7 mM solution in water) and enzyme as required (usually in 5 - 25 µl). Addition of hydrogen peroxide started the reaction which was run at 25°. The amount of enzyme taken was adjusted so that the activity lay in the range 50-100 units. The 'unit' of enzyme activity used throughout is defined as the activity required to produce an increase in absorbance at 470 nm of 0.01 in 5 minutes in the above guaiacol assay. Thus, with 50-100 units of activity added per cuvette, progress curves were recorded from which meaningful rate data could be taken. A tangent was fitted to the steepest slope of the progress curve to determine the rate. With garbanzo enzyme preparations at all stages of purity, the initial rate was always the rate taken. However, this was never the case with HRP enzyme since a lag phase was always observed in product formation catalysed by this enzyme. After the short lag phase a linear rate of product formation was observed and was taken as the rate of reaction. Elimination of the lag phase was observed if 2 µl

of 26.7 mM hydrogen peroxide was first added to cause a limited preliminary reaction (which stopped when all the hydrogen peroxide was used), before the normal addition of hydrogen peroxide (10 µl of 26.7 mM solution) was made to determine reaction rate. The rate obtained following this pre-treatment to eliminate the lag was the same as that observed when the lag phase was ignored and the rate determined from the subsequent linear section of the progress curve.

4-6 Preparation of cell-free enzyme extract of garbanzo seedlings

All operations in the following procedure were carried out at 0-4° using materials and equipment cooled to this extent.

The 5-8 day old garbanzo seedlings which had been grown in the dark under a continuous light spray of tap water were rinsed with distilled water and ground in 0.01 M tris buffer pH 7.5 (about 7 ml per 10 g of seedlings) in a Waring blendor for short periods (10-20 seconds) at a time until a smooth homogenate was prepared. This homogenate was immediately enclosed in several layers of cheesecloth, squeezed and the liquid expressed was collected. The solid residue in the cheesecloth was discarded. Centrifugation of the expressed liquid at 5000g for 30 minutes provided a supernatant (about 7.5 ml per 10 g of seedlings) which was decanted and used as the crude cell-free enzyme.

This preparation was the starting material used in preliminary fractionation studies (see part 2A) and also for the partial purification of peroxidase (Figure 12 and Table 2). It was similar to the preparation used originally

by Wong (17-19).

4-7 Fractionation of cell-free garbanzo enzyme

In the preparation of a purified peroxidase extract from the cell-free enzyme, the procedures summarised in Figure 12 were used, all operations being again performed at 0-4°.

Acid treatment to pH 4.5 was used to precipitate much protein and to clarify the milky cell-free extract (see Figure 12). After acid treatment, the supernatant, titrated back to pH 7.5, was fractionated at 0° (in ice) with acetone, also at 0°. The acetone was added to 50% final concentration (v/v) at a steady rate over 4 minutes and stirring was continued for a further 5 minutes. Most peroxidase activity was precipitated between 35-45% acetone content. Little protein was precipitated until the acetone concentration exceeded 30%. Precipitated protein was recovered by centrifugation (Figure 12) and the supernatant was discarded. After suspension of the recovered protein in a small volume of 0.05 M tris buffer pH 7.5 (about 0.6 ml per 10 g seedlings), the insoluble denatured protein was spun down and the clear, light amber supernatant was then weighted with sucrose and applied to a Sephadex G-75 column (3 x 3[illegible] cm).

This column was packed at room temperature under a pressure head of about 15 cm of buffer with Sephadex G-75 swollen in 0.05 M tris buffer pH 7.5. After running the column with the same buffer and pressure head for about 12 hours in the cold the acetone-precipitated enzyme was layered on top of the gel bed which had been protected with

a thin layer of Sephadex G-25. The absorbance of the effluent at 254 nm was monitored using LKB 'Uvicord' equipment and fractions were collected at 30 minute intervals and assayed for enzyme activity and protein content (Figure 13).

Further purification of the four most active fractions (pooled) from Sephadex G-75 filtration was achieved by ion exchange chromatography on DEAE A-50 Sephadex. This column (1 x 10 cm) was packed under a 15 cm head with a slurry of DEAE A-50 Sephadex pre-equilibrated in 0.05 M tris buffer pH 7.5. The column was then washed in the cold with further 0.05 M pH 7.5 tris buffer flowing under the same pressure head before the protein sample was applied. The active fractions were found to be among the first to come off the column (Figure 14); most of the protein in the applied sample was firmly bound on the top of the column and moved only on the introduction of a salt gradient.

The Sephadex G-75 column was used repeatedly without repacking but a fresh DEAE-Sephadex column was used each time.

Besides these fractionation procedures finally adopted for the purification of garbanzo peroxidase, the cell-free enzyme was initially fractionated for preliminary studies in a number of additional ways (see part 2A).

Thus, in the heat treatment procedure, the enzyme was agitated in flasks in a water bath at 50^{o} for normally 60 minutes during which time considerable protein denaturation occurred and the chalcone-flavanone isomerase activity (15, 16) in the crude preparation was destroyed. After rapid cooling, centrifugation of the heat-treated enzyme removed the denatured protein and yielded a clarified

supernatant (heat-treated enzyme) which was suitable for further fractionations.

Ammonium sulphate fractionation was performed by adding the solid salt to a stirred, heat-treated enzyme at 0-4^{o} at about the rate of solution of the salt. Stirring was continued for 15 minutes after the required addition of salt had been completed. The suspension was then centrifuged to recover the precipitated protein and provide the starting material (supernatant) for the next precipitation step upon incremental (10%) increase in ammonium sulphate concentration. No adjustment of the pH of the solution was made during this procedure. Assays with guaiacol on the redissolved protein precipitates obtained by ammonium sulphate treatment showed no evidence of any clear-cut fractionation and concentration of the peroxidase activity.

Acetone fractionation of heat-treated or ammonium sulphate precipitated enzyme was performed in the manner previously described except several increments in acetone concentration were utilised to achieve stepwise precipitation of the bulk of the protein. Stability of the peroxidase activity in acetone was sufficient to allow this extended manipulation as judged by the presence of enzymic activity (guaiacol and chalcone assays) in all fractions.

4-8 Polyacrylamide gel electrophoresis

A vertical thin-slab, analytical apparatus was used (142). The gel slab was poured from a solution prepared by deaerating 15 ml of 15% w/v acrylamide solution, in water, containing 0.3% N,N'-methylenebisacrylamide and 15 ml of buffer (0.1 M in both tris and glycine) and then adding

with mixing, 0.3 ml of 10% w/v N,N,N',N',-tetramethylenediamine (TEMED) followed by 0.3 ml 10% w/v ammonium persulphate solution freshly prepared in water.

For analytical purposes, six separate wells were formed in the top end of the gel, but in cases where enzyme was to be recovered a single well extending across the gel was formed. The 0.1 M tris-glycine buffer used in the preparation of the gel was also used in anodic and cathodic compartments. Protein samples weighted with sucrose were applied under buffer to the bottom of separate wells in the end of the gel. The sample volume for each well was between 10 and 50 µl and the peroxidase activity between 100 and 400 units. A voltage gradient of 14 V/cm was applied for 1.5 hours under room temperature conditions to separate the proteins after which time the gel was removed from the mould and examined. In cases where net-negatively charged isoenzymes were being investigated, the gel was pre-run for 1.5 hours to remove persulphate, and the buffer in the electrode compartments was changed before the protein samples were applied.

The gel was stained to detect peroxidase activity by immersion in a solution of benzidine dihydrochloride in acetate buffer in the presence of hydrogen peroxide (143). Alternatively, the gel was stained yellow by soaking for 10 minutes in a solution of the disodium salt of chalcone in 0.05 M tris buffer pH 7.5 (concentration approximately 1 mg/ml). Then the gel was transferred to either a clean dish in which a moist atmosphere was maintained for a time (about 30 minutes) to permit the slow decolorisation of the yellow chalcone to occur ($\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ formation) in the zones

of peroxidase activity or, alternatively, the chalcone-stained gel was immersed in a dilute solution of hydrogen peroxide in pH 7.5 tris buffer to promote very rapid decolorisation of chalcone in the loci of peroxidase activity.

4-9 Preparation of Y_1 and Y_2-promoting and OC-inhibiting factors from cell-free enzyme

To obtain a preparation of the factor(s) which promoted Y_1 and Y_2 and inhibited OC formation in the enzymic oxidation of chalcone in the absence of added hydrogen peroxide, cell-free enzyme was heated at 100^{o} for 15 minutes, cooled at room temperature and centrifuged to provide a clear, light amber solution devoid of peroxidase enzyme activity. The yield of supernatant on a volume basis was typically found to be 70% of the cell-free extract.

4-10 Incubation mixtures and conditions

In preliminary work which explored the nature of the enzymic activity in the garbanzo preparations (see part 2A), the incubation mixture typically contained enzyme preparation (up to 3 ml in 0.05 M tris buffer pH 7.5 and equivalent to several hundred to several thousand units of peroxidase activity), chalcone (0.5-1.0 mg freshly dissolved as the disodium salt at a concentration of 1 mg/ml in 0.05 M tris buffer pH 7.5) and buffer (0.05 M tris pH 7.5) to make a total volume of 4 ml. In these early investigations, hydrogen peroxide was not added. The reaction mixture was incubated at 37^{o} for 1.5 hours. Since only a slow consumption of chalcone occurred, the reaction was not deliberately stopped before the product extraction step which immediately followed.

The volume of enzyme added was determined empirically; with crude and heat-treated preparations, a 3 ml volume corresponding to 4-5 g of seedlings was always used while a lesser volume (down to 0.1 ml) was taken with ammonium sulphate and acetone precipitated preparations. A wide variation, perhaps 10-fold, in the peroxidase activity in the aliquot of precipitated enzyme taken did not affect results significantly because the reaction rate was very slow in the absence of added hydrogen peroxide and qualitative effects only were being observed. Further, the consistent use of a standard 3 ml volume of crude and heat-treated enzyme ensured the key qualitative effects of heat-stable dialysable factors on OC and Y_1 and Y_2 formation were detected.

Later, when the garbanzo enzyme had been identified as a peroxidase and partially purified preparations were used, in addition to HRP enzyme, the incubation mixtures and conditions were changed dramatically from those noted above to those detailed in the appropriate sections in the Results. In general the reaction mixture contained in a 4 ml total volume of 0.05 M tris buffer pH 8.0, enzyme (less than 1.0 to more than 100 units), hydrogen peroxide (0-10 μmoles) and chalcone (0.2-2 μmoles). For spectrophotometric observations, reaction mixtures were reduced to 2 ml total volume. The chalcone was normally added as an ethanolic solution (concentration 1-5 mg/ml). The final concentration of ethanol in the reaction mixture was restricted to 5% maximum which was observed to be without effect on reaction rate or product composition. Addition of hydrogen peroxide was routinely used to start the reaction.

Incubation time was limited to a few minutes at either room temperature (about 20-25^{0}) or at 25^{0} under controlled conditions and was normally restricted to the time taken for the decolorisation of the chalcone solution. Products were usually extracted after the reaction had gone to completion or had reached a slow rate so that a technique for arresting the reaction was not routinely required.

For spectrophotometrically monitored reactions relevant to Figures 25-28, the concentrations of chalcone were sufficiently low for chalcone disappearance to be recorded at the absorption maximum (296 nm in pH 8.0 tris buffer). Since the rates of many of these reactions were rapid, an external recorder (Sargent) operated with a chart speed of 5 inches per minute was used to obtain progress curves to which tangents could be fitted more accurately.

4-11 Extraction of reaction products

Reaction products were routinely extracted into ether and the importance of the pH of the aqueous layer in determining the observed product pattern from the enzymic reaction has been stressed in the Results. In this connection it is emphasised that the final volume of ether extract was never dried before removal of the solvent on a rotary evaporator. Hence some small amount of the aqueous layer at the appropriate pH was deliberately retained in the etherial solution to exert important effects on the stability of the recovered product.

Acceptable quantitative recovery of the compounds dealt with was obtained upon two extractions of the aqueous phase with approximately an equal volume of ether each time.

Recovery of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ was found to be satisfactory with the aqueous layer pH up to 7.5, but other compounds were best extracted from slightly acid pH media.

Analytical scale reactions were standardised at 4 ml aqueous reaction volume and were conducted in 10 ml stoppered centrifuge tubes in which the ether extraction (2 x 5 ml) was performed immediately after the reaction time had elapsed. Each volume of ether was effectively removed using a Pasteur pipette.

Preparative scale reaction products were recovered by equivalent ether extraction in a separating funnel which served also as the reaction vessel.

Most reaction mixtures required only a pH adjustment of the aqueous layer (see Results for specific details) prior to immediate and direct extraction in the reaction vessel. However, when crude garbanzo enzyme preparations were used in the enzymic reaction, as in the preliminary investigations (part 2A), removal of protein was a pre-requisite to product extraction. To remove protein, the reaction mixture was made 50% ethanolic and placed in a boiling water bath for several minutes. Denatured protein was removed by filtration, aided by added celite, and ethanol was removed (rotary evaporator) from the filtrate before the aqueous solution (pH 7.5) was ether extracted to recover products and unchanged chalcone.

4-12 Anaerobic reaction procedures

The reaction under anaerobic conditions was run normally in Thunberg tubes or occasionally in Thunberg-type spectrophotometric cuvettes. Chalcone and enzyme were mixed with buffer in the tube and hydrogen peroxide in a

small volume of water was placed in the bulb section of the stopper. The complete assembly was then evacuated for 10 minutes using a high vacuum pump. After this, oxygen-free nitrogen was cautiously introduced to less than atmospheric pressure and the evacuation recommenced for about 15 seconds to remove most of the gas. Three further similar cycles of flushing with nitrogen and evacuation were performed before the Thunberg assembly was filled with nitrogen to slightly above atmospheric pressure, sealed off and disconnected from the nitrogen and vacuum line. The stopper was firmly held against a slight positive pressure of nitrogen by a rubber band anchored to the side-piece of the tube. Before the contents of the assembly were mixed, the tube was partially immersed in a water bath at 25^{o} for 10 minutes to warm the main volume of sclution. Incubation at 25^{o} continued after mixing the contents.

Oxygen-free nitrogen was provided by passing a slow stream of commercial oxygen-free nitrogen through a tube packed with copper turnings and heated at 400^{o}. Hydrogen was passed through the heated tube to ensure reduction of the copper to the metallic state before it was used to remove traces of oxygen.

4-13 Manometric experiments

Standard procedures (144) were employed in manometry using Braun equipment. The reaction mixtures given in Table 3 were used, each in a total volume of 4 ml in 0.05 M tris buffer pH 8. Chalcone was dissolved as the disodium salt in this buffer a short time before use. The concentration of chalcone in the stock solution was checked

spectrophotometrically immediately before each manometric run since in the buffered stock solution, unlike in ethanol, significant chalcone isomerised to the flavanone. (Separate spectrophotometric studies showed that added flavanone, to 10% of the concentration of chalcone initially present, was without effect on the enzymic reaction.) An aliquot of the chalcone stock solution was placed in the main well of the manometric flask together with the additional buffer required to make the total reaction volume of 4 ml. In the sidearm was placed enzyme in 0.4 ml total volume of buffer pH 8.0. The hydrogen peroxide was supplied as 5 µl aqueous solution and was deposited as an isolated drop in the entrance to the sidearm. When the system had been equilibrated at 25° and sealed off, the enzyme solution in the sidearm was tipped into the flask collecting as it flowed the drop of hydrogen peroxide. Readings of the manometers were continued until uptake of gas had ceased which was normally about 20-25 minutes after the start of the reaction. In the calculation from the observed pressure change of the volume of oxygen absorbed the solubility of oxygen was taken as 0.02822 ml/ml of solution.

4-14 Methods for estimation of hydrogen peroxide

(a) Volumetric. The stock solution of approximately 30% w/v hydrogen peroxide from which known concentrations were prepared for addition to enzyme reaction mixtures was standardised periodically by titration against a standard solution of potassium permanganate in the presence of sulphuric acid (145).

(b) Spectrophotometric. A method was developed to

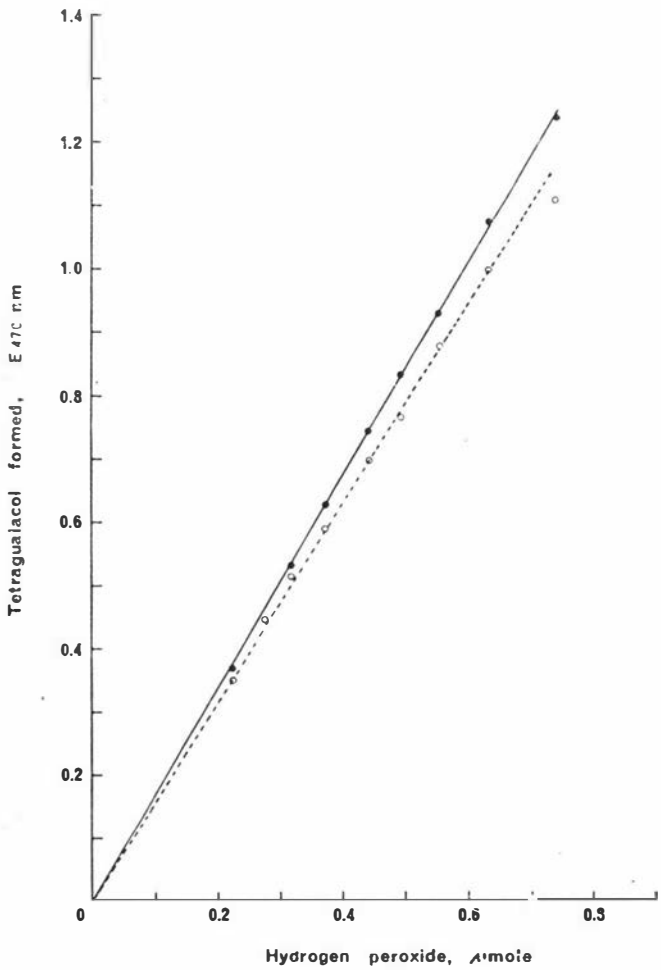
permit rapid and accurate estimation of hydrogen peroxide in the concentration range 0.1-0.5 mM. This method was applied to the estimation of residual hydrogen peroxide in 2 ml reaction volumes after the reaction of chalcone had gone to completion (see section 2B-7). The method utilised the quantitative peroxidatic oxidation of guaiacol by hydrogen peroxide to the coloured product, referred to as tetraguaiacol, the formation of which was monitored at 470 nm.

Initially, a calibration curve was constructed for enzymic tetraguaiacol production (absorbance at 470 nm) as a function of the amount of hydrogen peroxide added to 0.05 M tris buffer pH 8, total volume 2 ml (see Figure 62). The reaction mixture contained HRP enzyme (400 units added in 5 μl tris buffer pH 7.5), guaiacol (5 μl of 130 mM (0.65 μmole) with amounts of hydrogen peroxide up to 0.4 μmole and 10 μl (1.30 μmoles) with higher amounts) and buffer to make to 2 ml, temperature 25^{o}. Addition of known amounts of hydrogen peroxide (0.22-0.88 μmoles in 10 μl of water) started the reaction. At the lower hydrogen peroxide concentrations the lower concentration of guaiacol was found necessary to prevent significant depression of tetraguaiacol formation. The high amount of HRP enzyme used gave a rapid reaction, complete within 100 seconds. This feature was important since tetraguaiacol was unstable and its absorbance at 470 nm dropped continuously.

While the above calibration curve provided accurate estimations of hydrogen peroxide in pH 8.0 tris buffer, a further curve was found necessary for determinations of residual hydrogen peroxide in the presence of products of

Figure 62

Calibration curves for the estimation of H_2O_2 by tetraguaiacol formation in HRP-catalysed reaction



●——● Pure system in 0.05 M tris buffer pH 8.0

o----o In presence of products of chalcone reaction

See text for details of reaction mixtures and procedures.

the chalcone reaction since tetraguaiacol was more unstable in this system. A further calibration curve (Figure 62) was therefore prepared for known amounts of hydrogen peroxide added to a reaction mixture which contained, in addition to the components specified in the previous calibration above, the freshly-formed products of a standard chalcone reaction. The standard chalcone reaction system for the calibration recorded in Figure 62 contained chalcone (0.37 μmole), HRP enzyme (1 unit) and hydrogen peroxide (0.22 μmole) in pH 8.0 tris buffer, 2 ml total volume, temperature 25^{o}. This reaction went almost to completion in about 100 seconds and was monitored at 470 nm by the decrease of the chalcone absorption which was initially 0.7 at this high wavelength on account of the high concentration present. The hydrogen peroxide added (0.22 μmole) was near the minimum required for the reaction to go effectively to completion. When the reaction was virtually complete, a known additional amount of hydrogen peroxide was added and followed immediately by a combined addition of HRP enzyme and guaiacol as given for the previous calibration. Tetraguaiacol formation, which occurred even more rapidly than before, gave a measure of the total hydrogen peroxide content of the final reaction mixture. However, this total hydrogen peroxide consisted of a fixed amount carried over from the chalcone reaction in addition to the amount finally added. Addition of the HRP-guaiacol mixture, without further hydrogen peroxide, at the end of the chalcone reaction led to reproducible tetraguaiacol formation. Hence, the total tetraguaiacol formation observed was reduced by the amount equivalent to the hydrogen peroxide carried over from the

chalcone reaction to give the net values plotted in Figure 62 for the final amounts of hydrogen peroxide added. The changeover point for guaiacol addition from 5 µl to 10 µl (see previous calibration above) was when the total hydrogen peroxide content of the reaction mixture exceeded 0.4 µmole, as indicated by tetraguaiacol formation.

4-15 Paper chromatography

All paper chromatography was done on Whatman 3 MM paper which was washed, prior to use, in 5% acetic acid and rinsed in distilled water before drying. The standard solvent pair for 2-D work was benzene: acetic acid:water (125:72:3 by volume) in the first direction followed by 10% aqueous acetic acid in the second. No equilibration time was allowed prior to running in either solvent.

Rather than use the full dimension of sheets of Whatman paper (46 x 57 cm), a much more convenient procedure was developed to handle the large number of analytical 2-D chromatograms which were run. This consisted of reducing the size of the chromatogram so that four small 2-D chromatograms could be obtained per full-size sheet. The area potentially available for spot migration was about 20 x 20 cm on each small chromatogram. Advantages of this reduction in scale were the greater speed with which chromatograms could be developed thus minimising changes in the compounds under study and also the increased capacity of the chromatography tanks which allowed more samples to be handled at a time. Little or no disadvantage accrued with the reduced dimensions of the chromatogram. Separation of compounds was satisfactory for most purposes and was nearly as good as on

the full-size sheets, provided that the spot applied at the origin was kept small, preferably 0.75 cm diameter or less and containing not more than 250/ug of solid material.

The procedure used with the small-size chromatograms was to cut the full-size sheet in half, across the shorter dimension. On each half-size sheet, two spots were applied, each 4.5 cm from the mid-point of a line drawn parallel to and 7 cm in from the freshly cut edge on each half sheet. Each half sheet carrying two separate samples was then chromatographed (descending) in BeAW and dried. Then the bulk of the 7 cm wide strip across the top of the 1-D chromatogram which had dipped into the BeAW solvent trough was cut off and the remainder of the sheet was folded suitably about that mid line equidistant from the two lines of spots which arose from the first direction development. In this folded form the connected chromatograms were immersed along the folded mid-line in the second direction solvent and the descending alignment of the body of each chromatogram, clear of the edge of the solvent trough, was ensured by appropriate folds parallel to and suitably separated from the mid-line fold. When finally dry after second direction development, the individual chromatograms were separated by cutting along the mid-line fold. The area of paper which had been finally immersed in the HA solvent proved convenient for recording details of the sample chromatographed.

Times required for running these small chromatograms at about 25^{o} were, 1 hour in BeAW, 20-30 minutes 'drying' time between solvents and 1-1.25 hours in 10% HA. With full-size chromatograms, running times were at least three times

as long. Occasionally, where the maximum separation was required in the first dimension only, as in the resolution of flavonol and aurone, full-length chromatograms were employed but two spots were placed on each sheet as for the small-size chromatograms, so that the second direction could be run (adequately) over the small-size distance only.

Special mild treatment was applied in the chromatography of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ in particular, to minimise the transformation to OC and flavonol and the extent of other losses of these compounds. Hence these compounds were applied to the chromatograms in acetone solution (ethanol was also used with other more stable compounds) and the solvent was removed with a minimum exposure to a cold air stream. Minimum delay was aimed for at all stages of the chromatographic procedure and low temperatures (down to 4^{o}) during the chromatographic separation were found advantageous if good quality eluates of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ were sought.

4-16 Diazotised sulphanilic acid spray reagent

Diazotised sulphanilic acid chromogenic reagent was prepared immediately before use by mixing equal volumes of a saturated solution of sulphanilic acid in HCl and of 0.07% sodium nitrite. A few minutes later, the solution volume was doubled by adding aqueous sodium carbonate (5% w/v anhydrous salt) and the prepared reagent was then used immediately. Stock solutions used in the preparation of this spray reagent were held at $0\text{-}4^{o}$ in order to lower the temperature of the prepared reagent and thus enhance the stability of the diazonium product formed. The stock solution of sulphanilic acid was prepared from sulphanilic

acid (4 g), concentrated HCl (40 ml) and water (400 ml).

4-17 Preparative method of OC production

The preparative scale reaction was carried out at room temperature (20-25°) in a 500 ml separating funnel. The reaction mixture contained chalcone (50 mg (182 μmoles) in 5 ml 85% ethanol), HRP enzyme (800 units in 10 μl of a 1 mg/ml solution of enzyme in 0.05 M tris buffer pH 7.5), hydrogen peroxide (50 μl (430 μmoles) of 30% w/v solution in 5 ml of water) and 0.05 M tris buffer pH 8.0 (190 ml). Enzyme and buffer were mixed and the chalcone solution was thencarefully added with swirling followed by rapid addition and mixing of the hydrogen peroxide to start the reaction. Air was bubbled steadily through the solution during the incubation time for complete reaction which was 3.5-4 minutes. Decolorisation of the chalcone was visually followed to determine accurately the incubation time in each run. The final solution was faintly yellow in contrast to the orange-yellow when chalcone was present. Alkali (2.5 ml of 2.5 N NaOH) was immediately added with rapid swirling once the enzymic reaction was complete. Two minutes later, the alkaline solution was acidified (5.2 ml of 2 N HCl) and the product (largely OC) was recovered without delay by ether extraction (2 x 200 ml). The combined wet ether extract was evaporated on a rotary evaporator to yield crude OC as a yellow solid.

4-18 Preparative method of $\emptyset Y_1$ and $\emptyset Y_2$ production

These precursors of OC and flavonol were readily prepared when the reaction system detailed above for preparative OC production was used. The required modification was

restricted to acidification (3.56 ml of 1.44 N HCl) immediately the reaction was complete. Ether extraction as before, then proceeded immediately. The wet ether extract was taken to dryness on a rotary evaporator with the water bath at 30^{o} and water contacting only a small area on the evaporating flask so that the solution was evaporated close to 0^{o}. Once the volatile ether had been removed, evaporation of the small volume of aqueous liquid which remained was assisted by the addition of a little ethanol. In drying this final volume, the evaporating flask was normally held out of the water bath in order to prevent damaging application of heat as drying was slow and consequently little evaporative cooling occurred. The dried sample could be successfully held at -10^{o} for a few days if necessary.

4-19 Preparation of ØOC

In the course of preliminary attempts to obtain crystalline $ØY_1$ or $ØY_2$ from solutions of reaction product mixtures it was discovered that from these solutions ØOC could be obtained instead. Thus, a freshly made reaction product mixture of $ØY_1$ and $ØY_2$ solid was taken up in a minimum volume of either acetone or ethanol at room temperature. A little water (about 10-20%) was then added cautiously and the solution made more aqueous by blowing off mainly organic solvent under an air jet. During this treatment the solution became a brownish-red colour (initially slightly yellow) and after being concentrated to a small volume it was placed in ice. Aggregates of a white solid separated out over the next few hours. This

solid was filtered off and washed clean of mother liquor with aqueous acetone at 0^{o}. From its u.v. spectrum in ethanol and on the addition of alkali, the solid was identified as ØCC. The deposition of ØOC always occurred when reaction product mixtures of ØY compounds were treated as described here. A quantity of ØOC was prepared from a number of reaction product samples of ØY compounds which were put through the above procedure.

The originally deposited ØOC solid was further purified by deposition from hot aqueous acetone. On only one occasion was a truly crystalline product observed and then the long fine colourless needles formed in but very small amount. Repeated attempts at recrystallisation of ØOC to achieve greater purity were discouraged when some samples were lost through spontaneous and very extensive conversion to OC. This compound was much more soluble in acetone or ethanol than ØOC and consequently remained in solution at the concentrations present.

4-20 Purification of OC

Crude OC solid from the preparative scale reaction was dissolved in a minimum volume of dry acetone. The small amount of tris buffer salt which had been carried over in the wet ether extract was removed as insoluble white solid by this treatment. The OC solution in acetone was slowly concentrated under an air jet which cooled the remaining solution to well below room temperature. When a suitably high concentration was reached, solid OC began to be deposited from this solution. The removal of solvent was continued for a short time after the deposition of solid commenced. Then the sample was set aside in ice for a

few hours before the total solid was recovered by filtration and rinsed clean with ice-cold acetone. This solid was then used as the purified OC which served as starting material in various reactions. The sample used in spectroscopic examinations was purified by a more extensive process which included prior crystallisation from hot solvent (see section 2A-7).

The instability of OC limited the recovery of purified solid even with the described procedure of deposition from a concentrating solution in acetone to a maximum of about 60% of the compound present in the crude sample. The rest remained in acetone as a very impure solution. Further manipulation just increased the amount of impurity and correspondingly decreased the OC content. Conventional recrystallisation of crude OC from hot solvent (aqueous acetone or ethanol) provided OC solid but of inferior purity and in lower yield than from the adopted low temperature process.

4-21 Separation and purification of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$

The main problem faced in the isolation of $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ was the instability of these compounds. Separation of the compounds from reaction product mixtures was easily achieved by paper chromatography in dilute acetic acid solvents. However, usual methods of recovery of the separate compounds following chromatography gave unacceptable levels of impurities in the isolated products compared with the low levels of impurity in the reaction product mixture (compare Figures 17 and 18). By giving attention to all factors which were found to contribute to the presence of

impurities in the chromatographically isolated samples, it was eventually possible to isolate ϕY_1 and ϕY_2 with a minimum of associated impurities.

Thus, in the procedure finally adopted, the reaction product mixture was chromatographed on full-size sheets of Whatman 3 MM paper in 0.5% HA solvent at 4^{o} for several (5-8) hours in darkness or in dim light to clearly separate ϕY_1 and ϕY_2 as bands. For this chromatography, product from 10 mg of chalcone was banded across each sheet from acetone solution and the chromatograms were run immediately. After development, the chromatograms were removed from the solvent troughs but still left suspended for their final 30 minutes within the tank.

To prevent breakdown of ϕY_1 and ϕY_2 which would occur during any drying time, the bands were then detected on the wet chromatograms immediately they were removed from the tank. (The final period in the tank out of contact with the solvent reservoir kept excess solvent on the chromatograms to a minimum and restricted movement of especially the faster moving band of ϕY_1 after it was detected and before it was cut out.) On the wet chromatograms the bands were not immediately visible in long wavelength u.v. light. However, on irradiation for about 1 minute, a blue fluorescence developed in the location of each band. This colour was also immediately noted on examination in u.v. light of (cool) air-dried chromatograms, from which impure eluates only were obtained. Thus the colour was produced due to the instability of the ϕY compounds. A narrow guide strip on each wet chromatogram was 'developed' by exposure to u.v. light, the rest of the chromatogram being protected by a

blank sheet of chromatography paper. The bands containing $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ were then marked out and separately excised from the chromatogram, loosely rolled up and placed immediately in the deepfreeze. Bands were also located by alternative methods of removing a guide strip which was then either dried rapidly in an oven at 110^{o} and sprayed with diazotised sulphanilic acid, or heated longer to reveal the bands directly in visible light as yellow zones. Neither method was as convenient as the u.v. irradiation procedure.

The bands were rapidly eluted, after thawing, using acetone at room temperature. The procedure adopted was to fold the band in concertina fashion, with folds about 2 cm apart and running across the band, and then to saturate the loosely packed 'sandwich' of paper with acetone. Immediately, the free solution in the sandwich was expressed using a hand-held vice arrangement. A piece of plastic lined the space between the jaws of the vice and channeled the solution into a receiver held in ice. Further acetone was used to rinse the compressed paper block and then the pressure was released. The paper was again irrigated with acetone to saturation and the liquid again expressed. Tests showed most of the compound was recovered in the first two cycles. Normally three elution cycles were performed before the paper was discarded.

Acetone was selected as the eluting solvent for several reasons. Firstly, it was more readily removed than ethanol under reduced pressure to recover the eluted compound Secondly, it penetrated the folded paper very efficiently and quickly at the outset and also after the paper had been released from compression. Thirdly, it was a good solvent

for the ØY compounds. Equivalent bands from two chromatograms were normally eluted concurrently when a quantity of compound was desired.

The combined acetone eluate was reduced in volume on a rotary evaporator at 0^{o} to the residual aqueous acetic acid solution which had been present in the bands of moist chromatography paper eluted. The same care as noted in the drying of the crude preparation of ØY compounds extracted from the enzymic reaction was taken to avoid overheating of the solution in all solvent removal steps using the rotary evaporator.

The aqueous solution which contained the ØY compound together with many paper fibres and other impurities eluted from the chromatography paper was bulked up if necessary with distilled water and extracted twice with an equal volume of ether. The combined ether extract was concentrated to half volume and washed with water before being finally taken to dryness in the rotary evaporator. A little alcohol was added near the end of this step to assist removal of the small amount of water carried over purposely with the wet ether. The small amount of acetic acid which had been carried through this entire elution and final solvent extraction process of purification was vital for the prevention of general degradation and also transformation of ØY compounds to OC.

An alternative method whereby $ØY_2$ could be directly isolated from the reaction product mixture without the requirement for chromatography was also investigated. This process utilised the property noted with chromatographically isolated samples that $ØY_2$ tended to form crystalline aggregates

while $\emptyset Y_1$ always remained as a glassy solid. Thus, while the reaction product mixture of $\emptyset Y_1$ and $\emptyset Y_2$ was always obtained, on removal of the solvent following ether extraction, as a faintly yellow glassy solid, areas of granulation characteristically developed within a film of this solid when the flask was stored between zero and -10^{o} overnight. In a particular case where very coarse granules developed, a sample of the granular solid was subsequently recovered in free-flowing form by careful treatment with ether to dissolve preferentially the glassy matrix within which it formed. Subsequent tests (u.v., i.r., 5% HA chromatography) showed the strongly yellow product isolated was a substantially pure preparation of $\emptyset Y_2$. However, on account of the low amount of $\emptyset Y_2$ recovered in terms of the reaction product present and of the variability of the granulation process between preparations, this method was not pursued further as a means of preparation of $\emptyset Y_2$.

4-22 Synthesis of chalcone

Isoliquiritigenin was synthesised by condensation of resacetophenone and p-hydroxybenzaldehyde under alkaline conditions. Resacetophenone (6.3 g) was dissolved in 25 ml of 50% potassium hydroxide and p-hydroxybenzaldehyde (5.10 g) was added with stirring until the compound dissolved. The flask was then flushed with nitrogen, tightly stoppered and incubated in a water bath at 60^{o} for 21 hours. Ice (about 20 g) was then added, followed by concentrated HCl (20 ml) to precipitate the crude product. This solid was recovered by filtration and washed with water. It was then dissolved in a minimum volume of warm ethanol and chromatographed on two large (5 x 40 cm) polyamide columns using 95% ethanol

as the eluting solvent. The chalcone band was recovered following the elution of faster moving components, mainly unchanged starting materials. Chalcone (5.9 g) was recovered on drying the combined chalcone eluate from both columns. The product was further purified by recrystallisation from aqueous ethanol and acetone to yield yellow needles m.p. 202-204°. Several further recrystallisations provided the sample which was used as substrate in the detailed biochemical studies. This preparation was chromatographically pure and melted sharply at 204-205°.

Chalcones, besides isoliquiritigenin, were available in the laboratory in small amounts having been previously synthesised by Dr. E. Wong in connection with previous studies (15). ^{14}C- and ^{3}H-labelled isoliquiritigenin were also available from this source.

4-23 Purification of chalcones by paper chromatography

The chalcones used in the study of the substrate specificity of the enzymes (see Figure 30) were mostly available in only very small quantities from previous work of Wong (15). An amount of about 1 mg of each compound was subjected to 1-D chromatography as a band 3 cm long on full-length papers in the solvent systems BeAW, 30% HA and 30% isopropanol in water. The finally eluted (85% ethanol) compound was dried, taken up in 5 ml of ether which was then washed with an equal volume of water before the organic layer was again evaporated to provide the sample used in the enzymic reaction. This solution of the alcohol-eluted chalcone in ether followed by water washing removed visible amounts of whitish material eluted from the chromatography

paper. Such contaminating material was recovered in alcohol elution despite the use of specially pre-washed paper (85% ethanol wash after regular washing in 5% HA followed by distilled water rinse) for the purification of these chalcones. The presence of paper-derived impurities in the final chalcone samples was indicated by the inhibition of the reaction of isoliquiritigenin which was noted when a chromatographically prepared sample was used. This sample had been run through the above purificiation procedure with the other chalcones, as a control, starting with the sample purified by repeated recrystallisation and routinely used as substrate in the enzymic reaction.

4-24 Synthesis of hispidol

Hispidol was synthesised by alkaline ferricyanide oxidation of isoliquiritigenin as previously detailed by Wong (17) and purified by recrystallisations from aqueous ethanol and acetone. The melting point of the sample used as fluorimetric standard was 289-291°.

4-25 Preparation of trimethylsilyl derivatives

For each of the compounds silylated ($\emptyset Y_1$, $\emptyset Y_2$, OC and flavonol), a sub-milligram amount of the dry solid was added to a vial sealed with a serum cap. The silylating reagent N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was then added (50-100μl per vial). When the solid had dissolved in this added reagent (after 1-2 hours at room temperature), the compound was considered to have been silylated. Samples (2-5μl) of the solution were withdrawn

and submitted to analytical and preparative GLC. The remaining reaction solution for each sample was observed to keep satisfactorily at 0^{o} for a number of days.

4-26 Gas-liquid chromatography of trimethylsilyl ethers

A Packard gas chromatograph fitted with a flame ionisation detector and column temperature programmer was used in the analysis of the trimethylsilyl ether derivatives prepared from βY compounds, OC and flavonol (see section 2D-5). Glass columns were used; the preparative column (4 mm ID x 2 m) and the analytical column of similar dimensions were packed with silylated 80-100 mesh Gas Chrom Q coated with 3% and 10% SE-30 respectively. The flow rates used were, nitrogen carrier gas 50 ml/min, hydrogen 60 ml/min and air, 250 ml/min. The inlet temperature was 230^{o} while the outlet and detector temperatures were set at 240^{o}. Oven temperature was programmed to rise from 200^{o} at sample application to 240^{o} at 1^{o} per minute.

With the analytical column (10% SE-30), 5 µl samples of each silylation reaction mixture were injected. Binary mixtures of these were also prepared in various combinations and similarly injected to check relative retention properties of the major products. The main TMS product from each silylation reaction was trapped and sealed in a capillary tube for later mass spectral examination as the appropriate peak emerged from the 3% SE-30 column operated at a fixed 240^{o} oven temperature. This trapping was accomplished by fitting a stream splitting device between the column outlet and the detector so that when the detector recorded the start of the peak of interest a clean capillary

tube was inserted in the outlet of the main stream of column effluent to trap the vaporised compound in a short section of the air-cooled tube which was then immediately sealed off in a small flame. Sealed samples were held at 0^{o} until examined (within 72 hours) by mass spectrometry.

4-27 Preparation of diazomethane

An etherial solution of diazomethane was prepared from p-toluenesulphonylmethylnitrosamide ('Diazald' from Aldrich) immediately before use following established procedures (146). To prepare 0.3 g of diazomethane, the distillation flask was charged with 0.5 g potassium hydroxide (pellet), 0.75 ml of water and 2.5 ml of 95% ethanol. A dropping funnel fitted in the neck of this flask contained 2.15 g of Diazald dissolved in 15 ml ether. To the stirred contents of the distillation flask held at 65^{o} in a water bath, the Diazald solution was added slowly over 20 minutes. Liberated diazomethane was led with the ether vapour from the distillation flask through a water cooled condenser to a receiver where it was collected in ethereal solution over sodium sulphate. A small additional volume of ether was added to the distillation flask after the last of the Diazald solution and until no further diazomethane distilled over.

4-28 Methylation of OC using etherial diazomethane

Dry OC (90 mg) was dissolved in 100 ml of dry ether containing 5% methanol (to increase the solubility of OC) and the solution was cooled to 0^{o}. Diazomethane (300 mg theoretically) in ether was added and the mixture was left for 1 hour at 0^{o} and then a further hour at room temperature

before the solvent was evaporated. A clean yellow solid, poorly soluble in ethanol but soluble in acetone, was obtained. Crystallisation from acetone-ethanol gave the OC-methyl ether in 83% yield. Further amounts of the product were similarly prepared.

4-29 Catalytic hydrogenation of OC and OC-methyl ether

Both compounds were hydrogenated at atmospheric pressure in 95% ethanolic solution over a 10% palladium on charcoal catalyst at room temperature. The catalyst was dispersed in ethanol and pre-saturated with hydrogen before the organic compound was added in solution and the reaction started. Uptake of hydrogen occurred very rapidly (section 2D-3 (c)) and was followed manometrically.

A typical reaction system for OC contained 120 mg purified OC in 60 ml ethanol and separately 200 mg of catalyst in 40 ml ethanol (for initial saturation with hydrogen before the solution of OC was added). For OC-methyl ether, 90 mg of compound in 60 ml of ethanol was added to catalyst prepared as for OC. When either one or two mole-equivalents of hydrogen had been taken up, the catalyst was quickly filtered off to interrupt further reaction. The filtrate was dried and the products were examined chromatographically.

4-30 Quantitative estimation of ϕY_1 and ϕY_2

This estimation was normally performed spectrophotometrically on a suitable aliquot of an alcoholic stock solution of the ϕY compound diluted to 2 ml in 85% ethanol. Rather than taking a direct measurement of the absorbance of the solution of the ϕY compound, alkali (5 μl of 1.25 N NaOH)

was added to transform in 1 minute the compound to OC anion as quantitatively as possible. On acidification (10 µl of 1.44 N HCl) of this solution, the OC product was then estimated from the absorbance at λ max. 325 nm ($E^{1\%}_{1cm} = 0.845 \times 10^3$). The ØY compound equivalent to this calculated OC content was obtained by multiplying by the ratio of the molecular weights of the compounds, that is 288/270. This procedure had the advantage that the effect of impurities which were frequently present and which tended to absorb maximally at 275 nm. (λ max for ØY compounds also) but minimally at 325 nm, could be substantially overcome since exposure to the alkali and acid treatment required to obtain OC did not alter their absorbance. When the ØY compounds were pure, for example in the accurate estimations of the proportions in enzymic reaction product mixtures, the quantities of ØY compounds could be directly obtained from absorbance readings at 275 nm using the appropriate $E^{1\%}_{1\ cm}$ values, see section 4-32.

A source of limited error in the estimation of ØY compounds as OC after alkali transformation, was the unavoidable proportion of flavonol in the product even when minimal amounts of alkali for efficient transformation were added (see Figure 31). The $E^{1\%}_{1cm}$ value for flavonol at 325 nm was calculated as 0.42×10^3 (compare value of 0.78×10^3 at λ max 357 nm (147)). Hence, the observed absorbance at 325 nm of alkali transformed ØY compound would be lower the greater the proportion of flavonol in the product. Under the carefully controlled conditions of transformation as described above and employed throughout the present work, the flavonol content was estimated (Figure 31) to lie in the range 5-10%. Therefore the

estimated OC, equivalent to the ØY compound, would have been low by about 2-5% since the presence of this flavonol component with lower specific absorption was ignored.

4-31 Optical activity measurements

Measurements were made with a Bendix automatic polarimeter at the wavelength of the sodium D line at room temperature, approximately 20°. The compound was dissolved in 2 ml 85% ethanol and this solution used to fill the cell of pathlength 2 cm. From the observed angle of rotation, α, the specific rotation, [•], was calculated from the relationship (148):

$$[\alpha] = \frac{100\alpha}{l\ (dm) \times c\ (g/100ml)}$$

where l is the cell path length in decimetres and c is the concentration of solute expressed in grams per 100 millilitres. For the ØY compounds, the concentration of solute was calculated from the equivalent OC content determined spectrophotometrically. Thus, an aliquot of solution from the cell was diluted in 85% ethanol and transformed to OC by controlled addition of alkali and then acid. The concentrations of ØY compounds present usually fell within the range 0.1-0.6 g/100 ml.

4-32 Determination of $E^{1\%}_{1cm}$ values for $ØY_1$ and $ØY_2$

The $E^{1\%}_{1cm}$ values for $ØY_1$ and $ØY_2$ (as separate $ØY_2a$ and $ØY_2b$ forms), were measured indirectly by determining the ratio of the absorbance for each ØY sample at 275 nm (λ max) to the absorbance of the equivalent OC at 325 nm (λmax), formed on controlled addition of alkali (5 µl 1.25 N NaOH per 2 ml sample in 85% ethanol) followed by

acidification (10 µl of 1.44 N HCl). The absorbance ratios (ØY sample:equivalent OC) thus recorded were 0.604, 0.622 and 0.619 for $ØY_1$, $ØY_2a$ and $ØY_2b$ samples respectively. From the $E^{1\%}_{1cm}$ value for OC at 325 nm, 0.845×10^3, and these ratios, the corresponding $E^{1\%}_{1cm}$ values for the ØY compounds, at 275 nm and of molecular weight 286 compared with 270 for OC, were then calculated as $ØY_1$, 0.480×10^3, $ØY_2a$, 0.495×10^3 and $ØY_2b$, 0.495×10^3.

4-33 Determination of pK'_a of ØY compounds, OC and flavonol

The apparent pK_a (pk'_a) was calculated from the relationship given by the Henderson-Hasselbalch equation:

$$pH = pK'_a + \log \frac{[salt]}{[acid]}$$

For each compound, the ratio of the concentration of salt (conjugate base or ionised form) to acid (unionised form) was determined spectrophotometrically at a known pH where the concentrations of these forms were similar. This was possible with each of the compounds studied since at selected wavelengths the absorbance of the acid (unionised) and conjugate base (ionised) forms differed widely. Hence from the absorbance at an intermediate pH where a mixture of acid and conjugate base forms existed, the ratio of these forms at the known pH could be readily calculated using the absorbance data determined for the fully acid and fully conjugate base forms at the same wavelength and concentration.

A stock solution of each compound in 85% ethanol was prepared so that a 50 µl aliquot added to 1.95 ml of 0.05 M tris buffer in the pH range employed gave a suitable

absorbance reading at the wavelengths of interest. The spectrum of the unionised form was recorded at about pH 3 which was attained on addition of 10 µl of 1.44 N HCl to 1.95 ml 0.05 M tris buffer pH 7. To obtain the spectrum of the fully ionised form, the pH of the buffer was increased until the absorbance due to the ionised species reached maximum intensity while the isosbestic point of interest was retained. If eventually at higher pH the spectrum no longer included the established isosbestic point, the presence of an additional ionic species was indicated and the spectrum at the highest pH at which the isosbestic point persisted was taken as that of the monoionised form. For example, in the case of OC, at pH 9 the spectrum of the fully ionised form was observed, isosbestic point 345 nm, while at pH 11, the absorbance at 345 nm dropped appreciably with further apparent ionisation and limited red shift in the absorption above this wavelength. With $\emptyset Y_1$ and $\emptyset Y_2$, maximum ionisation was apparent at pH 9. The instability of these compounds meant that the absorption at the wavelength of interest (335 nm in all cases) was recorded immediately on rapid mixing of the aliquot of ethanolic solution and the buffer. These compounds showed an isosbestic point at 300 nm. For ØOC also, maximum ionisation was obtained at pH 9. In the case of flavonol, the first ionisation (isosbestic point 365 nm) was apparently complete at slightly above pH 8. Each compound was found to give rise to a suitable mixture of ionised and unionised forms in pH 7 buffer. Thus the absorbance at this pH and at appropriate wavelength, OC, 380 nm, ØY compounds, 335 nm, ØOC, 335 nm and flavonol 386 nm, was used, in conjunction

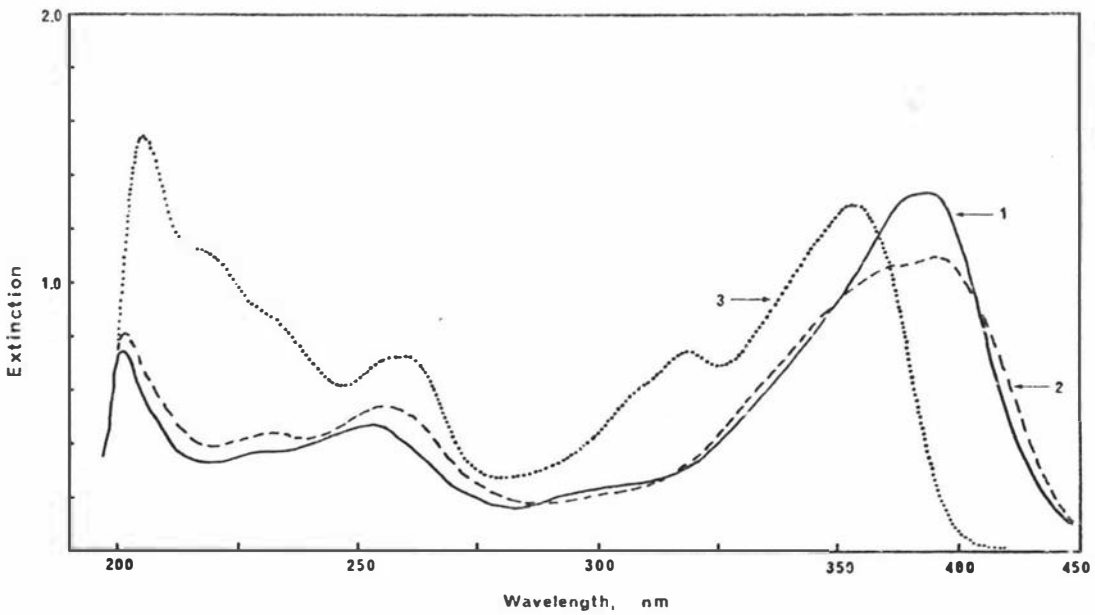
with the absorbance at the same wavelength of the unionised and fully ionised forms, in the calculation of the data recorded (Table 10).

4-34 Light sensitivity as a factor in estimation of aurone

A complicating factor in the accurate quantitative estimation of hispidol (aurone) was the discovery that exposure to light affected the absorbance of a solution of the compound. This is illustrated by the absorption spectra presented in Figure 63 of the compound freshly dissolved in 85% ethanol and of a portion of the same solution after standing 45 minutes or longer on the laboratory bench in bright daylight to become fully light equilibrated. The spectrum of flavonol in 85% ethanol is also included for reference purposes in the Figure. This compound in solution was stable to light. The light-induced change in the spectrum of hispidol was fully reversible during subsequent storage in darkness for 15 hours. Further recycling of light and dark treatments reproduced the expected spectral changes without the appearance of any anomalies. Freshly dissolved solid always gave a spectrum unchanged by subsequent dark storage of the solution. Chromatography of hispidol in 30% HA in darkness, starting with light-equilibrated and dark-stored solutions, showed that dumbell-shaped spots were obtained from the light-equilibrated sample in contrast to homogeneous eliptical spots from the other sample. Most of the material in the dumbell-shaped spot was in the faster-moving section which corresponded in R_f with the dark-stored sample. On BeAW chromatograms, the same series of samples gave rise to only single spots of identical

Figure 63

U.v. absorption spectra of hispidol, before and after light equilibration, and of flavonol



1 = Freshly dissolved hispidol or dark-stored solution, in 85% EtOH

2 = Solution as in 1 but after equilibration in light

3 = Flavonol in 85% EtOH.

size and R_f. This light induced change was thought to involve cis-trans isomerism of the hispidol molecule. Similar irradiation-induced isomerisation of an aurone has been recently reported (149). At equilibrium in light, the absorbance at 390 nm was invariably 83-84% of the absorbance for the freshly dissolved sample. For convenience, quantitative work was therefore done with light-equilibrated solutions. The $E^{1\%}_{1cm}$ value for hispidol in 85% ethanol was also reassessed on account of this effect of light. A new value of 1.26×10^3 (388 nm) was found for freshly dissolved solid and for the light-equilibrated solution, a value of 1.05×10^3 (390 nm) was recorded; compare previous value reported 1.08×10^3 (17).

4-35 Fluorimetric estimation of aurone and flavonol

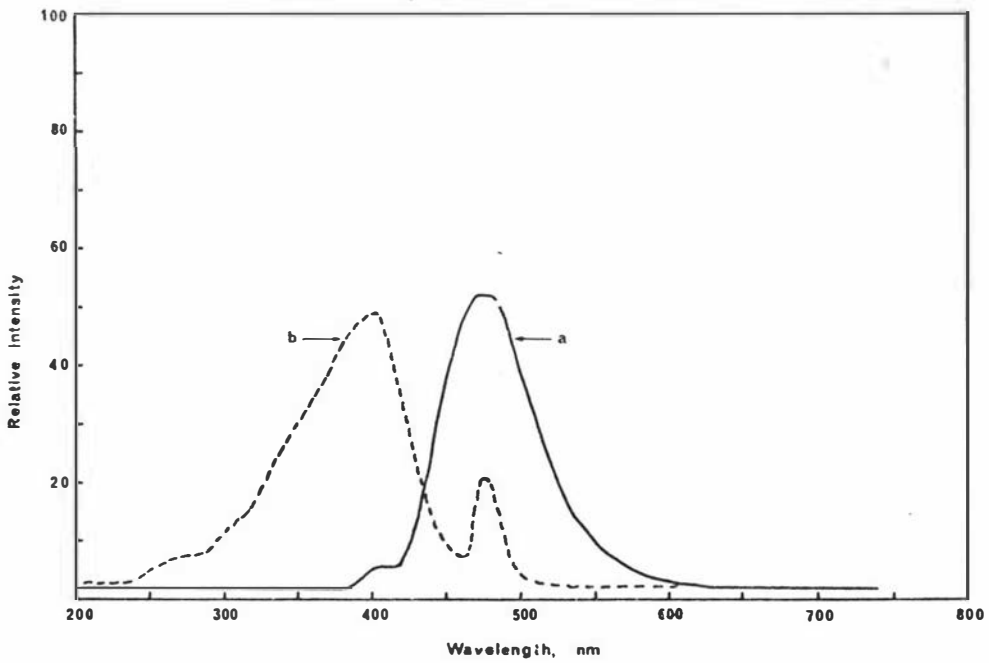
A fluorimetric method was developed to permit estimation of the low amounts of aurone (hispidol) and flavonol (4',7-dihydroxyflavonol) which were encountered in products of the chalcone reaction.

Fluorescence emission and excitation spectra of aurone (Figure 64) and flavonol (Figure 65) were specially determined with a spectrofluorimeter (Aminco-Bowman) to guide filter selection in the development of methods for experimental estimation of these compounds using a filter instrument (Farrand). The hispidol solution used was light-equilibrated.

Thus, using the filter instrument, both compounds were excited satisfactorily when a primary filter (Turner filter number 7-60) with a pass band maximal at 360 nm in the long wavelength u.v. was used. Specific secondary filter combinations for aurone and flavonol with pass bands

Figure 64

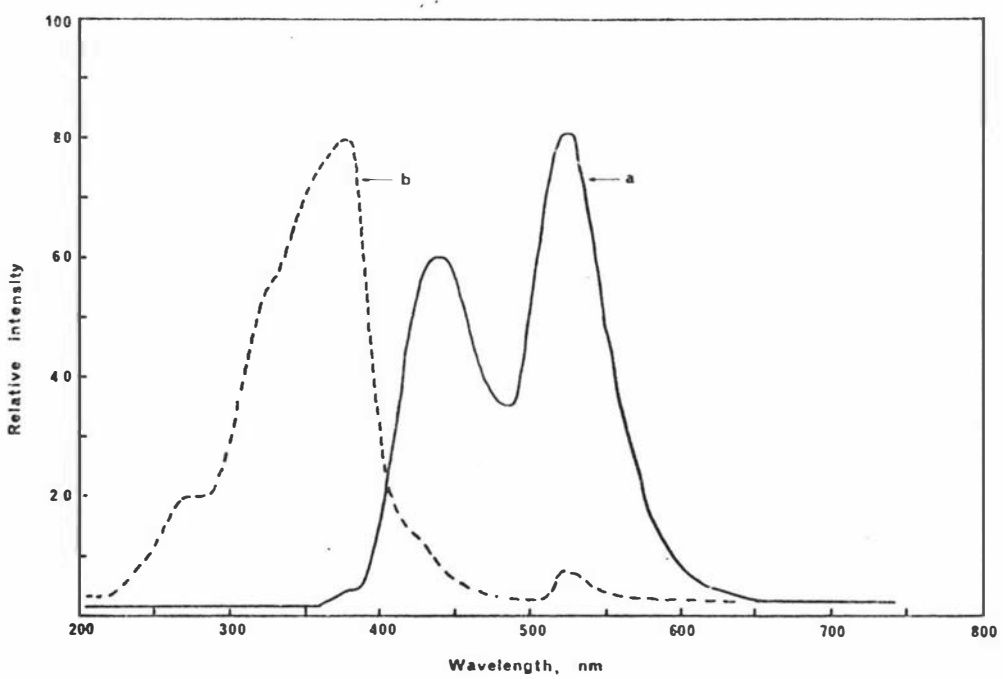
Fluorescence emission and excitation spectra of hispidol[x] in 85% EtOH



[x] Light-equilibrated solution in 85% EtOH, concentration 8.5 µg/ml
Excitation wavelength 400 nm for emission spectrum (a)
Detector wavelength 480 nm for excitation spectrum (b)

Figure 65

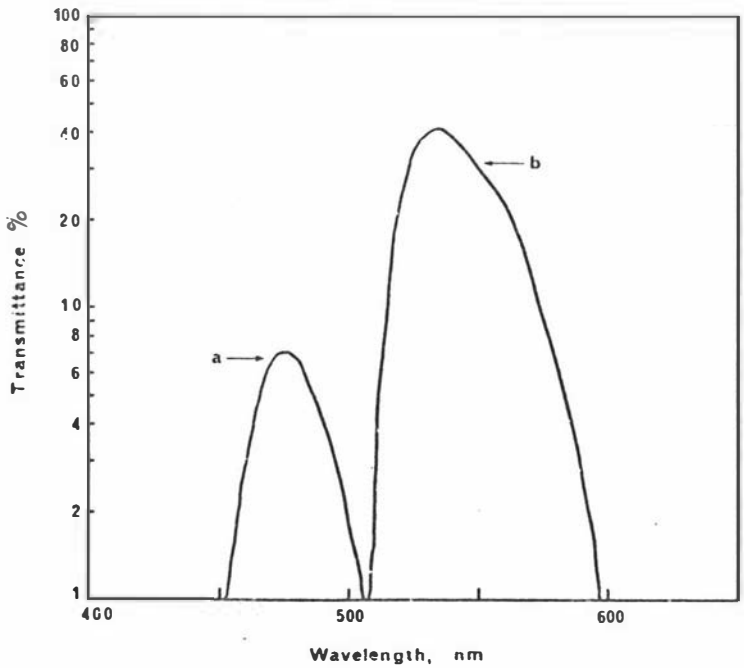
Fluorescence emission and excitation spectra of flavonol in 85% EtOH



Concentration of flavonol 12.4 µg/ml
Excitation wavelength 375 nm for emission spectrum (a)
Detector wavelength 525 nm for excitation spectrum (b)

Figure 66

Pass bands of secondary filter combinations used in the fluorimetric estimation of hispidol and flavonol.



(a) Filter combination for hispidol, Turner filters number 3 plus 48 plus 65A

(b) Filter combination for flavonol, Turner filters number 2A-15 plus 58.

shown in Figure 66 were used to sample the fluorescent light. Since the method was very sensitive for flavonol, a considerable part of the fluorescent light could be filtered out with impunity. This also increased the specificity of detection and restricted interference possibly by traces of aurone not separated from flavonol on chromatography.

Calibration curves were constructed for aurone and flavonol dissolved in a mixed solvent which consisted of 85% ethanol and 0.02 M sodium phosphate buffer pH 7.0 (3:2 v/v). A suitable sample concentration range was 0-1 µg/ml for aurone and 0-0.1 µg/ml for flavonol, within which fluorescence was a linear function of concentration. Sample volumes were between 1 and 2 ml. Reference concentrations for fluorimetry were prepared from newly made stock solutions of the compounds in 85% ethanol, the concentrations of which were determined spectrophotometrically. The ethanol-phosphate concentration in the fluorimetric sample affected the fluorescence readings for both compounds. Thus volumes of ethanolic stock solution greater than 5% of the final volume were avoided in the preparation of the fluorimetric solution as a precaution against increased readings which were found to accompany increased amounts of ethanol in the fluorimetric solvent mixture.

In order to check the fluorimetric method on eluates from paper chromatograms, standard amounts of aurone and flavonol were separately chromatographed in 1-D in BeAW and the 85% ethanol eluates of spots were estimated fluorimetrically and also spectrophotometrically where possible. For the two methods, estimates of recoveries agreed to

within 5%. Because of this general agreement at all levels where absorption measurements could be applied as a check, the fluorimetric method was considered to give valid results also at lower standard amounts (under 5 µg per spot) where no independent check was possible. For aurone chromatographed in the range 1-30 µg/spot, recoveries measured fluorimetrically increased gradually from 70 to 87% in going from the lowest to the highest amounts spotted. Similarly, flavonol chromatographed at 0.2-45 µg/spot was recovered in 70 to 85% measure. During this work it was also found that the weakest spots of both compounds could be eluted most conveniently in the fluorimetric solvent directly rather than in 85% ethanol initially.

In the course of developing the fluorimetric method and applying it to the estimation of aurone and flavonol eluted in 85% ethanol from chromatograms, even very small amounts of residual acetic acid normally present from the chromatographic solvent were found to have adverse effects on the fluorescence of samples. Thus when fluorescence measurements were made in 85% ethanol solvent on aliquots of eluates from paper chromatograms which could also be checked spectrophotometrically, it was found that the recoveries estimated fluorimetrically fell to as low as 50% and 85% of the spectrophotometrically determined values for hispidol and flavonol respectively. That these discrepancies were probably caused by variable carry-over of acetic acid from chromatography was shown when added traces of acetic acid correspondingly depressed the fluorescence of standards of aurone and flavonol. Deliberate addition of a low amount of acetic acid to all 85% ethanol used, in

order to standardise the effect, was tried since tests showed that fluorescence was insensitive to small variations in acetic acid strength above a minimum. However, the measurements recorded were less accurate and more variable than with the finally selected ethanol-phosphate solvent.

4-36 Estimation of the yield of Y_1 and Y_2 in the enzymic reaction

The procedure employed was to convert to aurone the Y_1 and Y_2 formed in the reaction mixtures given below, by alkali treatment of the initial reaction product prior to ether extraction. This aurone, unavoidably contaminated by some flavonol, was isolated by 2-D chromatography. The recovered sample was then prepared for fluorimetric estimation by preferential destruction of the contaminating flavonol to non-fluorescent products upon warming in dilute alkali (see below).

The reaction mixtures in which Y_1 and Y_2 formation was measured contained chalcone (0.73 μmole), hydrogen peroxide (1.2 μmoles or 0.6 μmole with HRP enzyme (13 units) or garbanzo enzyme (25 units) respectively) and enzyme (given) in 0.05 M tris buffer pH 8.0, total volume 4 ml, at room temperature. Each reaction went to completion in less than 1 minute at which stage alkali (50 μl 2.5 N NaOH) was added, with mixing, to convert the Y_1 and Y_2 formed to aurone (OC and flavonol were also formed from the $\not$Y compounds). After standing for 10 minutes at room temperature to ensure complete conversion of Y_1 and Y_2 to aurone, the alkaline solution was acidified (150 μl 1.44 N HCl) and the products recovered by

ether extraction for 2-D chromatography. Duplicate runs were made for each reaction mixture. Each sample was chromatographed on a full-length paper in the BeAW first direction solvent to achieve maximum resolution of aurone and flavonol, while half-length development in 10% HA in the second direction separated most of the OC from the aurone and provided a check that all Y_1 and Y_2 had been converted to aurone.

The prominent aurone spot on each chromatogram was excised to include about half of the adjacent weak but prominent flavonol spot into which it merged (compare Figure 19 (ii)). The excised area of each chromatogram was eluted in 5 ml of 0.01 N sodium hydroxide for 1 hour at 60^{o} while under agitation in a water bath. Preliminary work with aurone and flavonol standards had shown that this treatment preferentially destroyed flavonol while leaving about 80% of the aurone intact. Hence destruction of the amount of flavonol present in each sample was expected. This was necessary since the amount of flavonol present, low in absolute terms but relatively large in relation to the aurone content, would have caused, by its very strong fluorescence, serious error in the estimation of aurone. Eluates were then quickly cooled to room temperature and half the volume of each was removed to a separate stoppered tube, acidified and the aurone then extracted quantitatively into ether. On removal of the ether, each sample was dissolved in 2 ml of the ethanol-phosphate solvent (section 4-35) for fluorimetry and allowed to stand in the light for 30 minutes to equilibrate. The aurone content of each sample was then determined fluorimetrically.

Readings were corrected for the fluorescence of a paper blank which had been put through the same treatment as the samples. From the results of duplicate determinations, which were in close agreement, the total aurone finally present was calculated at 0.88 and 1.09 /ug for the HRP and garbanzo catalysed reaction respectively of 200 /ug (0.73 /umole) of chalcone. Based on separate studies with standards, cumulative losses of aurone through all steps in processing of the samples were estimated to be about 50%. Hence the initial production of Y_1 plus Y_2 in the reaction was about 1% of the chalcone consumed.

4-37 Determination of radioactivity in garbanzol

The garbanzol product from the enzymic oxidation of chalcone-(3,5-T) was isolated chromatographically after the addition of sufficient carrier to enable the spot to be visually detected in u.v. light, excised and eluted with 15 ml of the liquid scintillator cocktail consisting of 6 g PPO, 0.275 g POPOP and 168 g naphthalene per litre of dioxan. The activity was determined for each sample from the time required to accumulate 5,000 counts in a Packard model 2002 Tri-carb liquid scintillation spectrometer. The counting efficiency was 30%.

In the second experiment on garbanzol formation in which the isotope used was chalcone-(carbonyl-^{14}C), the garbanzol product was isolated chromatographically after the addition of sufficient carrier to enable the spot to be visually detected in u.v. light, excised and eluted in 85% ethanol. The eluate was dried and then taken up in a small volume of ethanol for counting. In this case, each sample

was then dried on a planchette and the activity was determined from the time required to accumulate 5,000 counts in a Beckman Low Beta II counter. The counting efficiency was 23.5%.

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