THEORETICAL
The study of naturally occurring compounds has developed concurrently with organic chemistry. In common with the studies of natural products of all kinds, flavonoid chemistry has emerged from the undirected search of new compounds, and the establishment of their structures by conventional means. Substantiation and confirmation of the structures of flavonoid compounds by total synthesis has continued to develop. A variety of ingenious manipulations of functional groups have provided convenient methods for the synthesis of flavonoid compounds with a wide range of functional substitution patterns. Significant progress has also been made in the solution of problems relating to the biological origins and inter-relationships of flavonoid compounds.

The flavonoids, an important group of naturally occurring plant pigments, are generally polyphenolic compounds having \( C_6-C_3-C_6 \) carbon skeleton. Among flavonoids, the term assigned to this large class of natural substances, derivatives of 2-phenylchroman occupy an important position.

The flavonoids are important to man not only because they contribute to plant colour but also because many members are physiologically active. The importance of flavonoidic compounds in the tanning of leather, the fermentation of tea, the manufacturing of cocoa and in the flavour qualities of foodstuff is well established\(^{1a,b}\). Certain flavonoids are among the earliest known
natural dyestuffs. They are widely used as antioxidants for fats and oils. Among the physiological activities of flavonoids include vitamin P activity, diuretic action, treatment of allergy, protection against X-ray and other radiation injuries, cure of frostbite, antibacterial activity, prophylactic action, oestrogenic activity, antitumour effects and anticancer property.

The term 'flavonoid' derives from the most common group of compounds, the flavones, where an oxygen bridge between the ortho position of ring A and the benzylic carbon atom adjacent to ring B forms a new γ-pyrone type ring. Such heterocycles, at different oxidation levels, are present in most plants. The flavane corresponds to the lowest oxidation level of ring C, and is taken as the parent structure for the rational nomenclature of this group of compounds.

An oxygen bridge involving the central carbon atom of the C₃-chain occurs in a rather limited number of cases, where the resulting heterocycle is of the benzofuran type. The aurones belong to this structural group. The oxygen bridge is absent in chalcones and dihydrochalcones. Principal structural groups of natural flavonoids are shown in Fig.-1. The oxidation levels concern the three central carbon atoms: [±10 or ±2H]= +1 oxidation unit.

Besides the carbon atom link, the flavonoids also have typical oxygenation pattern in their benzene rings. Ring A generally
Fig. 1

1. Flavan
2. Dihydrochalcone
3. Chalcone
4. Flavone
5. Flavanol
has three alternate oxygens at position 2', 4', and 6' in the open formula or, in other words, ring A generally derives from phloroglucinol. Compounds having more or less oxygens in their A ring are very seldom encountered. The ring A can be occasionally alkylated with methyl groups, prenyl or prenyl derived units or with glycosides. In contrast, ring B has, in most cases, a para-oxygen substituent, or two oxygens, para- and meta- with respect to propane chain. Compounds with non-oxygenated B ring or with one ortho-oxygen function are very rarely found. Compounds bearing three oxygens, one para- and two meta- are less frequent. Typical oxygenation pattern in flavonoid is shown in Fig.-2, and Fig.-3 shows substitution pattern of the rings A and B in some less common flavonoids.

![Fig.-2](image-url)
Flavone

Sulfuretin

Nobiletin

Karanjin

Pinocembrin

Pinoquercetin

Morin

Robinetin

Fig.-3
A recent addition to the flavonoid class is 'biflavonoids'. Biflavonoids, which are generally derived from two apigenin or two naringenin or naringen-apigenin units, have mostly been isolated from gymnosperms. Depending upon the nature and the position of the linkage of the constituent monomeric units, all the C-C and C-O-C linked biflavonoids are classified into various families. Representatives of various biflavone families, depending on the position of the linkage of two flavone units, are shown in the Fig.-4.
Cupressuflavone

Amentoflavone

Agathisflavone

Hinokiflavone

Succedaneaflavone

Ochnaflavone

Fig.-4
BIGENESIS OF FLAVONOIDS

Chemical speculations on the mode of formation of the carbon skeleton of this large class of natural products stimulated the interest in the biosynthesis of flavonoids. Although the origin of the carbon atoms of flavonoid is well known \(^7-^9\), the actual compounds that condense to yield the \(C_{15}\)-skeleton and the sequence of changes which result in the formation of a relatively diverse group of compounds, based on variation in the oxidation level of the \(C_3\)-portion of the molecules, are less well understood.

Basically ring A is formed by head-to-tail condensation of three acetate units while ring B as well as \(C_3\)-chain arise from a phenylpropanoid precursor derived from the shikimic acid pathway \(^10\). The involvement of acetic acid and substituted cinnamic acid has been confirmed through studies with labelled compounds, notably by Grisebach \(^11\) and Geissman \(^12\) (Fig.-5).

\[
3 \text{CH}_3\text{COSCoA} + \begin{array}{c}
\text{Ph}
\end{array} \downarrow \begin{array}{c}
\text{Ph}
\end{array} \\
\text{Fig.-5}
\]
However, a more detailed knowledge of this reaction and of the chemical nature of the immediate precursors obtained from the enzymic studies support the proposal that CoA ester of malonic acid and cinnamic acid are the substrates of an enzyme-mediated condensation (Fig.-6)\textsuperscript{13,14}. While no experimental evidence has been obtained, so far, regarding the possible intermediates in the formation of ring A from acetyl CoA, direct evidence for the reaction mechanism formulated in Fig.-6 has been obtained from the chemical degradation of the overall product formed from p-coumaroyl CoA and \textsuperscript{14}C-labelled malonyl CoA with an enzyme preparation from cell suspension cultures of parsley\textsuperscript{15}.

![Chemical structure diagram](image)

Fig.-6
There are still some doubts about the actual structure of the phenylpropane unit used by the plants as a starter for the process of polyketide condensation and then ring A. Most chemists now believe that the cinnamic acids (p-coumaric, and more rarely caffeic, ferulic and sinapic acids) are obligatory intermediates in the biosynthesis of most flavonoids.

It has been repeatedly demonstrated, using labelled chalcones and flavanones, that these compounds are central intermediates from which most, if not all, other flavonoids originate. Conclusive evidence has not, so far, been obtained to answer the question whether chalcones or flavanones are the more direct precursors of the various flavonoids. There is good evidence for the in vitro and in vivo existence of an equilibrium between chalcones and the corresponding flavanones. The chalcone-flavanone interconversion is catalysed in vivo by an enzyme, chalcone-flavanone isomerase, isolated from various plant sources. The more important naturally occurring flavonoids are at the same or a higher oxidation level than flavanones, and many special hypotheses have been proposed to explain their genesis.

The first oxidative hypothesis for flavonoid biosynthesis was proposed by Grisebach, who also made a detailed experimental study into the chemistry and biochemistry of flavonoids. The main feature of Grisebach's hypothesis was the formation of an epoxide.
chalcone (I), which could lead to flavonols, aurones, flavones and isoflavones, through plausible chemical mechanisms (Fig.-7).

The weakest point of the epoxide hypothesis is that natural chalcone epoxides are as yet unknown. Although, synthetic 2'-OR chalcone epoxides are known\textsuperscript{24}, their epoxidation with H\textsubscript{2}O\textsubscript{2} requires strongly alkaline conditions which are conditions totally different from those occurring \textit{in vivo}\textsuperscript{25,26}.

There are alternative oxidation paths, which involve either the enolic form of the flavanone (II) followed by an attack by the equivalent of \textit{OH} and/or direct oxidation of a flavanone, to afford a cation at C-3 (III) which could be transformed to a flavone, flavanonol or isoflavone (Fig.-8)\textsuperscript{27,28}. These hypotheses also give satisfactory explanation for the biogenetic correlations amongst the various flavonoids and, specially, for the very frequent presence of an oxygen atom in position C-3. However, the mechanisms proposed for the direct oxidation of flavanone to give a flavanone C-3 cation followed by reaction with \textit{OH}, and enolisation of the flavanone followed by attack by equivalent of \textit{OH} are doubtfully feasible \textit{in vitro}. 
Fig. - 7
S

Flavan-3,4-diol
Catechin
Anthocyanidin

Fig.-8
Pelter made a detailed experimental study into the chemistry of flavonoids and put forward a hypothesis, based on the phenolic oxidation of 4-hydroxychalcone (IV), which is supported by a large number of in vitro chemical analogies. Pelter suggested that a hydrogen (or hydride) abstraction from the 2- or 4-hydroxyl group in chalcone generates a radical (or cation) (V) which induces cyclisation as depicted in Fig.-9 to give isoflavone.

Fig.-9

\[
\begin{align*}
\text{(IV)} & \quad \Rightarrow \quad \text{(V)} \\
\text{Fig.-9}
\end{align*}
\]
Formation of flavones and aurones can be explained from the radical (V) as shown in Fig.-10. The formation of flavonols can be explained in terms of further oxidation of 4'-hydroxy group of either flavanonols or flavones (Fig.-11).

Fig.-10
Fig. 11
The production of the flavonoids devoid of a hydroxyl group on the B-ring and above the flavanone oxidation level is not explained by Pelter's hypothesis. Pelter suggested that these compounds are probably produced directly from chalcones as shown in Scheme-I. Cyclisation of the 2'-hydroxychalcone (VI) is initiated by a metal ion to yield the metal enolate (VII) followed by an oxidative loss of the metal to yield flavone. If attack on the C-3 of metal enolate (VII) were by water, then flavanonol would result.
Roux and Ferreira\textsuperscript{30} have proposed another hypothesis for the flavonoids biosynthesis from \(\alpha\)-hydroxychalcone (IX) which probably orginates from \(p\)-hydroxyphenylpyruvic acid (VIII)\textsuperscript{31,32} and malonate (or acetate) units. The Roux theory is supported by the large natural distribution of \(\alpha\)-hydroxychalcones\textsuperscript{33,34}.

\[
3\times \text{CH}_3\text{COOH} +
\begin{array}{c}
\text{HOOC} \\
\text{HOOC}
\end{array}
\xrightarrow{\text{VIII}}
\begin{array}{c}
\text{HOOC} \\
\text{HOOC}
\end{array}
\]

The formation of both 2,3-cis- and 2,3-trans-flavanonols can be explained by the cyclisation of the enolic form of \(\alpha\)-hydroxychalcones. Subsequent reduction of these flavanonols leads feasibly to 2,3-cis- and 2,3-trans-flavan-3,4-diols and eventually to corresponding flavan-3-ols (Fig.-12). These classes of compounds form flavonols and anthocyanidins by oxidation and elimination reactions, respectively.
2,3-trans-Flavanonol  \[\xrightarrow{\text{Red.}}\] 2,3-trans-Flavan-3,4-diol  \[\xrightarrow{\text{Red.}}\] 2,3-trans-Flavan-3-ol

2,3-cis-Flavanonol  \[\xrightarrow{\text{Red.}}\] 2,3-cis-Flavan-3,4-diol  \[\xrightarrow{\text{Red.}}\] 2,3-cis-Flavan-3-ol

Fig.-12
2-Hydroxy-2-benzylcoumaranones may be formed by the alternative method of cyclisation to the \( \alpha \)-position of the enolic form of the \( \alpha \)-hydroxychalcone (IX) or more likely to the corresponding carbonyl group of the keto-isomer. Such a cyclisation requires acid conditions for the enolic ether form\(^\text{33}\), while those for the keto form are as yet unestablished. The formation of 2-hydroxy-2-benzylcoumaranone (X) can not be explained by the epoxide induced biosynthesis where the alternate method of cyclisation\(^\text{35}\) provides (hydroxybenzyl) coumaranone (XI) and not (X).

\( \text{IX} \) \hspace{1cm} \text{X} \\
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{O} & \quad \text{CH}_2 \\
\text{OH} & \quad \text{CH}_2 & \quad \text{OH}
\end{align*}

\( \text{XI} \) \\
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{O} & \quad \text{CH} \\
\text{OH} & \quad \text{CH} & \quad \text{OH}
\end{align*}
A recent hypothesis (Fig.-13)\textsuperscript{36} postulates a simultaneous phenol oxidation of the two aromatic rings of the chalcone intermediate, followed by an intramolecular coupling, according to a scheme very common in plants. Intermediates such as (XII) and (XIII) (oxonium salts) should be formed, which could lead to aurone, flavone, flavanonol and isoflavone.

Reduction of flavanonols is assumed in the biogenesis of flavonoid structures in low oxidation level but other possibilities also exist. Thus, as shown by Clark-Lewis and co-workers\textsuperscript{37,38}, sodium borohydride reduction of chalcones gives flavenes which can conceivably also serve as precursors of leucoanthocyanidins, anthocyanidins and catechins\textsuperscript{39}. 

\begin{align*}
\text{HO-} & \quad \text{OH} \\
\text{HO} & \quad \text{O} \\
\text{OH} & \quad \text{OH}
\end{align*}

\begin{align*}
\text{NaBH}_4 & \quad \rightarrow \\
\text{HO} & \quad \text{OH} \\
\text{HO} & \quad \text{O} \\
\text{OH} & \quad \text{OH}
\end{align*}

\begin{align*}
\text{Flavene} & \quad \rightarrow \\
\text{HO-} & \quad \text{O} \\
\text{HO} & \quad \text{OH}
\end{align*}
Isoflavone

Fig. - 13
The mechanism of formation of ring C in anthocyanidins has still many obscure points. Haslam's hypothesis$^{40}$ on the anthocyanidin and catechin biosynthesis is the most widely accepted one and strictly correlates the biosynthesis of anthocyanidins with that of catechins (Fig.-14).

The formation of biflavonoids, despite the range of biflavonoids now known, may be explained in terms of oxidative coupling of two chalcone units and subsequent modification of C$_3$-chain$^6a$. Abstraction of an electron from the C-4 anion of naringeninochalcone (XIV) affords a radical which may be represented by the canonical formulae $R_\alpha$, $R_\beta$ and $R_\gamma$.

![Diagram of the mechanism of formation of ring C in anthocyanidins and biflavonoids](image.png)
Fig. 14
While the abstraction of an electron from the C-4' anion of (XIV) will give another radical which may be represented by several canonical formulae. However, the only canonical formulae which are important in the biosynthesis of most of biflavonoids are $R_8$. The formation of precursors of all naturally occurring biflavonoids can be explained by the appropriate pairing of these radicals.

An alternative to the radical pairing process is the possibility of electrophilic attack of one of the above mentioned radicals upon the phloroglucinol nucleus of a chalcone or corresponding flavanone$^{41}$ (Fig.-15) which would account for the fact that in most known naturally occurring biflavonoids at least one 6- or 8-position is involved in the interflavonoidic linkage.
Fig. 15
STRUCTURAL ELUCIDATION OF FLAVONOIDS

The structural elucidation of flavonoids has been discussed in detail in some recent reviews and monographs. However, some of the techniques frequently used by us and included in the discussion of this thesis need mention here.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The application of the n.m.r. spectroscopy proved to be the most powerful tool in the structure determination of flavonoids. The valuable contribution in this field has been made by Batterham, Mabry, Massicot, Clark-Lewis, Kawano and Pelter and Rahman. Early work on the H-n.m.r. spectroscopy of flavonoids was hindered by lack of their solubility in CDCl₃ and CCl₄. Progress was made following the introduction of Me₂SO-d₆, but the most significant advance arose from the conversion of flavonoids into their more soluble trimethylsilyl ethers.

The chemical shifts of the protons of rings A and B prove to be independent of each other, but are affected by the nature of ring C. The peaks arising from ring A in most flavonoids occur upfield from other peaks and can readily be recognised.

The most commonly occurring hydroxylation pattern in natural flavonoids is 5,7,4'-trisubstituted system (XV) in which,
owing to the symmetrical substitution, ring B protons appear as superimposed doublets ($J=9$ Hz) corresponding to an $A_2B_2$ system and ring A protons as $AB$ doublets ($J=2.5$ Hz). In other cases, however, the interpretation is not so simple owing to the superimposition of signals and appearance of complex multiplicities of protons of an ABX or ABC system.

\[
\text{HO} \quad \text{O} \quad \text{OH}
\]

\[
\text{OH} \quad \text{O} \quad \text{OH}
\]

(XV)

Considerable variations are generally found for the chemical shifts of the ring C protons among various flavonoid classes. For example, the C-3 proton in flavones gives a sharp singlet near $\delta 6.3$, the C-2 proton in isoflavones is normally observed at about $\delta 7.7$, while the C-2 proton in flavanones is split by C-3 protons into a doublet of doublet ($J_{\text{cis}}=5$ Hz, $J_{\text{trans}}=11$ Hz) and occurs at about $\delta 5.2$. The signals for the two C-3 protons appear as a pair of quartets ($J_{H-3a-3b}=17$ Hz) near $\delta 2.7-3.0$. However, they often appear as two doublets since two signals of each quartet are of low intensity. The C-2 proton in flavanonols appears near $\delta 4.9$ as a doublet ($J=11$ Hz) coupled to the C-3 proton which comes at about $\delta 4.2$ as doublet.
Solvent induced shift has been used for assigning the position of methoxyls in methoxyflavones. By measuring the n.m.r. spectra first in CDCl$_3$ and then in C$_6$D$_6$, Wilson et al.\textsuperscript{52} found that the size of the benzene induced shift ($\Delta$) of certain methoxyl signals was to some extent indicative of the position of the methoxy group in the flavone nucleus.

A more recent innovation in this field is that of lanthanide induced shift\textsuperscript{48}. The technique is extremely helpful in establishing the internuclear linkage of biflavonoids and also for the distinction of A and B ring methoxyl signals.

**MASS SPECTROMETRY**

Electron impact mass spectrometry serves as a valuable tool in the structure determination of flavonoids, especially when only very small quantities of the compounds are available. It has been applied successfully to all kinds of flavonoids.

Most flavonoid aglycones yield intense peaks for the molecular ion (M$^+$). In addition, peaks for (M-H)$^+$ and, when methoxylated, (M-CH$_3$)$^+$ are usually the major peaks. The most useful fragmentations in terms of flavonoid identification are those which involve cleavage of intact A- and B-ring fragments\textsuperscript{6b}.

Two common fragmentation patterns of flavonoids are, an exception being chalcones which undergo direct fission on each side
of carbonyl group, retro-Diels-Alder (RDA) cleavage, pathway I and pathway II (Chart-I). Pathway I (RDA) process affords two different ions designated as $A_1^+$ and $B_1^+$, the ratio of the two being indicative of the charge distribution within the parent ion. In contrast, pathway II yields predominantly a single charged species, $B_2^+$. These two fragmentation processes are competitive and the combined intensities of the $B_2^+$ and $[B_2-CO]^+$ ions are approximately inversely proportional to those of $A_1^+$ and $B_1^+$ and the series of ions derived from them (Chart-I).

Flavones were among the first flavonoids to be analysed by mass spectrometry\textsuperscript{53,54}. Although molecular ion, $M^+$, is the base peak for most of the flavones, the fragment $(M-CO)^+\cdot$ and pathway I fragments $A_1^+$ and $B_1^+$ are usually prominent. An ion $(M-1)^+$ is often found in the mass spectra of flavones, its origin, however, is obscure. Pathway II fragment, $B_2^+$, though usually found is not much intense.

In the case of flavanones, typical fragmentation by RDA process yields ions which correspond to the same $A_1^+$ and $(A+1)^+$ ions, observed for flavones (Chart-I); however the B-ring ion contains an ethylene group\textsuperscript{55,56}. 
Pathway I with H transfer

Pathway II

Pathway I

(A_1+H)^+

A_1^+

B_1^+

B_2^+

(M-CO)^+

A_2^+

B_3^+

(B_3-CO)^+

Chart-I
Another diagnostic fragmentation, that helps in structure determination of flavanones, is the loss of either a hydrogen or an aryl radical to produce \((M-1)^+\) and \([M-(B\text{-ring})]^+\) ions.
A moderately intense B-ring ion (XVI) is found in the case of 4'-methoxyflavanones which is formed by the fission of the B-ring from the molecular ion accompanied by a hydrogen transfer.

![Chemical structure of XVI](image)

The presence of a hydroxyl or a methoxyl group at C-4' facilitates, by the enhanced resonance stabilisation of the molecular ion, the formation of p-hydroxybenzyl or p-methoxybenzyl cation (XVII), respectively. The prominence of this ion may be associated with its rearrangement to a tropolium structure (XVIII).

![Chemical structure of XVII](image)

\[ R = \text{H, CH}_3 \]

![Chemical structure of XVIII](image)
Chalcones give strong ions for $M^+$, $(M-H)^+$ and $(M-\text{CH}_3)^+$ (for methoxychalcones). However, most diagnostic fragments result by the fission on either side of the carbonyl group. The relative intensities of these ions, designated as $A^+_2$ and $B^+_3$ (Chart-I) and of those derived from them, depend upon the substitution pattern of the chalcone$^{56,57}$.

It has been established, in the case of 2'-hydroxy-chalcones, that an equilibrium exists between chalcone and flavanone and the ions derived by fragmentation of both the chalcone and its corresponding flavanone are found. In some cases, however, the cleavage of the chalcone adjacent to the carbonyl group is much faster than the isomerisation to flavanone and thus the spectrum of the chalcone predominates. It has been emphasised that, in most cases, it is difficult to determine with certainty from the mass spectral data whether chalcone or flavanone was originally present.

Mass spectrometry has also been very useful in the structure elucidation of biflavonoids. Biflavonoids have mostly been studied as their permethylated derivatives$^{42}$. In general, two flavonoid units of a C-C linked biflavonoid fragment by the pathways which are well defined for the corresponding monoflavonoids. Some A- and B-ring fragments are exactly the same as those observed for the monoflavonoids, while others are typical A- and B-ring fragments except that they have an intact flavonoid unit attached to them. The
C-O-C linked biflavonoids undergo fission on both the sides of the ether linkage to yield ions which undergo further fragmentation. Doubly charged ions are usually present.

The modes of fragmentation of amentoflavone hexamethyl ether (XIX), a C-C linked biflavone, and hinokiflavone pentamethyl ether (XX), a C-O-C linked biflavone, are shown in Charts–II and III, respectively

**AMENTOFLAVONE HEXAMETHYL ETHER (XIX)**

Main peaks: m/z 622 (100), 621 (33), 592 (8), 576 (10), 312 (2), 311 (5), 245 (5), 181 (2), 180 (3), 135 (16) and 132 (3).

**HINOKIFLAVONE PENTAMETHYL ETHER (XX)**

Main peaks: m/z 608 (39), 607 (12), 593 (36), 580 (4), 529 (11), 578 (11), 576 (6), 431 (7), 327 (23), 313 (100), 312 (22), 311 (22), 304 (2), 297 (29), 296 (25), 281 (22), 181 (11), 180 (3), 135 (19) and 132 (18).
Chart-II
Chart-III