AUTOXIDATION OF CATECHIN

by

J. W. T. Seakins

"Regnum Scientiae ut regnum Coeli non nisi sub persona infantis intratur". Francis Bacon

Thesis submitted to the University of London for the
Degree of Doctor of Philosophy

March 1953

The British Leather Manufacturers' Research Association,
Egham, Surrey.

Battersea College of Technology,
London, S.W.11.
F.G.S.

I.B.S.

optimis parentum.

ACKNOWLEDGMENTS

The author wishes to thank the Director of Research and the Council of the British Leather Manufacturers' Research Association for permission to submit this work and to record his thanks to Dr. B. E. Hathaway and Dr. J. Kenyon, F.R.S., for their advice and continued encouragement, to Dr. W. B. Elstow for a gift of 3',4'-dihydroxyflavan and to Dr. W. F. Reid for the acetone-dried powder of Liscodia tabacum.
Summary of Thesis

An exploratory study of those reactions of catechin which lead to polymer formation has enabled the conditions for autoxidation to be defined. The formation of polymers and hydrogen peroxide during the autoxidation at 35° and pH6-8 of catechin and of related 3':4'-dihydroxyflavans has been studied by measurement of oxygen uptake, and by absorption spectra, colour reactions and chromatographic properties of the polymers, and by elementary analyses of the electrodialysed polymers (1). The evidence obtained together with that provided by spectroscopic study of intermediates produced by silver oxide oxidation (2) support the theory of quinone polymerisation for the autoxidation of catechin. Whereas quinone polymerisation of catechin and 5:7:3':4'-tetrahydroxyflavan involves the phloroglucinol residue, oxidative coupling of 5:7-di-O-methylcatechin and 3':4'-dihydroxyflavan resembles that of catechol.

Further evidence for head-to-tail quinone
polymerisation of catechin has been found in studies of the autoxidation of mixed phenolic substrates.

Enzymic oxidation by mushroom, potato and tobacco polyphenoloxidases proceeds at a faster rate and lower temperature than the autoxidation of catechin, and gives a product precisely similar to the autoxidation polymer. The rate of oxygen uptake is proportional to the enzyme concentration. The significance of the autoxidation and enzymic oxidation of catechin to the formation of phlobatannins is considered. Oxidation polymers of catechin are comparable with the tannins which have now been isolated in relatively large amounts from amongst tannin extracts of Acacia catechu and Uncaria gambir, plants valued for their phlobatannins (3).

Special sections summarise the chemistry of catechin, the physical techniques on which much of this work depends, and the application of the theory of condensation polymerisation to the polymerisation of catechin.

Three flavonols have been identified in extracts from Acacia catechu.
Publications Submitted

(3) " " , Biochem. J. 1957, 67, 239.
# THE AUTOXIDATION OF CATECHIN

## CONTENTS

### PART ONE

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>The Chemistry of Catechin</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Theories of the Catechin-Tannin</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Transformation</td>
<td></td>
</tr>
</tbody>
</table>

### PART TWO

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>Empirical Study of the Catechin-Tannin</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Transformation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The Vanillin Reaction</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Results and Discussion</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Conclusion</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>An Initial Comparison of the Autoxidation of Catechin and Catechol</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Absorption Spectra</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Manometry</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Silver Oxide Oxidation</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Conclusion</td>
<td>31</td>
</tr>
</tbody>
</table>
### PART THREE

<table>
<thead>
<tr>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>74</td>
</tr>
<tr>
<td>Chromatography</td>
<td>74</td>
</tr>
<tr>
<td>Manometry</td>
<td>76</td>
</tr>
<tr>
<td>Spectrophotometric Experiments and Analytical Samples</td>
<td>73</td>
</tr>
<tr>
<td>Preparation of (+)-Catechin and (-)-Epicatechin</td>
<td>80</td>
</tr>
<tr>
<td>4:5-Dianilino-1:2-benzoquinone</td>
<td>81</td>
</tr>
<tr>
<td>5:7-Dihydroxyflavan</td>
<td>82</td>
</tr>
<tr>
<td>5:7-Dihydroxy-3':4'-dimethoxy- and 5:7:3':4'-Tetrahydroxyflavans</td>
<td>82</td>
</tr>
<tr>
<td>Attempts to prepare 5:7:3':4'-Tetrahydroxy-6':dimethyflavan</td>
<td>85</td>
</tr>
<tr>
<td>Attempted Preparation of 5:7:3':4'-Tetrahydroxy-6':methyflavan</td>
<td>87</td>
</tr>
<tr>
<td>Attempted Preparation of 5:7-Di-O-methylcatechin</td>
<td>83</td>
</tr>
<tr>
<td>Attempted Preparation of 5':4'-Dihydroxy-5:7-dimethoxyflavan</td>
<td>90</td>
</tr>
<tr>
<td>5:7-Di-O-methyl-(+)-catechin</td>
<td>93</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>94</td>
</tr>
<tr>
<td>4:5-Dimethylcatechol</td>
<td>95</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Tri-C-methylphloroglucinol</td>
<td>96</td>
</tr>
<tr>
<td>5-Methoxy-4-methylresorcinol</td>
<td>97</td>
</tr>
<tr>
<td>Experiments with the Autoxidation Polymer of Catechin</td>
<td>100</td>
</tr>
<tr>
<td>Enzyme Preparations</td>
<td>102</td>
</tr>
<tr>
<td>Fractionation of Tannin Extracts</td>
<td>104</td>
</tr>
<tr>
<td>The Flavonols of Acacia Catechu</td>
<td>108</td>
</tr>
<tr>
<td>References</td>
<td>110</td>
</tr>
<tr>
<td>Figure</td>
<td>List of Figures</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Silver Oxide Oxidations</td>
</tr>
<tr>
<td>2</td>
<td>Infrared Spectra</td>
</tr>
<tr>
<td>3</td>
<td>Autoxidation of Flavans</td>
</tr>
<tr>
<td>4</td>
<td>Mixed Autoxidations (1)</td>
</tr>
<tr>
<td>5</td>
<td>Mixed Autoxidations (2)</td>
</tr>
<tr>
<td>6</td>
<td>Spectra of Acetyl Derivatives of Catechin and its Autoxidation Polymer</td>
</tr>
<tr>
<td>7</td>
<td>Enzymic Oxidations by Mushroom Polyphenoloxidase</td>
</tr>
<tr>
<td>8</td>
<td>Comparison of Spectra</td>
</tr>
<tr>
<td>9</td>
<td>Spectra of some Condensed Tannins</td>
</tr>
<tr>
<td>10</td>
<td>Chromatograms of Uncaria gambir and Acacia catechu</td>
</tr>
</tbody>
</table>
PART ONE
Chapter 1

Introduction

The vegetable tannins form a class of phenolic substances produced during the metabolism of plants and trees. Howes (71) records some 300 different species of plants which contain in some part at least 10% of tannin. Tannin producing plants and trees are distributed throughout the world, but countries with tropical or subtropical climates supply the bulk of the tannins of commerce. Tannins occur in different parts of the plant depending on the species; they have been found in the bark (mangrove) and heartwood (quebracho) of trees, in the leaves (dhawa), roots (rhatany), rhizomes (canaigre) of plants, and in the fruits (myrabolams) and galls (oak) of trees. The phenolic character of the tannins is responsible for their colour reactions with iron salts and is partly responsible for their feeble acidity and for their astringent taste. Tannins have been used by mankind for several thousand years, chiefly in the manufacture of leather, during which the tannins transform the collagen of raw animal
pelt into a durable, fibrous product, leather, which resists putrefaction.

In vegetable tannage hydrogen bonds link together the tannin molecules by their phenolic groups to the receptor sites of the collagen chains to form a reasonably stable cross-linked structure. Salt links may also be formed between carboxyl groups in certain tannins and basic groups present in the collagen. The product, leather, shrinks at a higher temperature in water than does the untanned pelt. Whilst simple phenols, including catechin are absorbed by collagen, their molecules are not large enough to link together neighbouring collagen chains and do not tan. In fact simple phenols lower the shrinkage temperature of pelt.

Because most tannins are amorphous, the classical methods of separation and criteria of purity are not applicable. Consequently, although the tannins were amongst the earliest classes of substances to be investigated (see for example Nierenstein's "Incunabula of Tannin Chemistry") little fundamental progress has been made. The classical work of Emil Fischer and his collaborators at the beginning of this century gave a great impetus to the subject;
but apart from Freudenberg's elucidation of the structure of catechin, it is only in the last decade that further progress has been made. Even so, little or nothing is known about many tannins.

Freudenberg (33) divided the natural tannins into two classes according to their behaviour when boiled with dilute mineral acids. The hydrolysable tannins are broken down into simpler substances - phenolic acids and sugars, for example, myrabolam extract yields mainly gallic (I), chebullic (II), and ellagic (III) acids and glucose:

The phlobatannins, on the contrary, resist breakdown under these conditions and are transformed into red or brown insoluble materials which are known as "phlobaphenes" or "tanner's reds". The phlobatannins also rapidly react with formaldehyde and concentrated hydrochloric acid to give insoluble buff-coloured products. The phlobatannins are sometimes called "condensed" or "flavo-tannins". This classification does not imply that the tannins of one class have
identical structures, but does indicate that they possess common structural features. Some plants produce both types of tannin; for example, oak bark contains phlobatannins, whilst the heartwood contains hydrolysable tannins.

More than half of the tanning materials of commerce contain phlobatannins (total annual production approximately 400,000 tons). Chief sources are the heartwood of Schinopsis lorentzii (Quebracho), bark of Acacia mollissima (Wattle or Mimosa), leaves and twigs of Uncaria gambir, heartwood of Acacia catechu (Burma Cutch). Often associated with these amorphous phlobatannins are crystalline (+)-catechin and its epimer, (-)-epicatechin (IV, R=H).

Thus the leaves and twigs of Uncaria gambir contain considerable amounts of (+)-catechin. Rao and Seshadri (102) have found 3% of (-)-epicatechin in the heartwood of Acacia catechu. Mergert and Kurth (64,65) report
the presence of about 5% of (+)-catechin and 
(-)-epicatechin in white fir bark (Abies concolor),
which contains some 8% of phlobatannins. Roux (118)
has found (+)-catechin and (-)-epicatechin in wattle 
bark, and Mayer (92, 93) has found (+)-catechin and 
(+)-gallocatechin (V, R=H) in the barks of oak and 
sweet chestnut. (+)-Catechin, (+) gallocatechin, 
(-)-epicatechin, (-)-epigallocatechin and the gallates 
of the epimers (IV & V, R=3:4:5 (HO)3C6H2CO-) have 
been identified in green tea leaves (109, 15).

Catechin has the characteristic reactions of 
phlobatannins and may be transformed into a tannin 
under a variety of conditions (Chapter III). These 
facts suggest that (epi)-catechin and related substances 
may be the precursors of the tannins with which they 
are associated. Indeed since 1827 (96) a relationship 
between catechin and phlobatannins has been suspected.
A knowledge therefore of the ways by which catechin 
may be transformed into a tannin and of the structure 
of the tannin may shed light on the constitution of the 
phlobatannins, compounds which have proved difficult to 
degradate by classical methods.
Chapter 2

The Chemistry of Catechin

Catechin is a crystalline material which occurs in the leaves, barks, fruits and flowers of several trees and plants where it is often associated with tannins (Chapter 1). The two main sources of (+)-catechin and its epimer are respectively the leaves and twigs of Uncaria gambir (Rubiaceae) a climbing shrub of Malaya, and the heartwood of Acacia catechu (Leguminosae) a tree growing extensively in India and Burma.

Since a knowledge of the chemistry of catechin is fundamental to the work herein described, a brief account of the experiments leading to the elucidation of its structure is given. The polemics between Freudenberg and Mierenstein, 1920-1936, however, have been omitted. Unless specified "catechin" refers to (+)-catechin from Uncaria gambir.

Berzelius (1837) was the first to isolate a pure specimen of catechin, although several workers had previously isolated impure specimens. He converted the crude catechin into its lead salt, which he subsequently decomposed with hydrogen sulphide and
Although Zwenger in 1841 and Hlasiwetz in 1865 had respectively proved the presence of catechol and phloroglucinol nuclei in catechin, it was not until 1902 that Kostanecki and Tambor deduced the correct formula, \( C_{15}H_{14}O_6 \cdot 4H_2O \), for the hydrate. These authors prepared a tetramethyl ether, a monoacetyl-tetramethyl ether and also a pentaacetyl derivative, which lost four acetyl groups on boiling with alcoholic potassium acetate. These facts indicated that catechin contained five hydroxyl groups - four phenolic and one alcoholic - and that the other oxygen atom was probably ethereal or heterocyclic.

Later Kostanecki and Krembs prepared the mono- bromo derivatives of tetra-O-methylcatechin and its acetate. As the tetramethyl ether and its monobromo derivative both gave veratric acid on oxidation with potassium permanganate, substitution had occurred in the phloroglucinol nucleus; but since they were unable to introduce a second bromine atom they erroneously concluded that there was only one free position in the phloroglucinol nucleus.

Later Kostanecki and Lampe reduced tetra-O-methylcatechin with sodium and ethanol and recrystallized the liberated catechin from water.
methylated the reduction product, but they were unable to determine the structures of these products. In 1920 Freudenberg\textsuperscript{(39)} repeated these reduction experiments and showed that the methylated reduction product was 2:4:6:3':4'-pentamethoxy-\(\alpha\):\(\beta\):diphenylpropane which was synthesised as follows:

\[
\begin{align*}
\text{MeO} & \quad \text{COCH}_3 + \text{H} & \quad \text{OMe} \\
\text{OMe} & & \\
\text{MeO} & \quad \text{OMe} & \quad \text{alk. KON} & \quad \text{MeO} \\
\text{COCH} = \text{CH} & & \\
\end{align*}
\]

This result was later confirmed when Freudenberg and Cohn\textsuperscript{(46)} ethylated the reduction product and showed that it was identical with 6-ethoxy-2:4:3':4'-tetramethoxy-\(\alpha\):\(\beta\):diphenylpropane, which was synthesised in the same manner as (I).

A.G. Perkin and Yoshitake\textsuperscript{(99)} had previously shown that the catechin of \textit{Acacia catechu}, "Acacatechin", was different from that of \textit{Uncaria gambir}, and that from the latter material a third catechin could be isolated. All these catechins gave protocatechuic acid and phloroglucinol on alkali fusion. Freudenberg, Bohme and Beckendorff\textsuperscript{(43)} showed that tetra-O-methy lacacatechin
on reduction with sodium and ethanol and subsequent methylation gave (I), and since the derivatives of catechin and acacatechin had similar optical properties they concluded that these catechins were structurally similar.

A large scale investigation of acacatechin by Freudenberg and co-workers (44, 52) revealed the presence of (\(\pm\))-catechin, and smaller amounts of (-)-catechin and two new (\(\pm\))- and (-)-forms. It was found possible to convert (\(\pm\))-catechin to the new (\(\pm\))-form by boiling in brine for 24 hr. These authors therefore assumed that the new (\(\pm\))-form was an epimer of (\(\pm\))-catechin, (\(\pm\))-epicatechin. Similarly epimerisation of (-)-catechin gave (-)-epicatechin, and (+)-catechin gave (+)-epicatechin. A mixture of equal quantities of (+)- and (-)-epicatechin gave the (\(\pm\))-epicatechin, identical with that derived from acacatechin.

When however Freudenberg and Furrman (53) carefully extracted fresh heartwood of *Acacia catechu* with ether, they obtained nearly pure (-)-epicatechin. Acacatechin was therefore a mixture of stereoisomers formed from (-)-epicatechin during the primitive native method of extraction. Freudenberg and co-workers had thus
demonstrated the existence of six isomers, viz (+)-, (-)-, (±)-catechin, (+)-, (-)-, (±)-epicatechin, and hence catechin contains two asymmetric carbon atoms.

There are thus four structurally isomeric formulae for tetra-O-methylcatechin which would give the α,β-diphenyl-propane structure on reduction and methylation, and which contain two asymmetric carbon atoms:

Freudenberg, Fikentscher and Harder (47) attempted to eliminate the elements of water from tetra-O-methylcatechin by heating the p-toluenesulphonic ester with hydrazine, but only degradation products were obtained. When the experiments were repeated with tetra-O-methylepicatechin an optically inactive anhydro-compound was obtained which was hydrogenated to give the desoxy derivative. These compounds were isomeric but not identical with the corresponding anhydro- and desoxy-tetra-O-methylcatechin, obtained by heating the p-toluenesulphonic ester with quinoline.
Since the desoxy-compound is inactive, (V) is excluded. Reduction with sodium and ethanol, followed by methylation, of the tetra-O-methylamphlo compounds gave two isomeric products, that from epicatechin was identical with (I). Hence the pendant phenyl group does not migrate in the elimination of the elements of water from tetra-O-methylcatechin.

Thus there remain two structural possibilities for tetra-O-methyldeoxyepicatechin:

These workers synthesised (VI) and found that it was not identical with tetra-O-methyldeoxyepicatechin, which was therefore (VII). Therefore (III) is the formula of tetra-O-methylenicatechin.

That year (1925), Freudenberg and co-workers reduced cyanidin and its pentamethyl ether (VIII. R=H and Me respectively) (which had been synthesised by two independent routes) to (-)-epicatechin and its pentamethyl ether respectively (III).
Later Freudenberg, Carrara and John (45) showed tetra-O-methyldesoxy catechin to be 5:7:3:4'-tetramethoxy isoflavon which was synthesised in the following way:

$$\text{MeO} \text{CHO} + \text{OH} \text{C} \text{CH}_2 \text{OMe} \rightarrow \text{MeO} \text{H} \text{O} \text{C} \text{CH}_2 \text{OMe}$$

The product which had been obtained by reducing tetra-O-methylanhydro catechin with sodium and ethanol followed by methylation, was also synthesised as follows:

$$\text{MeO} \text{CH}_2 \text{COCl} + \text{MeO} \text{OMe} \rightarrow \text{MeO} \text{CH}_2 \text{CO} \text{OMe}$$

(1) $\text{MeMgI}$
(2) $-\text{H}_2\text{O}$
(3) $\text{H}_2$

The results of the action of hydrazine on the p-toluenesulphonic esters of tetra-O-methyl catechin and its epimer have been interpreted as follows by King, Clark-Lewic and Forbes (76). Formation of tetra-O-methylanhydrocatechin is attributed to the stereospecific trans-elimination ($E_2$) of
p-toluenesulphonic acid from the epicatechin ester, in which the four participating centres C(2), C(3), H(2) and O(3) are planar. The configuration of the corresponding groups in the catechin ester is unfavourable, and the observed disintegration products may arise through the intermediate flav-3-en (IX). The formation of the isoflavan by the action of quinoline on the catechin ester may be interpreted as a slow heterolysis of the C-2-O bond and rearrangement of the corresponding carbonium ion. Thus catechin is the trans-isomer (2:3) and epicatechin the cis-isomer. Further corroboration of these assignments is to be found in the formation of (2)-epicatechin and its pentamethylether by the catalytic hydrogenation of planar cyanidin and its pentamethylether and also planar penta-O-methylqueretcin.

By reductive ring-opening using sodium and ethanol in liquid ammonia followed by methylation, Birch, Clark-Lewis, and Robertson obtained from
(+)-catechin and (-)-epicatechin tetramethyl ethers the enantiomorphous 1-(3:4-dimethoxyphenyl)-3-
(2:4:6-trimethoxyphenyl)propan-2-ols (X). It follows that the 3-hydroxyl groups in (+)-catechin and
(-)-epicatechin are of opposite configurations. This fact coupled with Freudenberg's work on the epimerisation of the catechins shows that epimerisation occurs by inversion of the 2-aryl group. These authors used Prelog's atrolactic acid method (cf 101) to determine the absolute configuration of the catechins. In this method, the optically active alcohol is converted into its phenylglyoxylic ester, which is treated with methyl magnesium iodide. The resulting atrolactic ester is hydrolysed and the sign of rotation of the acid is determined, which is related to the configuration of the carbinol atom. An examination of the infrared absorption of the hydroxyl groups of the tetramethyl ethers of (+)-catechin and (-)-epicatechin indicated strong intramolecular bonding of axial hydroxyl groups, which confirms the conclusions arrived at on theoretical grounds by Whalley (139).

These combined results lead to the assignment of the following formulae to (+)-catechin and (-)-epicatechin:
Chapter 3

Theories of the Catechin-Tannin Transformation

Neubauer in 1855(95) showed that catechin is converted into a tannin when its aqueous solution is boiled for some time. Later in 1873 Löwe(86) found that an aqueous solution of catechin was unchanged after heating at 110° for 3 hr. in the absence of oxygen, but when heating was continued for 3 days nearly all the catechin was converted into a brown tannin. Ettii(30) confirmed Neubauer's observation that an aqueous solution of catechin slowly reddens on exposure to air and that this change is accelerated by heat. Ettii also found that catechin can be transformed, in acid and alkaline solutions, into brown substances which possessed tanning properties.

Considerable differences are on record as to the nature of the catechin-tannin transformation; these are summarised in the following.

Freudenberg(40) reported in 1924 that catechin 3-acetate was more stable than catechin, but in
collaboration with Maitland, he found that 
7:3:4'-trihydroxyflavan(I) was even more sensitive
to the action of acids, alkali or atmospheric oxygen
than was quebracho catechin(II).

Freudenberg and Maitland also observed that quebracho
catechin(II) exhibited a more marked tendency to
undergo self-condensation than catechin(III) did;
these authors therefore attributed a stabilising effect
to the 5-hydroxyl group in catechin. Freudenberg and
Maitland demonstrated that in the acid-catalysed
polymerisation of quebracho catechin(II) fission of
the pyran ring occurred to give a secondary benzyl
alcohol, and carbon atom C(2) then condensed with either
carbon atom C(6) or C(8) of another molecule to give a
bifunctional dimer capable of further polymerisation
(chapter 9).

Clayton found that the unsubstituted flavan
nucleus was extremely stable. Flavan could be
recovered almost quantitatively after prolonged refluxing
in water, dilute aqueous acid and dilute methanolic
hydrochloric acid. The pyran ring was not cleaved by sodium in ethanol or in butanol-conditions under which tetra-O-methylcatechin is converted into an \( \alpha\beta\)-diphenylpropane derivative. Whilst this work was in progress Freudenberg and Weinges\(^{(55)}\) reported that hydroxyl groups in 7- and 4'-positions are the basic structural requirements for a flavan to undergo the phlobaphene reaction. Incidentally this basic flavan(IV) is a \( \alpha\beta\)-diphenylpropane derivative.

Reichel\(^{(106)}\) supported the views of Freudenberg on the mechanism of the catechin-tannin transformation and made the further suggestion, based on the earlier work of Roberts\(^{(103)}\), that catechin might be oxidised to the corresponding \( \alpha\)-quinone and further react to produce an ether (chapter 7). Reichel also thought that loss of water in the secondary stage of the condensation might be accounted for by ether formation between two of the secondary alcoholic groups in catechin, and was of the opinion that the catechin-tannin transformation may proceed by any or all of these routes. Until this work
was started there was little evidence for Reichel's first suggestion and no satisfactory mechanism for the change had been enunciated.

Bergmann and Pojarlieff\(^{(11)}\) found that tetra-o-methylcatechin formed a phlobaphene on boiling with dilute acid; these workers inferred that the 3-hydroxyl group was the only group which had a marked influence on the stability of the pyran ring.

Consideration of these facts and theories stated above leads the writer to conclude that catechin may be transformed into a tannin by several routes. The present work describes the application of different qualitative and quantitative techniques to the transformation, which has enabled the limits of the various reactions to be defined, and to the study of model flavans and simple phenols related to catechin, which has enabled a partial type formula to be assigned to the autoxidation polymer of catechin. Finally, comparisons are reported between the autoxidation and enzymic oxidation polymers of catechin and several typical phlobatannins.
PART TWO
Empirical Study of the Catechin-Tannin Transformation

Introduction. The purpose of this preliminary examination was to define the conditions of the various possible reactions by which catechin may be transformed into a tannin and which were discussed in the previous chapter. To this end oxygen or oxygen-free nitrogen was bubbled through solutions of catechin (10 mmolar) at various pH's which were maintained at 100°. Samples were withdrawn from the former and examined by one- and two-dimensional paper chromatography with various spray reagents, and from the results an approximate value of the period of reaction was obtained. Samples were also withdrawn from the anaerobic reaction after an interval equal to the period of the corresponding aerobic reaction and likewise examined.

Chromatography has proved a most useful tool both in following the course of the reactions, and in facilitating the examination of the products by affording a very convenient means of separating the reaction products from catechin. It has also helped considerably in the examination of the two tannin extracts. The chromatograms
were developed by specific spray reagents, and amongst several examined, the following three proved of value. Ethanolic ferric chloride with catechol groups gives a green coloration turning to mauve on exposure to ammonia. Ferric ferricyanide is a sensitive reagent for phenols with which it gives a blue colour. The vanillin reagent is a sensitive test for certain phloroglucinol and resorcinol derivatives with which it gives red colorations.

The Vanillin Reaction. From the interaction of vanillin and phloroglucinol in ethanolic hydrochloric acid Etiti isolated a yellow crystalline substance, $C_{20}H_{18}O_8$, to which formula(I) was assigned. Wenzel was unable to repeat Etiti's experiments and isolated an amorphous red product of composition $C_{14}H_{12}O_5$. Later Ciuliano confirmed Wenzel's observation and further suggested the structure(IV) for the red amorphous product.

As this reaction has proved most useful, an exploratory study of its scope and limitations was made, and from the results a tentative mechanism is suggested. A number of phloroglucinol and resorcinol derivatives were examined and the results are collected together in table I. An examination of the table reveals that the following groups of compounds do not react:— (a) derivatives
<table>
<thead>
<tr>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcinol</td>
<td>2:4:6-Triethylresorcinol</td>
</tr>
<tr>
<td>4-Ethylresorcinol</td>
<td>Tri-O-methylphloroglucinol</td>
</tr>
<tr>
<td>4:6-Diethylresorcinol</td>
<td>Tri-O-methylphloroglucinol</td>
</tr>
<tr>
<td></td>
<td>2:4:6-Triethylresorcinol</td>
</tr>
<tr>
<td></td>
<td>Di-O-methylresorcinol</td>
</tr>
<tr>
<td></td>
<td>Resorcinaldehyde</td>
</tr>
<tr>
<td></td>
<td>Resacetophenone</td>
</tr>
<tr>
<td></td>
<td>Phloroglucinaldehyde</td>
</tr>
<tr>
<td></td>
<td>Phloroglucinaldehyde</td>
</tr>
<tr>
<td></td>
<td>Phloroglucinaldehyde</td>
</tr>
<tr>
<td></td>
<td>Phloroglucinaldehyde</td>
</tr>
</tbody>
</table>
which contain a carbonyl group adjacent to the ring, (b) derivatives in which the 2, 4 and 6 positions are blocked, i.e. the phenol can no longer react in the keto form, and (c) derivatives in which ketolisation is prevented. The red colour was discharged when the solutions were diluted, but was restored when the solutions were made strongly acidic. When vanillin was replaced by veratric aldehyde or p-dimethylaninobenzaldehyde the results were qualitatively the same, except that with the latter the colour was produced in dilute acid solution.

Only amorphous products, which decomposed about 300° could be isolated from the interaction of phloroglucinol and vanillin in ethanolic hydrochloric acid at room temperature. The low solubility of the dark crimson product from a 1:2 mixture of phloroglucinol and vanillin respectively prevented an examination of its spectrum, but the product from a 1:1 mixture had a strong absorption band at 450mμ (ε 1% 1cm 8.9), which shifted to 490mμ (ε 1% 1cm 123) in strongly acid solution, whilst that from a 2:1 mixture of phloroglucinol and vanillin respectively had a very strong absorption band at 460mμ (ε 1% 1cm 240), which became even more intense in acid solution (ε 1% 1cm 313).

Analyses (C, H and O%e) indicated that the products are derived from phloroglucinol and vanillin units in
approximately the same proportions as the reactants.

The author's attention has been directed to the parallel between the vanillin reaction and the Ehrlich reaction for indole and pyrrole derivatives. It is suggested that the product for a 1:1 mixture of vanillin and phloroglucinol is (IV), which in strongly acid solution exists as the halochromic salt (V). It is further suggested that with excess of phloroglucinol the reaction takes a similar course to that postulated by Guerrero and Williams for Tollen's naphthoresorcinol test for uronic acids. In this reaction, aerial oxidation to the xanthone (II) would occur which in acid solution would exist as the xanthylum salt (III).
In agreement with these formulations reductive acetylation of these products gave colourless substances.

Results and Discussion. This empirical study of the fate of catechin in aqueous solution at 100° has revealed several different reactions. At pH < 2 a buff-coloured precipitate, "phlobaphene", was rapidly formed (period of reaction about 3 hr.), together with traces of catechol and phloroglucinol. The phlobaphene gave the colour reactions of catechol and phloroglucinol, and on exposure to air turned brown. A sample of the phlobaphene formed during the early stages of the reaction was soluble in some organic solvents, and standard chromatograms of the reaction mixture revealed in addition to catechin, phlobaphene (immobile) and traces of catechol and phloroglucinol, other mobile substances. The phlobaphene obtained after several hours boiling was insoluble in all common organic solvents, and hence it was not possible to determine its spectrum; however chromatography of the reaction mixture was able to show that only phlobaphene and degradation products were present. This reaction was also studied at pH (0.1NHCl) at 50°, 37° and 20°. No reaction occurred at 20°; a precipitate slowly formed at 37°, but catechin was still present after 14 days; at 50° phlobaphene was more
rapidly formed, but catechin could still be detected after 7 days. These results are in agreement with Freudenberg's interpretation of phlobaphene formation (chapter 9), which recalls the ready cleavage of certain benzyl phenyl ethers in acid solution, for example (VI) is debenzylated at 65° with concentrated hydrochloric acid(6), and the formation of phenol-formaldehyde resins.

Between pH 4-8, however, catechin underwent autoxidation to a brown tannin. Although the tannin had phenolic properties, it lacked the characteristic reactions of phloroglucinol and catechol. The rate of autoxidation increased with increasing pH. At pH4 and 100° the period of reaction was greater than 300 hr, but at pH 3 the period of reaction had fallen to 4 hr. Only epimerisation occurred in the absence of oxygen, except at pH8 and 100° when combination between the phloroglucinol residue of catechin and phosphate buffer readily took place. The product which lacked the characteristic reaction of phloroglucinol is probably similar to that reported between sodium hydrogen sulphite and resorcinol (or phloroglucinol) to give (VII)(130).
Sodium hydrogen sulphite arrested autoxidation, presumably by virtue of being a reducing agent.

Under more alkaline conditions, another oxidative reaction—possibly ring fission of the catechol groups\(^{(4)}\)—intervened, very rapidly yielding brown "humic acids" which were devoid of all phenolic properties.

The autoxidation of catechin was also studied in solvents of various dielectric constants (2-40), but owing either to insolubility or to the extreme slowness of reaction, no results of significance were obtained.

Tetra-O-methylcatechin could be recovered unchanged after prolonged boiling in aqueous ethanol in the absence or presence of oxygen, but in dilute ethanolic hydrochloric acid it was rapidly transformed into dark products, which did not lend themselves to fruitful examination.

**Conclusion.** This study has enabled the limits of autoxidation to be defined (pH\(^{4-8}\)). The autoxidation was chosen for further study because the reaction-mechanism of the phlobaphene reaction has been established\(^{(50,51,55)}\) and because it was held that the oxidative transformation was more likely to occur under physiological conditions.
An Initial Comparison of the Autoxidation of Catechin

and of Catechol.

Introduction. In the previous chapter it was shown that at pH3 and 100° catechin rapidly undergoes oxidation (4 hr.) to a brown tannin, and that the results therein described suggested that the phloroglucinol fragment of catechin was directly or indirectly involved in the process. As oxygen has been shown to be necessary for the transformation of catechin to a tannin between pH4 and 8 it became of interest to compare the autoxidation of catechol and of catechin. Oxygen uptakes are well-nigh impossible to measure at 100°, but by working at 35° measurements become feasible; it was further found that the complication of combination between buffer and catechin which takes place at 100° was obviated at this lower temperature.

Absorption Spectra. Solutions of catechin and catechol in phosphate buffer were transparent above 370 and 320μ respectively. Accordingly only spectral changes above 350μ were measured during this preliminary study. Both solutions during the early stages of the autoxidation turned pink, subsequently the solution of catechol became
dark brown, whilst the solution of catechin soon changed to a bright orange-brown. No intermediate could be detected spectroscopically in either solution. The spectrum of the solution of catechol showed no maximum in the range 320 - 600\(\mu\), but as the autoxidation proceeded the general absorption in this range increased in intensity. On the other hand the solution of catechin developed a pronounced maximum at about 420\(\mu\) and general absorption in the range 320 - 600\(\mu\), both of which increased in intensity as the autoxidation proceeded (Table 2).

**TABLE 2**

_Spectral Changes occurring during the Autoxidation of 0.01 molar solutions of Catechin and Catechol at pH8 and 35°._

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>0</th>
<th>0.5</th>
<th>4.5</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda_{\text{max}} (\mu))</td>
<td>-</td>
<td>435</td>
<td>430</td>
<td>422</td>
<td>410</td>
</tr>
<tr>
<td>(E_{\text{max}}^\text{Lcm}) (0.03)</td>
<td>0.11</td>
<td>2.96</td>
<td>54.5</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>Corresponding (E_{\text{Lcm}}) value (0.01) for catechol</td>
<td>0.3</td>
<td>0.75</td>
<td>4.0</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>
The autoxidation at pH 6 was slower but similar results were obtained except that the characteristic maximum for the solution of catechin was now at 400 μ. Campbell and Coppinger(22) found that the absorption maximum for phenol shifted from 273 μ in neutral or acid solution to 290 μ in alkaline solution, and similarly the absorption maximum of resorcinol shifted from 283 μ to 288 μ in alkaline solution. The work of Aulin-Erdtman(3) shows that the primary ultraviolet absorption bands of phenols undergo a marked bathochromic shift when the hydroxyl groups become ionized. It is therefore unlikely that the difference in absorption maxima of the products of autoxidation obtained under slightly acid and slightly alkaline conditions is due to the operation of different mechanisms of autoxidation.

**Manometry.** Exploratory experiments on the autoxidation of catechin at pH 8 and 35° showed that the reaction was slow. When simple manometers were employed to measure oxygen uptakes, changes in ambient pressure and temperature produced considerable errors, and accordingly use was made of Haldane's constant pressure respirometer(63) which has a compensating vessel. The maximum rate of oxygen uptake for catechol was ten times greater than that for catechin (Table 3). Chromatograms indicated the absence of the starting materials when oxygen uptakes
had ceased. The acidified reaction mixtures liberated iodine from potassium iodide solution, and distillation of these solutions under reduced pressure gave solutions which were indistinguishable from aqueous hydrogen peroxide. The products of autoxidation which interfered with the hydrogen peroxide determinations were conveniently removed from the acidified reaction mixture by adsorption on alumina. The hydrogen peroxide in the eluate was determined in the usual manner. At pH 6 and 35° the reaction was too slow to be followed manometrically.

**Silver Oxide Oxidations.** Hydrogen peroxide has been found during the autoxidation of duroquinol to duroquinone and 5,6-dihydroxyindoles to artificial melamins. Its occurrence in the autoxidation of catechin and catechol might therefore indicate that these autoxidations proceed via the corresponding o-quinones. As no intermediate o-quinones could be detected spectroscopically further evidence was sought. Previous evidence for intermediate o-quinone formation includes an attempted isolation by Lamb and Steerangachar of an aniline derivative (no analysis or m.p.) when tea polyphenoloxidase acts on catechin. Preliminary experiments have shown that 4:5-dianilino-1:2-benzoquinone was formed when catechol was autoxidised (pH 8) in the presence of aniline,
SILVER OXIDE OXIDATIONS

FIG. 1

KEY
- o-benzoquinone.
- Catechin o-quinone.
- Catechin o-quinone + $PO_4^{3-}$ buffer.
- Catechin in $PO_4^{3-}$ buffer oxidised by silver oxide.
but under the same conditions catechin gave a red solution from which no crystalline compound could be obtained. Silver oxide oxidations of catechin and catechol in non-polar solvents (acetone, dioxan, and ether) afforded solutions which had the same absorption maxima as authentic o-benzoquinone (Fig. I). Catechin-tannin was formed when a solution of catechin-o-quinone in dioxan was shaken with phosphate buffer (pH8) and by silver oxide oxidation of catechin in phosphate buffer (pH6 or 8, Fig. I).

Conclusion. These facts suggest that the main reaction sequence in the autoxidation of catechin involves o-quinone formation followed by oxidative condensation. Formation of o-quinone probably involves a free radical mechanism, since the reaction can take place in non-polar solvents. Campbell(20) found that a solution of 4:6-ditertbutylpyrogallol in ligroin rapidly developed an intense purple on exposure to air. Hydrogen peroxide is associated with quinone formation, and its accumulation results from the difference in velocity of radical processes(137) conveniently summarized as follows:

\[ \text{H}_2\text{Q} + \text{O}_2 \rightarrow \text{Q} + \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{Q} + \text{H}_2\text{O}_2 \rightarrow \text{Q} + \text{H}_2\text{O} \]

As the o-quinones are reasonably stable in non-polar solvents, the subsequent reaction is an ionic process.
requiring a dissociating medium. The results of the silver oxide oxidations make it unlikely that direct oxidation of the phloroglucinol residue is responsible for the characteristic absorption spectrum of catechin-tannin, but this possibility cannot be entirely excluded (compare the enzymic coupled-oxidations reported by Reid\(^{(107)}\) and Roberts\(^{(111)}\)). Further studies are required on model compounds to characterize fully this reaction sequence.
Chapter 6

Autoxidation of some Flavans related to Catechin

Introduction. In the previous chapter it was suggested that the profound difference in the absorption spectra of autoxidised catechin and catechol might be due to the participation of the phloroglucinol nucleus of the former in the reaction. This chapter is concerned with the autoxidation of some flavans related to catechin in order to test and extend this suggestion.

The Synthesis of Polyhydroxyflavans. Several methods are available for the synthesis of flavans. At first sight the catalytic reduction of flavylium salts is attractive since the intermediates are relatively easy to prepare, but in spite of special precautions yields are low (50). Elstow (27) was able to reduce in good yields certain simple flavylium salts in two stages, by lithium aluminium hydride followed by hydrogen in the presence of Raney nickel, but these flavans could not be demethylated. An attempt to extend this method to di- and tri-hydroxyflavylium salts was unsuccessful, as also was an attempt to prepare benzyloxyflavylium salts of the type required in this work. Elstow was able to synthesize 3':4'-dihydroxyflavan (III) by the following
method. Catalytic reduction of 2':3':4'-trihydroxychalcone, prepared by alkali condensation of salicylaldehyde and 3:4-dihydroxyacetophenone, gave the dihydrochalcone which was benzoylated. Reduction by lithium aluminium hydride of the tribenzoyloxydihydrochalcone gave the required flavan. This synthesis could not be extended to the preparation of alkylated or more highly hydroxylated flavans, as only starting materials or addition compounds were obtained in the attempted preparation of the intermediate 2'-hydroxychalcones.

The synthesis of flavanones from derivatives of phloroglucinol and cinnamoyl chloride has been extensively studied\(^{(124, 125)}\) and although the yields are low this reaction was used for the synthesis of 5:7-dihydroxy-, 5:7:3':4'-tetrahydroxy-, and 5:7-dihydroxy-3':4'-dimethoxy-flavanones. Improved yields were obtained more conveniently, however, by the use of ether instead of nitrobenzene as reaction medium. Clemmensen reduction at room temperature\(^{(115)}\) of these flavanones gave the corresponding flavans, (IV), (VI) and (VII). The attempted preparation of 5:7:3':4'-tetrahydroxy-5:8-dimethylflavanone and flavan (VIII) by either this method or by the alkaline condensation of di-C-methylphloracetophenone and protocatechuic aldehyde was unsuccessful. Similar difficulty in the isolation and preparation of certain
flavans, particularly those containing C-alkyl groups in 6- and 8- positions has already been described (27,112).

For the synthesis of 5:7:4':5'-tetrahydroxy-2'-methylflavan(IX), 4:5-dihydroxy-2-methylbenzaldehyde was required. It was hoped to prepare this intermediate by the action of phosphorus pentachloride on 6-methylpiperonal (19). However, application of the Gattermann reaction to 3:4-methylenedioxy toluene, prepared from piperonal by the Huang-Minlon method, gave only resinous materials, and the synthesis had to be abandoned.
Pioneer experiments to prepare 5:7-di-O-methylcatechin(II), via the 3':4'-carbonate\(^{(25,35)}\), were not promising. A paper chromatographic examination of the hydrolysed methylated carbonate indicated that various dicatechin carbonates were formed in addition to the required carbonate. It was later found that partial methylation of catechin(I) by methyl sulphate and aqueous alkali in the presence of excess sodium metaborate readily gave the required flavan. It was not found possible to separate 3':4'-dihydroxy-5:7-dimethoxyflavanone from the isomeric chalcone; reduction of the mixture gave an oil from which the flavan(V) could not be isolated. Paper chromatography of the oil indicated the presence of several substances.

**Manometry of the Polyhydroxyflavans.** Free hydroxyl groups in the 3'- and 4'- positions cause an accumulation of hydrogen peroxide (Table 3) which is associated with quinone formation. 5:7-Dihydroxy- and 5:7-dihydroxy-3':4'-dimethoxyflavans, which lack vicinal hydroxyl groups, can be recovered almost quantitatively after several days autoxidation. Oxygen uptake in excess of the theoretical 1 mole per mole of flavan may be attributed to (i) manometric difficulties associated with protracted experiments, (ii) two oxidative couplings per mole of \(\Phi\)-quinone, or (iii) some oxidation of the
<table>
<thead>
<tr>
<th>Catechin</th>
<th>Oxygen uptake (moles)</th>
<th>Residual peroxide (moles)</th>
<th>Maximum Oxygen balance rate (moles/min.)</th>
<th>Reaction period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:7-Di-O-methyl-flaven</td>
<td>1.59</td>
<td>0.38</td>
<td>$1.9 \times 10^{-4}$</td>
<td>&gt;7</td>
</tr>
<tr>
<td>5:7-Dihydroxy-3:4-Dimethoxy-flaven</td>
<td>2.00</td>
<td>0.39</td>
<td>$3.4 \times 10^{-3}$</td>
<td>&gt;7</td>
</tr>
<tr>
<td>3:4-Dihydroxy-5:7-Dihydroxy-flaven</td>
<td>1.90</td>
<td>0.37</td>
<td>$1.9 \times 10^{-3}$</td>
<td>&gt;7</td>
</tr>
<tr>
<td>5:7:3:4-Tetrahydroxy-flaven</td>
<td>2.00</td>
<td>0.30</td>
<td>$3.0 \times 10^{-3}$</td>
<td>1.25</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.25</td>
<td>5.6 x 10^{-3}</td>
<td>1.25</td>
<td>1.25</td>
</tr>
</tbody>
</table>
phloroglucinol and quinonoid residues, with incorporation of more oxygen in the polymer. The possibility of experimental error in the measurement of the total oxygen uptake, due to unavoidable changes in the temperature of the burette, is most likely; for multiple coupling (ii) in the autoxidation of catechin and 5:7:3':4'-tetrahydroxyflavan would lead to polymers exhibiting general light absorption like melanin, (see also following chapter). However, some oxidative degradation by hydrogen peroxide of the autoxidation polymer of catechin has been shown to take place even under the mild conditions employed in this autoxidation. Work by Swan and Wright (129) indicates that oxidation by hydrogen peroxide is responsible for the observed degradation of artificial melanins. Since the maximum rates of oxygen uptake were determined at an early stage in these experiments, their accuracy is not subject to the same limitation. These rates remained constant during the first couple or so hours, thereafter they slowly decreased. No period of induction was observed. These results contrast with those found for the autoxidation of 5:6-dihydroxyindoles under the same conditions, in which, after the initial period of induction, oxygen uptake was rapid, and the period of reaction was about an hour (10).
The similar manometric results for catechin and 5:7:3′:4′-tetrahydroxyflavan are consistent with a common mechanism of autoxidation. Autoxidation of the bifunctional catechin molecule is slow, since oxidation to quinone is retarded by phenols(73), and the faster rate of autoxidation of the 5:7-di-O-methylcatechin agrees with this principle. Since the rates of autoxidation of 5:7:3′:4′-tetrahydroxyflavan and 3′:4′-dihydroxyflavan are respectively faster than those of catechin and 5:7-di-O-methylcatechin, the secondary alcoholic group in catechin and its 5:7-dimethyl ether exerts a steric factor which lowers the rate of autoxidation(13).

Polymer Analyses. Acidification of the solutions of the 3′:4′-dihydroxyflavans which had undergone autoxidation gave fine suspensions from which the oxidation products could not be removed either by filtration or centrifuging. Dialysis, which was very slow, gave products containing considerable amounts of ash (ca 10%). Electrodialysis using Cellophane membranes was faster, but it was found that cations were preferentially removed(13). When ion-exchange membranes were employed deionisation was rapid(134), and the precipitated polymer, which migrated towards the anode compartment, could be recovered ash-free and almost quantitatively by centrifuging.
### Table 4

**Polymer Analysis**

<table>
<thead>
<tr>
<th>Polymers from:</th>
<th>Analyses 0%</th>
<th>H%</th>
<th>Atomic proportions</th>
<th>Empirical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C%</td>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>Catechin</td>
<td>52.6</td>
<td>4.6</td>
<td>15</td>
<td>15.7</td>
</tr>
<tr>
<td>5:7-3:1-0-methyl</td>
<td>57.7</td>
<td>5.5</td>
<td>17</td>
<td>19.5</td>
</tr>
<tr>
<td>3':4'-Dihydroxy-flavan</td>
<td>67.1</td>
<td>5.4</td>
<td>15</td>
<td>14.5</td>
</tr>
<tr>
<td>5:7:3':4'-Tetrahydroxy-</td>
<td>54.5</td>
<td>5.1</td>
<td>15</td>
<td>16.6</td>
</tr>
</tbody>
</table>
For the analyses of the dialysed autoxidation products (Table 4) to correspond with those of true polymers, the addition of 1.7 to 3.3 moles of water per mole of the starting materials must be made, but a similar assumption was made in the case of the melanins (10). Evidence for hydration was found in the persistence of a strong spectral band at 1627-1635 cm$^{-1}$ (Fig. 2) after prolonged drying of the autoxidation polymer of catechin. Hergert and Kurth (65) using similar evidence found that (+)-catechin retained water after being dried at 115$^\circ$ in vacuo for two weeks. Mayer (92) found that (+)-gallocatechin forms an extremely stable dihydrate. Unfortunately, retention of water by the polymers prevents an examination of the fundamental carbon-oxygen stretching vibration in the 1650-1720 cm$^{-1}$ region. The strong band at 3440 cm$^{-1}$ in the spectra of both catechin and its autoxidation polymer is assigned to hydroxyl stretching. The ill-defined region in the spectrum of the polymer below 1200 cm$^{-1}$ may indicate that the polymer is a mixture (of polymers of different molecular weights). Proteins, which may be thought of as mixtures of polymers, have diffuse infrared spectra. Since these polymers are hydrated and
difficult to analyse no acceptable conclusion may be drawn regarding secondary oxidation of some of the quinone units, or the extent of multiple coupling.

**Colour Reactions of Polymers.** Since the acid-catalysed polymer from catechin gives the vanillin, ferric, and ferric ferricyanide reactions, the application of these reactions to the present polymers is justified. The tests were carried out on the polymers after two dimensional paper chromatography. Unlike the corresponding monomers, the polymers from catechin and from 5:7:3':4'-tetrahydroxyflavan did not react with the vanillin or ferric reagents, but gave blue colours with ferric ferricyanide; whereas that from 5:7-di-O-methylcatechin gave only a strong vanillin reaction. 3':4'-Dihydroxyflavan, which gives positive ferric and ferric ferricyanide reactions, afforded an autoxidation polymer which did not react with either reagent. 5:7-Dihydroxy- and 5:7-dihydroxy-3':4'-dimethoxyflavans both react with the vanillin reagent, but on autoxidation they yielded products which did not react with this reagent. The information given by the vanillin reagent (Chapter 4) suggests therefore that in the autoxidation of catechin and 5:7:3':4'-tetrahydroxyflavan either both the 6 and 3 positions are involved or each phloroglucinol residue is conjugated with or


**TABLE 5.**

**Colour changes occurring during the Autoxidation of Flavans**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intermediate colour</th>
<th>Final appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>pink→yellow</td>
<td>red-brown</td>
</tr>
<tr>
<td>5:7-Di-O-methyl &quot;</td>
<td>yellow→pink</td>
<td>red, slight ppt.</td>
</tr>
<tr>
<td>5:7-Dihydroxyflavan</td>
<td>pink</td>
<td>pink, slight ppt.</td>
</tr>
<tr>
<td>5:7-Dihydroxy-3':4'-dimethoxyflavan</td>
<td>pink</td>
<td>pink, slight ppt.</td>
</tr>
<tr>
<td>3':4'-Dihydroxyflavan</td>
<td>yellow</td>
<td>green fluorescence</td>
</tr>
<tr>
<td>5:7:3':4'-Tetrahydroxyflavan</td>
<td>pink→yellow</td>
<td>red-brown</td>
</tr>
<tr>
<td>Catechol</td>
<td>pink</td>
<td>dark brown</td>
</tr>
</tbody>
</table>
linked to a carbonyl function; and that the phloroglucinol residue is not involved in the autoxidation of 5:7-di-O-methylcatechin.

**Absorption Spectra.** Absorption spectra of the solutions of the flavans were measured during and at the completion of autoxidation. Those of 5:7-dihydroxy- and 5:7-dihydroxy-3':4'-dimethoxyflavans showed a slow increase in absorption in the visible range, but because of their slow rate of autoxidation, these flavans were not further studied. Although all the other flavans exhibited intermediate colours (Table 5), no intermediate could be detected spectrophotometrically (compare the results of Mason (91) on the enzymic oxidation of catechol). A relative decrease in the intensity of the shoulder at 500μ in the spectrum of catechin undergoing autoxidation was observed, and this was possibly caused by hydrogen peroxide degradation, for the shoulder at 500μ in the polymer prepared by enzymic oxidation in which no hydrogen peroxide is formed was relatively stronger (Fig. 3).

The final absorption spectra (Fig. 3) of the polymers from the 3':4'-dihydroxyflavans may be arranged in two classes, those of 3':4'-dihydroxyflavan and 5:7-di-O-methylcatechin are almost coincident with
AUTOXIDATION OF FLAVANS

KEY
○ Catechin.
□ 3:5; 3':4'-Tetrahydroxyflavan.
● 3':4'-Dihydroxyflavan.
▲ 5,7-Di-O-methylcatechin.
△ Catechol.

FIG. 3
that of catechol and are compatible with that of a polymer with a repeated conjugated quinone unit, whereas those of catechin and 5:7:3':4'-tetrahydroxyflavan are consistent with that of a polymer containing a repeated isolated quinonoid unit. Substances in the first class exhibit absorption at 230\(\mu\), and general absorption between 300 and 600\(\mu\). Methoxyl groups in the 5- and the 7- positions of 5:7-di-O-methylcatechin therefore inhibit the head-to-tail polymerisation of the quinone, in the same way as they prevent the reaction of the electrophilic tetrazotised benzidine with 5:7-di-O-methyl- and tetra-O-methylcatechins. That this inhibition is due to deactivation and not to steric hindrance is shown by a consideration of the following facts. The phenyl diazonium ion will couple freely with tri-O-methylphloroglucinol, but not with the less reactive phenolic ethers (it will couple with phenol). The p-nitrophenyl diazonium ion will couple with di-O-methylresorcinol, though still not with the simple phenolic ethers. However the 2:4-dinitrophenyl diazonium ion will couple with anisole and phenetole. Substances in the second class exhibit well defined maxima at 410–430\(\mu\) and maxima at shorter wavelengths.
Discussion. An examination of the data accumulated on the autoxidation of the flavans clearly demonstrates that formation of an o-quinone is the first stage in the autoxidation of 3':4'-dihydroxyflavans, but that constitutional differences modify the rate of autoxidation and subsequent fate of the o-quinone.

The similarity of the rates of autoxidation of catechol, 5:7-di-O-methylcatechin and 3':4'-dihydroxyflavan, and of the spectra of the polymers obtained from them is consistent with a common mechanism of autoxidation viz, head-to-head polymerisation. It is suggested that the profound differences between the spectra of the polymers from catechin and 5:7:3':4'-tetrahydroxyflavan, and the spectra of the catechol polymers arises from the presence of head-to-tail units in the former. These units would be formed by coupling between one of the electrophilically activated sites of an o-quinone molecule and one of the nucleophilically activated sites of either another o-quinone or a catechin molecule, and followed by oxidation of the resulting diphenylol with either oxygen or hydrogen peroxide to a diphenoquinone. The colour reactions of the two polymers agree with this reaction sequence.
The data also show that the alcoholic hydroxyl group in catechin and 5:7-di-O-methylcatechin exerts only a steric effect, but does not otherwise participate in the autoxidation. The suggestion of Freudenberg and Ahlhaus\(^{42}\) that in acid solution catechin may polymerise as a reactive flavon does not therefore apply to the autoxidation of catechin (pH4-8).
Chapter 7

Autoxidation of Mixed Phenolic Substrates.

Introduction. In the previous chapter it was shown that autoxidation of catechin proceeds via the corresponding o-quinone, and it was further suggested that the characteristic absorption peak at about 420 m\(\mu\) of the polymer arose from the presence of a diphenoguinonoid group. In order to confirm and extend this suggestion, and to obtain the information that might have been forthcoming from the two flavans that could not be synthesized, a study of the autoxidation of mixtures of simple models related to the two phenolic nuclei of catechin was made. For this purpose equal amounts of the catechol and the phloroglucinol (0.1 m mole) in phosphate buffer (10 ml.) were shaken in an atmosphere of oxygen. For comparison the catechol was autoxidized alone. Colour changes etc. (Table 6) were noted, and absorption spectra were measured during and at the end of the autoxidation (Figs. 4 & 5).
<table>
<thead>
<tr>
<th></th>
<th>Alone</th>
<th>+ Chlorogluconol</th>
<th>+ Methoxy-methyleroscinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>pink→dark brown</td>
<td>green→black *</td>
<td>green→brown</td>
</tr>
<tr>
<td>4-Methylecatechol</td>
<td>pink</td>
<td>yellow→orange</td>
<td>pink→red</td>
</tr>
<tr>
<td>3-Methylecatechol</td>
<td>yellow→dark brown</td>
<td>green→black *</td>
<td>green→brown black ppt.</td>
</tr>
<tr>
<td>4:5-Dimethylecatechol</td>
<td>yellow</td>
<td>orange→pink</td>
<td>orange→pink</td>
</tr>
</tbody>
</table>

* Period of induction (about 20 min.)
Results and Discussion. Catechol, 3-methyl-, 4-methyl- and 4:5-dimethyl- catechols, when autoxidized alone gave products the absorption spectra of which are very similar to those of the polymers from 3′:4′-dihydroxyflavan and 5:7-di-O-methylcatechin, and exhibit weak general absorption between 300-600 μ. Standard two-dimensional chromatograms of these products gave single spots at the origin. The work of Erdtman(23) on the alkaline oxidation of pyrogallol and 4-ethylpyrogallol to 2:3:4:2′:3′:4′-hexahydroxydiphenyl and to 5:5′-diethyl-2:3:4:2′:3′:4′-hexahydroxydiphenyl respectively, and of Erdtman and co-workers(29) on the formation of humic acids from p-benzoquinone, together with that of Mason(90,91) on the enzymic oxidation of catechol and hydroxyhydroquinone indicate that the autoxidation products described above most likely arise in the following manner:—
FIG 4

MIXED AUTOXIDATIONS (1)

KEY
- 4-Methylocatechol.
- + phloroglucinol.
- + methoxymethylresorcinol.
- 4:5-Dimethylocatechol.
- + phloroglucinol.
- + methoxymethylresorcinol.
It is unlikely that they are formed by the Diels-Alder reaction described by Horner and Sturm(70) for the dimerisation of 4:5-dimethyl-1:2-benzoquinone in non-polar solvents viz:

\[
\begin{align*}
2 \text{CH}_3 \text{C} & \rightarrow \text{CH}_3 \text{C} \\
\text{CO} & \text{CH}_3 \\
\end{align*}
\]

Three of the four catechols studied undergo oxidative coupling with either phloroglucinol or 5-methoxy-4-methylresorcinol, as demonstrated by the colour changes and the spectra of the final products. 4:5-Dimethylcatechol does couple with phloroglucinol, whilst methoxymethylresorcinol could be recovered from a mixture of it and an excess of the catechol when autoxidation was complete. Similarly the autoxidation of 4-methylcatechol was not affected by the presence of an equimolecular amount of tri-3-methylphloroglucinol.

The "mixed autoxidations" of catechol or 3-methylcatechol with either phloroglucinol or methoxymethylresorcinol proceed in the same manner to give ultimately dark brown products, which have very similar absorption spectra, and which do not move on standard chromatograms. The green intermediates
FIG. 5

MIXED AUTOXIDATIONS (2)

KEY
- Catechol + phloroglucinol.
- 3-Methylcatechol.
- + phloroglucinol.
- + methoxymethylresorcinol.
- + methoxymethylresorcinol.

λµm

300 400 500
are probably quinhydrones. By contrast, autoxidation of 4-methylcatechol with either phloroglucinol or methoxymethylresorcinol affords red-brown solutions which exhibit pronounced maxima at about 460 μ similar in intensity and position to those of the autoxidation polymers of catechin and 5:7:3:4'-tetrahydroxyflavan. These products, which are mobile on standard chromatograms, are indicators (yellow in acids, red in alkali). They are reduced to colorless substances with zinc and dilute acid, and similarly yield colorless substances on reductive acetylation. Insufficient materials prevented further examination of these indicators, but the mode of formation and their colour reactions etc., suggest that they have structures analogous to that of the diphenooquinone described by Baker and Miles (5).

The formation of 4:5-dianilino-1:2-benzoquinone from aniline and o-benzoquinone (62), 5:6-dihydroxyindole-2-carboxylic acid from 3:4-dihydroxyphenylalanine (103) by the action of tryosinase, the diphenooquinone of Baker and Miles (5), and the work of Horner and Sturm (70) on the reactivity of 4:5-dimethyl-1:2-benzoquinone, together demonstrate that positions 4 and 5 are the most reactive electrophilic sites in o-benzoquinone. In agreement with this principle phloroglucinol does undergo
oxidative coupling with 4:5-dimethylcatechol, but the less reactive methoxymethylresorcinol does not.

It is therefore concluded that free 4- and 5-positions in catechol and in 3-methylcatechol permit multiple coupling with either phloroglucinol or methoxymethylresorcinol, but that only single coupling can take place between 4-methylcatechol and these phenols. The chromatographic and spectral properties of the products are in harmony with these conclusions.

Incidentally, these results show that the ether linkages which were postulated by Reichel (1) (106), Kursanov, Djemuhadze and Zaprometov (33), and Roux (117) in the formation of phlobatannins are untenable; for all catechols would thereby be expected to yield the same type of product with either phloroglucinol or methoxymethylresorcinol.

The fact that 4:5-dimethylcatechol does not undergo oxidative coupling with methoxymethylresorcinol indicates that in the head-to-tail units of the
autoxidation polymer of catechin the 6'-position of one catechin o-quinone residue is linked by a CC bond with either the 6- or the 8-position of another catechin o-quinone residue. The following observations of Kostanecki and co-workers\(^{80,82}\) indicate that the 8-position is the more favoured. Tetra-O-methylcatechin is oxidized by chromic acid to tri-O-methylcatechone, which on further oxidation affords veratric acid. Freudenberg interprets this reaction sequence (II,III,IV) as follows:

\[
\begin{align*}
\text{MeO} & \quad \text{OH} & \text{MeO} & \quad \text{OMe} & \quad \text{II} \\
\text{MeO} & \quad \text{OH} & \quad \text{Br} & \quad \text{MeO} & \quad \text{MeO} & \quad \text{IV} \\
\text{MeO} & \quad \text{OH} & \quad \text{OMe} & \quad \text{MeO} & \quad \text{III} \\
\text{MeO} & \quad \text{OH} & \quad \text{OMe} & \quad \text{MeO} & \quad \text{MeO} & \quad \text{V} \\
\end{align*}
\]

On the other hand the monobromo derivative, when similarly oxidized does not yield the intermediate tri-O-methylcatechone (III). Wawzonek\(^{133}\) concludes that substitution has occurred in the 8-position (V). A partial type formula (VI), which can exist in two tautomeric forms (VII) and (VIII), is appended.
Whilst many stable \( \text{p} \)-diphenoquinones have been isolated, steric hindrance between the 7-hydroxyl group or the heterocyclic oxygen atom of one unit and the 2-carbon atom of another is likely to prevent the forms (VII) and (VIII) from assuming a planar configuration; for this reason it is difficult to decide which form predominates in the autoxidation polymer of catechin.

In agreement with the above partial type formula (VI) for the autoxidation polymer of catechin, acetylation gave a brown coloured product, which had an absorption spectrum similar to that of the polymer possessing bands at 280, 370, and 460\( \mu \) (Fig.6). Reductive acetylation, however, gave a cream coloured product, which had negligible absorption in the visible and exhibited only one shoulder at 230\( \mu \). Such colour changes on reductive acetylation are characteristic of quinones.
Bradfield and Penney\(^{15}\) have shown that catechin may be considered to contain two chromophores, the catechol and phloroglucinol nuclei acting almost independently. Since these nuclei absorb in the same region of the spectrum, the value of \(\varepsilon_{\text{max}}(3,300)\) for (epi)-catechin (\(\lambda_{\text{max}} 280\)) corresponds closely to the sum of the values of \(\varepsilon_{\text{max}}\) for 5:7-dihydroxy-2:2-dimethylchroman and catechol (\(\varepsilon_{\text{max}} 624\) and 2,600; \(\lambda_{\text{max}} 272\) and 278\(\mu\) respectively). Acetylation of catechin reduces the contribution of the catechol nucleus (catechol diacetate \(\lambda_{\text{max}} 260\) and 266\(\mu\); \(\varepsilon = 394\) and 324), but increases the absorption of the phloroglucinol residue. Similar increases in absorption on acetylation of hydroxyphenyl ethers has been observed by Kirby and White\(^{73}\). Lindberg\(^{35}\) has shown that the absorption at 273\(\mu\) of 2:5:2':5'-tetraacetoxy-4:4'-dimethoxydiphenyl(IX) (\(\varepsilon = 7,310\)) is more than three times the absorption of methoxy-quinol diacetate(X) at its maximum (\(\lambda_{\text{max}} = 272\mu\); \(\varepsilon = 2,190\)). This increase and change in shape of the absorption curves is attributed to conjugation of the two 2:5-diacetoxy-3-methoxyphenyl groups in the former.
FIG. 6

SPECTRA OF ACETYL DERIVATIVES OF CATECHIN AND ITS AUTOXIDATION POLYMER

**WAVELENGTH**

- Catechin pentaacetate
- Reductively acetylated polymer
- Acetylated polymer
- Autoxidation polymer.
It may be inferred that the similar differences in shape and intensity of the absorption spectra of catechin pentaacetate ($\lambda_{\text{max}} = 270\mu$ ; $\varepsilon = 2.100$) and the reductively acetylated polymer ($\varepsilon = 4.900$ at $270\mu$) is due to the presence in the latter of a tetraacetoxy diphenyl group (XI), formed from the diphenoquinonoid groups of the original polymer.
Chapter 8.

The Molecular Weight of the Autoxidation Polymer of Catechin

Introduction. The methods available for the determination of molecular weights of polymers are very specialized, and it is doubtful whether they could be successfully applied to the autoxidation polymer of catechin, because of its physical properties — low solubility in water and insolvency in many other suitable solvents. However the following mathematical treatment gives results of interest and suggests reasons for the differences in the spectra of the autoxidation polymer and the two naturally occurring phlobatannins believed to be derived from catechin and epicatechin.

The Theory of Condensation Polymerisation. (Frith & Tuckett). The reaction by which catechin is oxidatively converted into a polymer is essentially step-wise, in contrast to the polymerisation of, for example, styrene which is a chain reaction (addition polymerisation). The first step obviously produces a dimer; and trimers, tetrarmers, etc., are
progressively formed if each oxidative coupling occurs between a monomer (catechin \( \alpha \)-quinone) and the growing polymer. However, coupling can occur between, for example, a dimer and a trimer to give a pentamer. Generally we may write:

\[
n\text{-mer} + m\text{-mer} \rightarrow (n + m)\text{-mer}
\]

It is a reasonable assumption to make that the reactivities of the \( \alpha \)-quinone and phloroglucinol groupings are independent of the size of the polymer; then \( p \), the fraction of catechol (or phloroglucinol) groups which have reacted is equally well defined as the chance that a catechol group, selected at random, has reacted. The oxidation of a catechol group leads to the formation of one CC link, \( p \) therefore equals

\[
\frac{\text{Number of CC links}}{\text{Number of reacted and unreacted catechol groups}} = (A)
\]

If at any time during the autoxidation there are \( N \) molecules of all sizes present and \( N_0 \) monomer units; \( \bar{n} \), the number average degree of polymerisation, is defined as \( \frac{N_0}{N} \). The total number of CC links formed is from \( (A) pN_0 \), and each catechol group which has reacted reduces the number of molecules by one, so that
Let $P_n$ be the probability that a molecule is an $n$-mer. This molecule contains $(n-1)$ CC links, and one unreacted catechol group. Since the probability that a catechol group has reacted is $p$ and not reacted $(1-p)$,

$$P_n = p^{n-1}(1-p) = \frac{N_n}{N}$$

But $N = N_0(1-p)$ from (B)

$$\therefore \quad N_n = N_0p^{n-1}(1-p)^2$$

The weight fraction of $n$-mers, $w_n$, is by definition

$$w_n = \frac{nN_n}{N_0}$$

if the small amounts of water lost in the reaction and end-group effects are ignored. It follows that

$$w_n = np^{n-1}(1-p)^2$$

Let $\bar{n}'$ be the number average degree of polymerization, monomers excluded, which equals

\[
\bar{n}' = \frac{\text{Number of monomer units present in dimers upwards}}{\text{Number of dimers upwards present}}
\]

\[
= \frac{N_0 - N_0}{\sum \frac{N_n}{2}} = \frac{N_0 - N_0}{\sum \frac{N_n}{2}} = \frac{1-(1-p)}{2} = \frac{2-p}{1-p}, \quad (F)
\]

from (B) and (D).
Equation (B) shows that as the polymerisation proceeds, i.e. \( p \to 1 \), the value of \( \bar{n} \), the number average degree of polymerisation, increases. There will always be present polymers of different sizes, the mole-fraction and weight fraction distributions of which are given by (C) and (E).

Experiments which are described in the next chapter show that the extract of *Uncaria gambir* contains approximately 50% of (+)-catechin, and the extract of *Acacia catechu* approximately 33% of (-)-epicatechin. These values of \( \bar{n} \) give the following corresponding values of \( p, 0.3 \) and 0.42; \( \bar{n}', 1.4 \) and 1.7; \( \bar{n}'' \), 2.5 and 2.7. The theory indicates that the average molecular weights of the extracts as a whole are 400 and 495, and of the residual extract after removal of monomers, catechin or epicatechin, 725 and 835 respectively. Even when 99% of catechin has been autoxidized the number average degree of polymerisation (\( \bar{n} \)) is only 10, which corresponds to an average molecular weight of 2,900 for the autoxidation polymer of catechin. These figures enable us to determine the fate of the catechol groups in the two extracts under consideration. 30% of the catechol groups will have reacted and will be present as diphenoquinonoid.
groups in the polymers, 50% will be present in the monomer (catechin and epicatechin), 20% will be present in the polymers as unreacted end groups. The corresponding figures for the autoxidation polymer (n=10) are as follows: 90% of the catechol groups will have reacted and will be present as diphenoquinonoid groups in the polymer, 1% will be present in the monomer and 9% will be present in the polymer as unreacted end groups.

To a first approximation it may be assumed that solvent extraction of the two phlobatannin extracts removes only monomers, catechin and epicatechin, then the composition of the residual fractions with respect to catechol groups is 60% present as diphenoquinonoid groups and 40% unreacted end groups. Early experiments showed that a solution of catechin undergoing autoxidation developed a maximum at 420 m\(\mu\) due to the formation of diphenoquinonoid groups, but that the maximum at 270 m\(\mu\), initially mainly due to the catechol group, remained unaltered in intensity. Hence it would be expected that these two fractions which are less polymerised than the autoxidation polymer of catechin would exhibit relatively smaller maxima at 420 m\(\mu\). This is indeed found to be the case (Fig. 9).
Further the definite but slight mobility, positive vanillin and ferric reactions of these two fractions are readily understandable. In agreement with these theoretical conclusions it was found that during the early stages of the autoxidation of catechin, one dimensional chromatograms of the reaction mixtures exhibited a trail from the origin to the catechin spot, which became less intense and finally disappeared as the reaction proceeded.

The theory of condensation polymerisation shows that quinone polymerisation of catechin can afford polymers whose average molecular weight is well within those recorded by Evelyn (32) for the phlobatannins of wattle (600-2,000). This theory also applies to the phlobaphene reaction and would apply to the extension of quinone polymerisation of galloocatechins and leucoanthocyanins, but steric factors may determine the size of the polymers formed in the latter reaction.
Chapter 9

Enzymic Oxidation of Catechin to a Polymer Structurally Related to some Phlobatannins

Introduction. Previous chapters have dealt with the autoxidation of catechin to a polymer which has been characterised, but there is little information relating to the enzymic oxidation and no information concerning the nature of the product (111). This chapter is concerned with the enzymic oxidation of catechin by various plant polyphenoloxidases. Since the leaves of Uncaria gambir and the heartwood of Acacia catechu are known to contain relatively large amounts of catechin and its epimer respectively, the tannins from these sources have been examined for the occurrence of phlobatannins related to the polymers from the autoxidation and polyphenoloxidase oxidation of catechin.

Results. Crude enzyme preparations were used in the enzymic studies, since in many cases the polyphenoloxidase activity has been found to be identical with that for the more purified enzyme (104).
FIG. 7

ENZYMIC OXIDATIONS BY
MUSHROOM POLYPHENOLOXIDASE

KEY
- 0.2 mmole catechin in P0 buffer, no enzyme.
- + 4x10^{-3} E.U., 4 second addition at P.
- + 8x10^{-3} E.U.
- + 16x10^{-3} E.U.
- 0.2 mmole catechol in P04 buffer + 8x10^{-3} E.U.
During the aerobic oxidation of catechin by mushroom polyphenoloxidase at 20°C and pH8, the rate of oxygen uptake rapidly diminished from the time the reaction commenced. The oxidation ceased before two equivalents had been taken up, unless relatively large amounts of enzyme were initially present. A second addition of enzyme to a partially oxidized reaction mixture resulted in an immediate acceleration of oxygen uptake (Fig. 7). The enzyme was only slightly inactivated during the aerobic oxidation of catechol, and no pink coloration was observed. Phloroglucinol did not undergo oxidation. The aerobic oxidation of the mixed phenolic substrates was also catalysed by mushroom polyphenoloxidase, to give products identical with those obtained in the autoxidation of these substrates, but no period of induction was observed. A plot of the initial rate of oxidation of catechin against enzyme concentration gave the usual straight line (Michaelis equation)94. The oxidation of catechin by potato polyphenoloxidase under identical conditions of temperature and pH was also studied; the results were similar, except that the rate of inactivation was higher. The progress curves obtained for the oxidation by tobacco polyphenoloxidase, under
FIG 8

COMPARISON OF SPECTRA

KEY
- Catechin oxidised non-enzymically.
- " enzymically.
- Catechol " enzymically.
the different (and optimum) conditions of temperature (35°) and pH (6), were similar to those for oxidation by mushroom enzyme.

The polymers obtained from the autoxidation and polyphenoloxidase oxidation of catechin remained around the origin in the standard two-dimensional chromatograms ($R_f$ values 0-0.1), gave negative or only faint reactions with ferric chloride and vanillin reagents, but gave strong ferric ferricyanide reactions; precipitated gelatin solutions; were retained on hide powder; and were precipitated on boiling with either formalin-hydrochloric acid mixture or N-sulphuric acid. The absorption spectra exhibited bands at 270 and 410 m$\mu$ and a shoulder at 500 m$\mu$, but the shoulder was less intense in the case of the autoxidation polymer (Fig. 3). Oxidation of catechin by hydrogen peroxide and horse radish peroxidase at pH 6 gave a yellow solution which exhibited a single absorption band at 360 m$\mu$.

The residual aqueous solutions of aqueous extracts (commercial and laboratory specimens) of the heartwood of Acacia catechu, and the leaves of Uncaria gambir were examined after exhaustive solvent extraction,
FIG. 9

SPECTRA OF SOME CONDENSED TANNINS.

Heartwood of *Acacia catechu*.

Leaves of *Uncaria gambir*.

Autoxidation polymer of catechin.

Commercial extract of *Acacia catechu*. 
and were found to contain approximately half of the original tannin. The absorption spectra of these phlobatannins at pH8 (Fig. 9) were similar to that of autoxidized catechin, exhibiting bands at 270, 410, and 550μ. A possible explanation of the differences in intensity of the maxima at 410μ is given in the chapter, "The Molecular Weight of the Autoxidation Polymer of Catechin". Elementary analyses of analytical specimens of these phlobatannins were similar to those for the autoxidation polymer of catechin of empirical formula C₁₅H₁₀O₆·3H₂O (Table 7). These phlobatannins also behaved similarly to the autoxidation polymer of catechin on acetylation and reductive acetylation. The other properties of these phlobatannins were identical with those recorded for the polymer obtained by autoxidation or by polyphenoloxidase oxidation of catechin.

The remaining phlobatannins present in the ethyl acetate extracts were shown by paper chromatography to contain mixtures of mobile substances which gave strong ferric and vanillin reactions (Fig. 10), and to have the characteristic properties of phlobatannins.
The absorption spectra of these substances were similar to those of the oxidation polymers of catechin. When acetylated these substances, which had the same elementary analyses as the phlobatannins, gave brown products, which on reductive acetylation gave cream coloured products.

**TABLE 7**

**Fractionation of Phlobatannin Extracts**

<table>
<thead>
<tr>
<th>Tannin</th>
<th>Et₂O extracted</th>
<th>EtOAc extracted</th>
<th>Residual aqueous layer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acacia catechu</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43% tannin</td>
<td>30%</td>
<td>23%</td>
<td>contained 58% of total tannin.</td>
</tr>
<tr>
<td>26% non-tannin</td>
<td></td>
<td></td>
<td>C, 54.2; H, 5.5</td>
</tr>
<tr>
<td><strong>Uncaria gambir</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40% tannin</td>
<td>45%</td>
<td>12.5%</td>
<td>contained 65% of total tannin.</td>
</tr>
<tr>
<td>33% non-tannin</td>
<td></td>
<td></td>
<td>C, 52.0; H, 5.7</td>
</tr>
</tbody>
</table>

A flavonoid fraction derived from *Acacia catechu* was found to contain in addition to quercetin (98) two flavonols chromatographically indistinguishable in two different solvents (3) from fisetin and quercetagetin.
This study on the course of the aerobic oxidation of catechin by different plant polyphenoloxidases suggests that catechin functions as a substrate for these enzymes which are widely distributed in plants, a suggestion which is confirmed by the following observations:

Forsyth (36) has shown that cacao beans contain some 3% of catechins of which 90% is (-)-epicatechin, and a powerful polyphenoloxidase. This enzyme is capable of removing 80% of the total polyphenols in one hour when the fresh beans are ground and exposed to air. However, Forsyth has indicated that it is unlikely that the "tannins" in commercial cacao are formed by polyphenoloxidase oxidation as fermentation is carried out under anaerobic conditions. Siegelman (127) has demonstrated that (-)-epicatechin, which is present in apples functions as a substrate for apple polyphenoloxidase. Roberts (111) has recorded similar results for tea polyphenoloxidase. The observation of Schmidt (122) is also relevant that the yield of (+)-catechin from the shells of the edible chestnut (Castanea vesca) was greatly reduced unless steps were taken to destroy enzymes present.

The products of polyphenoloxidase oxidation have
been shown to be closely similar to the polymer from the autoxidation of catechin which was formed by quinone polymerisation; and to have the analytical properties of a tannin\(^{(59,121)}\).

It is now appropriate to review past work on phlobatannins, in particular that from the Heidelberg School.

Sir Humphrey Davy (1803)\(^{(24)}\) noted that catechin was a non-tannin, and that an aqueous solution darkened on exposure to air. Later in 1855 Neubauer\(^{(95)}\) demonstrated that catechin is converted into a tannin when an aqueous solution is boiled for some time. Recently Ellis and Pankhurst\(^{(26)}\) have confirmed these observations by studying the effects of dilute solutions of catechin, autoxidized catechin, and various tannins on a monolayer of collagen. Lowe in 1873\(^{(86)}\) and Stti in 1877\(^{(30)}\) also observed that catechin could be transformed into a tannin under a variety of conditions.

When reference is made to the results recorded by, for example, Perkin\(^{(93)}\) or Rötsieper\(^{(116)}\) for alkali fusions of tannins the fact emerges that many yield as degradation products a di- or tri-hydric phenolic acid on the one hand and a di- or tri-hydric phenol on the other, and that these fission products correspond to
those given by the flavanols and the pigments—flavonols or flavones which accompany the tannins in plants. Thus quebracho tannin yields resorcinol and protocatechuic acid and is accompanied by fisetin (I); the tannin of heartwood of Acacia catechu yields phloroglucinol and protocatechuic acid and is associated with (-)-epicatechin (IV) and quercetin (II) (98).

(The recent application of paper chromatography by White (140), Roux (118), Roberts (109) and others, has demonstrated the complexity of phlobatannins, and in consequence the significance of alkali degradations is reduced, see also Fig. 10.)

A consideration of these facts led Freudenberg to suggest that all phlobatannins are derived from that 3-hydroxyflavan which gives on alkali fusion the same fission products as the tannin. For example, quebracho tannin was assumed to be derived from a hypothetical "quebracho" catechin (III), and the tannin from Acacia catechu from (-)-epicatechin (IV).
An investigation of the wood and leaves of quebracho (*Schinopsis lorentzii*) failed to reveal any traces of "quebracho" catechin. Accordingly Freudenberg and Maitland\(^{50,51}\) synthesised \(\dagger\)-quebracho catechin by catalytic reduction of the corresponding flavylium chloride. Quebracho catechin was reported to be more sensitive to atmospheric oxidation and to acids than is catechin itself. Analysis of the product obtained after four hours heating in acid solution revealed that it possessed the same empirical formula as the original quebracho catechin, but that its acetyl value had increased to an extent which indicated that another hydroxyl group had been brought into being. Freudenberg and Maitland\(^{50,51}\) suggested that opening of the pyran ring had occurred to give a secondary benzyl alcohol, and carbon atom \(\text{C}(2)\) then condensed either with carbon atom \(\text{C}(6)\) or \(\text{C}(8)\) of another molecule to give a bifunctional dimer capable of further polymerisation.
Recently Freudenberg and co-workers (54, 55) have demonstrated that the acid catalysed polymerisation of catechin and related flavans proceeds by the same route. Whilst the Heidelberg school (41, 54, 55, 93, 123) continue to maintain that many phlobatannins are catechins polymerised post mortem by plant acids, no satisfactory comparisons between artificial phlobaphenes and naturally occurring phlobatannins have been reported by them. Brown et al. (16) have supported Freudenberg's mechanism of phlobaphene formation by a study of the acid catalysed condensation of simple model compounds.

Russell's Theory. Russell suggested (119) that as no 3-hydroxyflavans had been found in many sources of phlobatannins, these latter may just as well be related to a 4-hydroxyflavan nucleus. By reduction
of various polyhydroxychalcones with zinc and dilute hydrochloric acid Russell claimed to have obtained two types of product - the corresponding 4-hydroxyflavan and a bimolecular substance designated a "flavpinacol" (V), for which the evidence was mainly indirect.

\[
\begin{aligned}
\text{V} & \quad \text{VI} \\
\text{C}_6\text{H}_5\text{C}=\text{CH}_2\text{C}=\text{CH}_2\text{C}_6\text{H}_5 & \quad \text{C}_6\text{H}_5\text{CCH}_2\text{CO}_2\text{C}_6\text{H}_5
\end{aligned}
\]

By reducing various polyhydroxychalcones (bearing hydroxyl groups in the positions suggested by the degradation products of individual phlobatannins) Russell obtained "flavpinacols" which he claimed were indistinguishable from these tannins. These products were absorbed quantitatively by hide powder, had elementary analyses similar to those of the corresponding tannins, and gave the same colour reactions.

Russell concluded therefore that the phlobatannins were beyond "reasonable doubt" polyhydroxyflavpinacols.
Russell's "proofs of identity" are only proofs that the tannins and the "flavpinacols" have the same phenolic pattern, which was to be expected from the method of synthesis. Furthermore, Finch and White have reduced chalcone(VI) under Russell's conditions and have shown the product to be a 1:6-dione(VII).

A high percentage of the phlobatannins of the leaves of Uncaria gambir and the heartwood of Acacia catechu, which are known to contain relatively large amounts of catechin and its epimer respectively, have now been shown to have identical tannin properties, similar absorption spectra, and similar elementary analyses to the polymers produced either by autoxidation or polyphenoloxidase oxidation of catechin. These phlobatannins behaved similarly to the autoxidation polymer of catechin on acetylation and reductive acetylation. A close structural relationship therefore exists between these substances and it is probable that these phlobatannins resulted from similar aerobic oxidation of catechin precursors during harvesting. The remaining phlobatannins present in the ethyl acetate extracts were shown to contain mixtures of mobile substances. The absorption spectra of these
substances were similar to those of the oxidation polymers of catechin, and they behaved similarly on acetylation and reductive acetylation; but solubility in organic solvents, mobility in chromatographic solvent systems, and strong vanillin and ferric reactions implied a lower degree of polymerisation. Formation of phlobatannins from catechin epimers by quinone polymerisation recalls the similar formation of artificial melanins from 5:6-dihydroxyindoles\(^{(10,13)}\), but no comparisons have been reported between these products and the melanins of animal or plant origin. The fact that these new phlobatannins may be accounted for by the polymerisation of catechin is in agreement with the suggestion of Freudenberg and Maitland\(^{(50,51)}\), but no evidence has been found for the acid-catalysed reaction which has been shown (chapter 4) to require low pH (<2) and high temperature (>50\(^{\circ}\)). Indeed such a reaction is a priori unlikely for the normal pH range of sap is 5 - 6\(^{(123)}\) and phlobatannin extracts have a pH of 3.5 or higher. The chromatograms of a solution of catechin which had been partially polymerised under Freudenberg's conditions differed considerably from those of several typical phlobatannin extracts.
Phlobatannins with different properties from these have been similarly isolated during the course of this work from extracts derived from the bark of wattle (Acacia mollissima) and oak and from black tea (Thea sinensis), extracts which have been shown to contain small amounts of catechin epimers. These phlobatannins gave strong vanillin and strong violet ferric reactions. It is therefore inadmissible to regard the phlobatannins as members of a closely related chemical family, but the presence of catechins in these plants, and of leucoanthocyanins in the barks of various eucalypts and Pinus radiata and many woody plants may indicate that the associated phlobatannins have arisen through quinone polymerisation. Indeed Roberts has recently shown the occurrence of head-to-head polymerisation (VIII) in the tea polyphenoloxidase oxidation products of (-)-epigallocatechin and its gallate, which are the main catechins of green tea.
PART THREE

EXPERIMENTAL
(a) **General Procedure.** Where necessary, solutions were concentrated in nitrogen under reduced pressure at \(<35^\circ\).

(b) **Chromatography.** For chromatographic examination of the products from the autoxidation of the flavans and of the fractions from the tannin extracts, the following solvents gave good separations:

- (A) H—Acetic acid,
- (B) Butan-1-ol:acetic acid:water::6:1:2 (v/v),
- (C) Acetic acid:water::4:1 (v/v),
- (D) tert-Butanol:water::4:1 (v/v) containing 1% formic acid.

Throughout this work two-dimensional chromatograms developed by solvent systems (A) and (B) in that order are referred to as "standard chromatograms". This solvent combination, (A) and (B), was useful in the separation of the more hydroxylated flavans from their products of autoxidation, whilst the alternative combination was employed with the other flavans.

(c) **Spray Reagents.**

- (A) 0.4% ethanolic ferric chloride.
- (B) 2 volumes of 10% ethanolic vanillin plus 1 volume 12N-hydrochloric acid mixed prior to use.
- (C) The ferric ferricyanide reagent was prepared by mixing equal volumes of 0.3% ferric
chloride and 0.3% potassium ferricyanide. After being dipped the papers were well washed in dilute hydrochloric acid and then allowed to dry.
Manometry.

Haldane's constant-pressure respirometer (63) was used to follow the oxygen uptake. In this, the reaction flask (50ml, Bl4 joint) was joined by small-bore pressure tubing to a gas burette (10ml) and to a manometer, the other limb of which was joined to a compensatory flask. Two taps communicating to the atmosphere were connected to the flasks and were used to fill the apparatus with oxygen. They were left open whilst the apparatus was reaching equilibrium. Butyl phthalate was used as the manometric fluid. A small tube containing the flavan (0.2m mole), and attached to a glass fibre, was placed upright in the reaction flask, surrounded by phosphate buffer, pH 3, (20ml) or phosphate buffer-methanol (1:1)(20ml). The compensatory flask contained the same amount of buffer. In this way the system could be equilibrated before the reaction was started by dislodging the tube and shaking the contents. Both flasks, immersed in a thermostat at 35° (20° for enzyme catalysed reaction), were shaken at sufficient speed to maintain the reaction mixture as a foam. The progress of the reaction was followed by direct reading of the gas burette, the movable limb of which was first adjusted so as to bring
the liquid in both arms of the manometer to the same level. When absorption of oxygen ceased, no flavan could be detected in the two-way paper chromatogram of the reaction mixture.

After acidification of the mixture, the polymer was adsorbed on a column of alumina (10 x 1.2 cm), prepared in 2N-sulphuric acid. Potassium iodide (1 g.) was added to the sulphuric acid eluate (130 ml.) which was then set aside (15 min.) in the dark, and the liberated iodine estimated with 0.01N-sodium thiosulphate. In another experiment, the oxidising agent was shown to be hydrogen peroxide, as the distillate gave a positive reaction with titanyl sulphate and was indistinguishable from distilled water between 300 - 700 μm.
Spectrophotometric Experiments and Analytical Samples.

(a) A solution of the flavan (1 m mole), or the catechol and the phloroglucinol (1 m mole each) in phosphate buffer (pH8; 100ml.) or its equivalent of phosphate buffer-methanol (1:1) was shaken with oxygen until no more was absorbed. Samples were withdrawn during the autoxidation for spectrophotometric examination. The solution was diluted where required with more buffer. For the isolation of the pure polymer phosphate buffer was removed by electrodialysis. The cell employed was constructed of "Pyrex" pipe-line and fitted with an anion-exchange membrane and a cation-exchange membrane. The cell was supplied with 100 volts D.C. until the resistance ceased to increase (about 10 hr.); the solution was then free of phosphate ion (molybdenum-blue test). During the electrodialysis the polymer was precipitated and generally migrated towards the anode. It was removed in the centrifuge, washed, and dried (3 days at 20° in vacuo, followed by 8 hr. at 70° in vacuo over phosphorus pentoxide). All the polymers were ashless on combustion.

(b) Anhydrous catechin (29mg.) and alkali-free silver oxide (50mg.) in dry dioxan (5ml.) were shaken for 2 hrs and then centrifuged. The spectrum of the supernatant solution was compared with an authentic
sample of \(\alpha\)-benzoquinone (\(\lambda_{\text{max}} = 370\mu m\), \(\log \varepsilon = 3.15\), Mason\(^{90}\) gives \(\lambda_{\text{max}} = 368\mu m\) and \(\log \varepsilon = 3.23\)).

The remainder of the supernatant solution was mixed with an equal volume of phosphate buffer, pH8, and the solution centrifuged to remove crystals of alkali phosphates precipitated by the dioxan prior to measuring its absorption spectrum.

Catechin (0.1m mole) in phosphate buffer (pH8, 10ml.) was shaken with silver oxide (50mg.) for 1 hour, and the spectrum of the centrifuged solution measured.
Preparation of (+)-Catechin.

Commercial catechin was ground with an equal weight of hyflo-supercel, and the mixture exhaustively extracted with ether. Removal of the solvent gave a pale yellow product, which was recrystallized twice from water (charcoal) to give colorless catechin tetrahydrate, m.p. 96\(^\circ\), anhydrous m.p. 219\(^\circ\)(65). The anhydrous catechin had \([\chi]^2_d + 12^\circ\) (c, 1.7 in acetone).

Preparation of (-)-Epicatechin(102)

Heartwood of Acacia catechu (75g.) was shredded and extracted with acetone (3 x 125ml.) over 10 days. The extract was taken to dryness and the residue crystallized from water (charcoal). Standard chromatograms showed that the product (2g.) still contained some catechin, but was sufficiently pure for use as a marker.
4:5-Dianilino-1:2-benzoquinone.

Catechol (2g.) was added to M/15 phosphate buffer, pH 3 (500ml.) containing aniline (5g.) and the solution aerated for 24 hr. The solution rapidly became red and the red precipitate formed was collected by filtration, washed with dilute hydrochloric acid, and dried. The dried material was extracted with acetone, and the acetone extract concentrated to give crude 4:5-dianilino-1:2-benzoquinone, which was recrystallized from acetone m.p. 191°, (1.5g.). This was identical with a specimen prepared by silver oxide oxidation of a solution of catechol in glacial acetic acid by the method of Hackman and Todd(62).

When the catechol was replaced by catechin in the above aerobic oxidation, the same red coloration was formed, but no crystalline material could be isolated from the buffer solution.
5:7-Dihydrorodinaphtyl Flavan

\[
\text{HO} \quad \text{O} \quad \text{OH} + \text{ClOCCH=CHC}_6\text{H}_5 \xrightarrow{\text{AlCl}_3} \text{HO} \quad \text{O} \quad \text{HO}
\]

\[
\text{Zn}/\text{Hg} \quad \text{HCl, AcOH.} \quad \text{HO} \quad \text{O} \quad \text{HO}
\]

This had m.p. 193° (Found: C, 74.2; H, 5.9. Calc. for C\text{15}H\text{14}O\text{3}; C, 74.4; H, 5.8). It was prepared by the method of Robertson, Shalley, and Yates (115).

The Formation of 5:7-Dihydrorodinaphtyl-3′:4′-dimethoxy- and 5:7:8′:9′-Tetramethoxy-Flavan

The corresponding flavonoids were prepared in an improved yield and more conveniently by modifying the method of Shinoda and Sato (124). The method is illustrated by the synthesis of 5:7-Dihydrorodinaphtyl-3′:4′-dimethoxy-flavonones and -flavan.

3′:4′-Dimethoxychinamic acid was prepared in excellent yield by the condensation of veratric aldehyde and malonic acid in pyridine with piperidine as catalyst, m.p. 180°. The acid was converted into the acid chloride by refluxing with thionyl chloride, m.p. 93° (from carbon tetrachloride). (Focdick and Stark (37)
give 80–82°). To a well stirred suspension in a dry ether (125 ml.) of the acid chloride (16.2 g.) and phloroglucinol (10 g.) was added dropwise aluminium chloride (9.5 g.) in ether (125 ml.). Next day the dark red reaction mixture was poured on to ice (200 g.) and conc. hydrochloric acid (100 ml.) and extracted with ether. Evaporation of the ethereal extract gave a red glass, which in the minimum volume of methanol was refluxed with 10% aqueous sodium hydroxide (20 ml.) under nitrogen for 3 hrs. The methanol was removed under reduced pressure and the solution then saturated with carbon dioxide, and exhaustively extracted with ether. The ether extract, after washing with saturated sodium bicarbonate and drying, was taken to dryness to give a yellow crystalline product (6 g.).

Two recrystallisations from aqueous ethanol (charcoal) and aqueous acetic acid gave the flavanone (3.5 g.) as white needles m.p. 202° (Lit 200°). (Found: C, 64.9; H, 5.2; OMe, 19.7. Calc. for C₁₅H₁₀O₄ (OMe)₂: C, 64.6; H, 5.1; OMe, 19.5%).

The flavanone (1.0 g.) was dissolved in glacial acetic acid (50 ml.) and amalgamated zinc dust (from 20 g. zinc dust) added. Conc. hydrochloric acid (20 ml.) was added portionwise to the cooled solution, with shaking, over 24 hrs. The solution was then filtered,
diluted considerably and neutralized with sodium carbonate. Evaporation of the ether extract gave the crude flavan (0.8g.) which crystallized in nodules from xylene m.p. 260°. (Found: C, 67.3; H, 5.9. C_{17}H_{18}O_{5} requires C, 67.5; H, 5.9%). The flavan, which gave a positive vanillin reaction, but a negative ferric reaction, slowly turned pink on exposure to air.

5:7:3':4'-Tetrahydroxyflavanone (eriodictyol) m.p. 257° (Found: C, 62.0; H, 4.0. Calc for C_{17}H_{12}O_{6}: C, 62.5; H, 4.2%) was prepared from dibenzoylaffeyl chloride and phloroglucinol. Caffeic acid, synthesised by the method of Vorsatz was benzoylated and converted into the acid chloride in the usual way. Reduction of eriodictyol by the method used for the 3':4'-dimethyl ether gave the flavan which formed felted needles m.p. 135° (from water) (Found: C, 62.1; H, 5.4. Calc for C_{17}H_{14}O_{5}.H_{2}O:C, 61.8; H, 5.5%) which were dried (P_{2}O_{5}) at 70°/0.1mm. for 3 hrs. The flavan gave a green ferric reaction and a positive vanillin reaction.
Attempts to prepare \(5:7:3^{'},14^{'},6\text{-tetrahydroxy-8-dimethylflavan}\).

Di-3-methylphloroglucinol was prepared in good yield by the method of Robertson and Whalley\(^{(114)}\) except that zinc cyanide was successfully employed instead of anhydrous hydrogen cyanide.

\[
\begin{align*}
\text{HO} & \text{COOMe} \quad \text{Zn}(\text{CN})_2 \quad \text{HCl, Et}_2\text{O} \\
\text{HO} & \text{COOMe} \quad \text{Zn/H}_2 \quad \text{HCl} \\
\text{HO} & \text{COOMe} \quad \text{Zn}(\text{CN})_2 \quad \text{HCl, Et}_2\text{O} \\
\text{HO} & \text{COOMe} \quad \text{Zn/H}_2 \quad \text{HCl} \\
\end{align*}
\]

Or more conveniently but in poorer yield from m-xylene by the method detailed for tri-3-methylphloroglucinol. Prepared either way di-3-methylphloroglucinol had m.p.161° (from xylene). Weidel and Wenzel gave 163°\(^{(135)}\).

(A) Interaction of di-3-methylphloroglucinol and dibenzoylceffeyl chloride by the method described above gave the flavanone in poor yield, which could not be obtained pure. Cold Clemmensen reduction of the crude flavanone gave a few crystals of the flavan which rapidly resinified on exposure to air. Two dimensional paper chromatography with the standard solvents of the reduction product indicated the presence of a small...
amount of flavan and larger amounts of several substances of unknown composition.

(B) Di-2-methylphloracetophenone. A solution of di-2-methylphloroglucinol (6.5 g.) and acetonitrile (3 ml.) in ether (250 ml.) containing zinc chloride (1 g.) was saturated with dry hydrogen chloride at 0°, and resaturated a day later. A day later the precipitated iminochloride was washed with ether and hydrolysed by boiling with water (100 ml.) for 2 hrs under nitrogen. The cooled solution deposited di-2-methylphloracetophenone (3.7 g.) m.p. 225° which was not raised on crystallisation from aqueous methanol. Campbell and Coppinger give 221-222° (21).

A solution of di-2-methylphloracetophenone (2 g.) and protocatechuic aldehyde (1.4 g.) in ethanol (10 ml.) was added to water (10 ml.) containing sodium hydroxide (10 g.) and the mixture refluxed for 30 mins under nitrogen. The ethanol was removed under reduced pressure and the solution acidified. Standard chromatograms of the red solution showed the presence of six coloured substances and a further four spots were revealed by ultraviolet light. Starting materials were identified by their colour reactions and R_F's. No spot having the anticipated colour reactions of the flavanone could be detected.
The Attempted Preparation of 5:7:3:4'-Tetrahydroxy-
--6':methylflavan.

3:4'-Methylenedioxytoluene was prepared in 95% yield
from piperonal by the Huang-Minlon modification of the
Wolf-Kishner reduction, as detailed in the preparation
of 3'-methylcatechol. It had $\eta^2_0 1.530$, lit.$\eta^1_0 1.530(120)$.

In the Catterman reaction as modified by Adam(1)
3:4'-methylenedioxytoluene gave only tarry materials.
The attempted Preparation of 5,7-Di-O-methyl-(+)-catechin (cf. 25, 35).

(A) To a solution of catechin (5.8 g.) in acetone (20 ml) at -5 ° was added successively with vigorous stirring 2N-sodium hydroxide (40 ml) and 12.5% phosgene in toluene (24 ml). Air was excluded by a rapid passage of nitrogen through the solution. After two minutes the solution was acidified to Congo red, and the layer of toluene removed. The solution was then extracted with ethyl acetate (5 x 200 ml), and the extract after being dried was distilled under reduced pressure. The residue (5.5 g.), and off-white glass, was not further purified, but a portion was methylated with a slight excess of diazomethane. The two way chromatogram of the hydrolysed methylated product showed the presence of unchanged catechin and various methyl ethers of it. No crystalline product could be isolated.
(B) To a stirred solution of catechin (5.8 g) in pyridine (20 mL) was added dropwise 12.5% phosgene in toluene (24 mL). When the initial reaction was complete, the mixture was gently refluxed for one hour. On cooling, the mixture separated into two layers. The upper toluene layer was discarded, and the lower syrupy layer poured into a slight excess of ice-cold dilute hydrochloric acid. The mixture was then extracted with ethyl acetate (5 x 200 mL), and the extract after being washed with dilute hydrochloric acid and sodium bicarbonate was taken to dryness. The product (5.5 g) was worked up as before with similar results.
Attempted Preparation of 3'-4'-Dihydroxy-5,7-dimethoxyflavan.

Di-O-methylphloroglucinol: Anhydrous phloroglucinol (26.0g.) in dry methanol (100ml.) was saturated with dry hydrogen chloride, and the solution then refluxed two hours. It was then resaturated with hydrogen chloride. Twenty four hours later excess methanol was removed, and the syrup poured into water (200ml.), which was then ether extracted. The ethereal extract was washed with aqueous sodium bicarbonate, dried and distilled. Fractional distillation gave the dimethyl ether (19.7g.; b.p. 138°/1.8mm.) and the monomethyl ether (5g.; b.p. 163°/1.8mm., m.p. 73°).

2:4-Di-O-methylphloracetophenone: A solution of the above dimethyl ether (19.5g.) in acetic anhydride (19.5ml.) was treated with boron trifluoride – acetic acid complex (30ml.) with cooling. A day later the yellow crystalline mass was added to water (250ml.) containing hydrated sodium acetate (30g.). The crude mixture of the isomeric phloracetophenones was filtered off, well washed with water and dried, and then chromatographed on alumina. The column was developed with light petroleum (b.p. 40-60°). Evaporation of the light petroleum eluate gave 2:4-di-O-methylphloracetophenone, m.p. 80 - 81° (10.7g.), lit. (88) 82°.
3:4:2'-Trihydroxy-4:6'-dimethoxychalcone. A solution of 2:4-di-O-methylphloracetophenone (4.9g.) and protocatechuic aldehyde (3.45g.) in ethanol (20ml.) was added to water (10ml.) containing sodium hydroxide (10g.). The mixture was refluxed under nitrogen for 30 mins when the solution was cooled and 4N-hydrochloric acid (75ml.) was added. The red precipitate formed was filtered off, well washed with water and dried. The dried material was thoroughly extracted with light petroleum in a Soxhlet and then twice crystallized from aqueous ethanol to give the chalcone as yellow prismatic needles m.p. 179°-180° (1.1g.), (Found: C, 64.6; H, 5.2; OMe, 13.6. C_{15}H_{10}O_4 (OMe)_2 requires C, 64.6; H, 5.1; OMe, 19.5%). Increasing the time of reaction or using sodium ethoxide in place of sodium hydroxide did not increase the yield.

Attempted Cyclisation of the Chalcone. A solution of the above chalcone (4g.) in ethanol (320ml.) and 2N-sulphuric acid (80ml.) was refluxed for 40 hr. Ethanol was removed under reduced pressure, and water (100ml.) added to precipitate the chalcone-flavanone mixture as a dirty yellow powder. It was not found possible to separate the flavanone from the chalcone either by fractional crystallisation or by partition chromatography on silica using wet ether or on magnesol-celite using wet ethyl acetate. Accordingly the chalcone-flavanone mixture was
reduced by the cold Clemmensen procedure described above, but only an intractable oil was obtained.
The Preparation of 5:7-Di-O-methyl-(+)-catechin

- Preparation of 5:7-Di-O-methyl-(+)-catechin (cf. 2)

\[
\begin{align*}
&\text{HO} & & \text{Me}_2\text{SO}_4 \\
&\text{OH} & & \text{NaOH} \\
&\text{B} & & \text{B}
\end{align*}
\]

\[
\text{dil. HCl}
\]

Aqueous 4-M-sodium metaborate (10ml.), methyl sulphate 
(2ml.) and 2M-sodium hydroxide (3ml.) were added 
successively to a solution of catechin (2.9g.) in ethanol 
(10ml.), and the mixture shaken for 15 min.; the reaction 
mixture was then poured into ice-water (200ml.) and 
extracted with ether. The acidified aqueous phase was 
extracted with ether (4 x 100ml.), and the ethereal extract 
washed free from mineral acid. Evaporation of the solvent 
after drying, left 5:7-di-O-methyl-(+)-catechin (2.6g.), 
forming needles from aqueous ethanol, m.p. 218-219°,
\[\alpha^o_D \text{I}^o (c, 1.1 \text{ in acetone}) .\] (Found: C, 64.0; H, 5.6; 
OMe, 19.1. C\text{17H}\text{13O}\text{6} \text{requires C, 64.0; H, 5.6; OMe, 19.1%}.\]
The flavan gave a green ferric reaction which became 
mauve on addition of ammonia.
The Preparation of 3-Methylcatechol.

\[ \text{CHO} + \text{MeSO}_3\text{H} \rightarrow \text{CHO} \]

To molten o-vanillin (50g.) was added dropwise with efficient stirring a solution of potassium hydroxide (28g.) in water (40ml.) over 20 mins. Methyl sulphate (54ml.) was added at such a rate that the solution remained alkaline. After 30 mins. the solution was cooled depositing 2:3-dimethoxybenzaldehyde, which was well washed with water and dried, m.p. 50-52° (50g.). Crystallisation from either ligroin (60-80°) or aqueous methanol gave the aldehyde m.p. 52°. Lit. 54-55° (97).

A mixture of 2:3-dimethoxybenzaldehyde (33g.), hydrazine hydrate (30ml. of 85%), and potassium hydroxide (40g.) in diethylene glycol (300ml.) was heated on a steam bath for 1 hr and then refluxed for a further 3 hrs, when evolution of nitrogen ceased indicating complete decomposition of the yellow hydrazone. The cooled mixture was poured into water (1 litre) and the product extracted with ligroin (60-80°). The extract was washed with dilute hydrochloric acid and then water, dried, and the solvent removed. Distillation of the residue gave 2:3-dimethoxytoluene b.p. 66°/2mm, \( n_D^{20} = 1.513 \) (27g.), Lit.: \( n_D^{20} = 1.512 \) (132).
A solution of 2:3-dimethoxytoluene (23g.) in glacial acetic acid (100ml.) and hydrobromic acid (150ml.) containing red phosphorus (1g.) was refluxed 7 hrs when evolution of methyl bromide ceased. The solution was filtered, and then concentrated (50ml) under reduced pressure, diluted with water (100ml), and ether extracted (5 x 200ml). The ethereal extract was washed with saturated sodium bicarbonate, dried and distilled. 3-Methylcatechol was obtained as an oil, b.p. 150°/40mm. (16g.) which crystallized on cooling. Recrystallisation from 1:1 benzene-ligroin (60-80°) gave 3-methylcatechol as hexagonal plates, m.p. 64-65°, lit. 63° (33).

4:5-Dimethylcatechol. This was prepared by the method of Bruce and Sutcliffe (17), m.p. 85° (lit 86-87°).
Tri-3-methylphloroglucinol. Three stage nitration of mesitylene (20 ml.) gave trinitromesitylene (29 g.) m.p. 232-233° from dioxan, lit. 232-233° (57). A solution of trinitromesitylene in dioxan, was quantitatively reduced with active Raney nickel and hydrogen at 80° to triaminomesitylene, which was isolated as the dihydrochloride by saturating the filtered dioxan solution with dry hydrogen chloride. A solution of the dihydrochloride (10 g.) in water (250 ml.) was refluxed under nitrogen for 18 hr., cooled, acidified and extracted with ether. Evaporation of the ether gave a red solid, which was crystallised from water (charcoal) and sublimed at 170°/1 mm., to give tri-3-methylphloroglucinol (2 g.) m.p. 181°. Weidel and Wenzel give 184° (136).
The Preparation of 5-Methoxy-4-methylresorcinol

Phloroglucinol dihydrate (81 g.) and potassium hydrogen carbonate (100 g.) were dissolved in water (300 ml.) and carbon dioxide passed through the warm (60°-70°) solution for 5 hrs. Soon after the solids dissolved, a white precipitate of potassium phloroglucinol carboxylate separated, which was filtered off, dried and washed with ether. The potassium salt was decomposed with ice-cold dilute hydrochloric acid, and phloroglucinol carboxylic acid recovered almost chromatographically pure by ether extraction (75 g.). The acid was quantitatively converted to the methyl ester on treatment with the theoretical amount of diazomethane. m.p. 172° (from methanol).

Herzig, Wenzel and Tölk give 174-176° (67).
Dimethyl sulphate (9.5 ml.) was added portionwise over 2 hrs to a refluxing solution of the above ester (13.4 g.) in acetone (150 ml.) containing anhydrous potassium carbonate (14.5 g.). After a further 2 hrs the solution was cooled and the solids filtered off. Evaporation of the solution gave crude methyl 2,6-dihydroxy-4-methoxybenzoate (20 g.) which was recrystallized from methanol (needles), m.p. 114-116°. Lit. 114-116° (66).

A solution of the above ester (14 g.) in ether (300 ml.) containing zinc cyanide (8.5 g.) was saturated at 0° with hydrogen chloride. 24 hrs. later the copious pale pink precipitate was washed with ether, and rapidly heated with water (500 ml.) to 100°. On cooling, methyl 3-formyl-2,6-dihydroxy-4-methoxybenzoate separated almost quantitatively, m.p. 177° after crystallisation from methanol. Lit. 176° (113).

A suspension of the above formyl derivative (15 g.) in hot methanol (500 ml.) and 12N-hydrochloric acid (200 ml.) were both added over 20 mins to well stirred zinc amalgam (150 g.). The reduction was completed by refluxing a further 10 mins. The methanolic solution was decanted from the residual amalgam, which was further extracted with boiling methanol (2 x 100 ml.).
The combined methanolic solutions were diluted with hot water (2 l.) and allowed to cool. Filtration gave the crude methyl 2:6-dihydroxy-4-methoxy-3-methylbenzoate (9g.) m.p. 120°. Lit. 132° (113).

The above ester (8g.) was refluxed with 10% sodium hydroxide (100ml.) for 2 hrs in an atmosphere of nitrogen. The ethereal extract of the acidified hydrolysate was washed with sodium bicarbonate solution and taken to dryness. Crystallisation of the crude product (5g.) from water gave the monohydrate m.p. 84°, anhydrous 116°, which on crystallisation from benzene gave 5-methoxy-4-methylresorcinol m.p. 120-121°. Robertson & Whalley give 119° (114).
Experiments with the Autoxidation Polymer of Catechin.

1) Dehydration by hydrogen peroxide. To a solution of the polymer (catechin-free) in phosphate buffer, pH 8 (5 ml.) was added hydrogen peroxide, 30% (1 ml.) and water (4 ml.). A blank was similarly prepared. Twenty four hours later the optical densities of the solutions were measured.

Results: - Blank $E_{1cm}$ 2.4; $\lambda_{max}$, 410 $\mu$
After treatment with peroxide $E_{1cm}$ at 410, 0.74 (no maximum).

2) Reductive Acetylations. The polymer (1 g.) dissolved in acetic anhydride (10 ml.) and triethylamine (1 ml.) was refluxed for an hour. One half of the dark brown solution was poured into water (100 ml.), and worked up in the usual way to give a brown amorphous powder. To the other half, zinc dust (1 g.) was added and the mixture refluxed for a further hour. The pale yellow solution was filtered and poured into water (100 ml.) to give ultimately a cream coloured powder. Under the same conditions of reductive acetylation catechin gave an excellent yield of the pentaacetate and p-benzoquinone gave an almost theoretical yield of p-diacetoxybenzene. The ethyl acetate and aqueous fractions of extracts of both Acacia catechu and Uncaria gambir behaved similarly.
on acetylation and reductive acetylation. Spectral determinations were made in dioxan.
"Acetone Powders" of Psalliota campestris and Nicotiana tabacum

Freshly picked mushrooms (350g.) were sliced under ice-cold acetone (21.), and homogenised in a top-drive macerator. The homogenate obtained was filtered through sintered glass and the solid (20g.) was immediately washed with ice-cold acetone (5 x 500ml.), and dried at 0° in vacuo. This powder (2.8 enzyme Units) had a polyphenoloxidase activity of Purpurogallin Number 0.14, when determined by Keillin and Mann's method (75), but purpurogallin was estimated spectrophotometrically in ethanol solution at 375 and 430μ. P.N. represents the mg. of purpurogallin formed from pyrogallol in 5 mins at 20° per mg. of dry weight of enzyme preparation. One E.U. corresponds to the amount of enzyme which produces 1g. of purpurogallin in 5 mins at 20°. The acetone powder of mushrooms was used without further treatment for enzyme catalysed oxidations, whereas the acetone powder of the leaves of the tobacco plant, P.N.0.0024, was first eluted with ice-water until free of amino acids.
Potato Juice

A chilled potato was minced and pulverized, and the expressed juice was filtered and centrifuged to remove starch (105). The freshly prepared juice had polyphenoloxidase activity of P.N. 0.0022, but quickly lost this activity and became coloured.
Isolation and Properties of Tannins derived from Acacia Catechu and Uncaria Gambir.

A 5% (w/v) aqueous solution (400 ml) of a commercial extract prepared from the heartwood of Acacia catechu was extracted for two days with ether and then for five days with ethyl acetate. The progress of the solvent extraction was followed by two dimensional paper chromatography of 5 μl samples using the standard solvents (Fig. 10). Extraction with ether was continued until catechin and epicatechin had been removed. The ether residue (6 g) contained in addition to catechin epimers, flavonols and substances of unknown composition of fairly high Rf values. Extraction with ethyl acetate was similarly continued until other substances of unknown composition of lower Rf values, had been removed (4.6 g). Paper chromatography of the residual aqueous layer showed a single phenolic component around the origin. An analytical sample of this tannin was prepared by electrodialysis as previously described.

A 5% (w/v) aqueous solution (400 ml) of a commercial extract derived from the leaves of Uncaria Gambir was similarly extracted with solvents. The ether residue (9 g) on crystallisation from water...
gave catechin tetrahydrate m.p. 96°. The ethyl acetate residue (2.5g) contained unknown phenolic substances of fairly low Rf values. Paper chromatography of the residual aqueous solution gave a single phenolic component around the origin.

The ethyl acetate fractions and the residual aqueous layers of both extracts gave precipitates with 1% gelatin. They also gave positive reactions in the formalin and condensation tests. These were respectively performed as follows:— An analytical solution of the tannin (50ml, 0.4% tannin), 40% formalin (10ml) and 12N-hydrochloric acid (5 ml) were boiled for 30 mins. A positive result was indicated by complete precipitation of the tannin. An analytical solution of the tannin was made 1N with respect to sulphuric acid and was boiled 3 hr. A positive result was indicated by almost complete precipitation of the tannin (cf. 33). The ethyl acetate phenols of both extracts gave strong ferric and vanilin reactions, whilst the phenols in the residual aqueous layers gave only faint reactions; all fractions gave strong ferric ferricyanide reactions.

An aqueous extract of acetone-extracted heartwood of Acacia catechu, after solvent extraction, gave a tannin solution which had properties identical with
those of a solution prepared from a commercial extract.

The tannin content was determined by the hide-powder method of Grassmann, Endisch and Kuntara (60).
FIG. 10

UNCARIA Gambir

1. Catechin

ACACIA Catechu

1. Catechin
2. Epicatechin
9. Quercetin
10. Fisetin
11. Quercetagetin
FIGURE 10

KEY TO CHROMATOGRAMS

Uncaria gambir.

1. Extracted with ether.

2, 3. Extracted with ethyl acetate; positive vanillin and ferric reactions.

4. Not extracted with organic solvents; faint vanillin and ferric reactions.

Acacia catechu.

1, 2, 3, 8, 9, 10, 11. Extracted with ether; 1, 2, 3, 8, positive vanillin and ferric reactions.

4, 5, 6, 7, 13. Extracted with ethyl acetate; positive vanillin and ferric reactions.

12. Not extracted by organic solvents, faint vanillin and ferric reactions.
Separation of the Flavonols from Acacia Catechu by

**paper Chromatography**

Spots (10µl.) of a 2% ethanolic solution of the ether residue were thickly applied to base lines 4 cm. from the lower edge of 5 sheets of Whatman No.3 paper. Single-way ascending chromatography was effected by N-acetic acid. After the papers had been developed for 4 hrs, they were dried, and given a second development with N-acetic acid to ensure complete migration of the catechins. The transverse zones containing the flavonols were located in U.V. light, cut out and extracted with boiling ethyl acetate.

Spots (5µl.) of a 0.5% solution of the ethyl acetate residue (3mg.), and marker spots (5µl.) of 0.5% solutions of fisetin, myricetin, quercetagetin, quercetin and robinetin respectively were applied to Whatman No.1 filter papers which were developed for 24 hrs by the ascending method with the following solvent systems:

- m-cresol-acetic acid-water (25:1:24, v/v; upper phase)
- butan-2-ol-acetic acid-water (14:1:5, v/v).

Papers which had been chromatographed with the solvent system containing m-cresol were dried at 60°C. Individual flavonols were detected by their fluorescences in U.V. light.
Authentic quercetagetin was isolated from the flowers of *Tamates erecta* (89).
REFERENCES

11. Bergmann & Pojarlieff, Collegium, 1931, 233, 244.
22. Campbell & Coppinger, J.A.C.S., 1951, 72, 2708.
25. Einhorn, & Pfeiffer, Ber., 1904, 77, 106.
34. Finch & White, J.C.S., 1950, 3367.
35. Fischer & Freudenberg, Ber., 1913, 46, 1121.
38. Freudenberg, Ber., 1920, 53, 1416.
40. Freudenberg, Collegium 1924, 655, 418.
42. Freudenberg & Ahlhaus, Monat., 1956, 87, 1.
43. Freudenberg, Böhme & Beckendorff, Ber., 1921, 54, 1204.
44. Freudenberg, Böhme & Purrman, Ber., 1922, 55, 1734.
45. Freudenberg, Carrara & Cohn, Ann., 1925, 446, 87.
46. Freudenberg & Cohn, Ber., 1923, 56, 2127.
51. Freudenberg & Maitland, Collegium, 1934, 275, 656.
52. Freudenberg & Purrman, Ber., 1923, 56, 1185.
54. Freudenberg, Stocker & Porter, Ber., 1957, 90, 957.
58. Giuliano, Annali di Chimica Applicata, 1939, 22, 86.

64. Hergert & Kurth, TAPPI. 1953, 36, 137.


67. Herzig, Wenzel & Tulk, Monat., 1902, 23, 86.


73. James, Snell & Weissberger, J.A.C.S., 1938, 60, 2084.

74. James & Weissberger, J.A.C.S., 1938, 60, 98.


82. Kostanecki & Tambor, Ber., 1902, 35, 1867.

34. Lamb & Sreerangachar, Biochem.J., 1940, 34, 1472.
37. Lovering & Smith, Chem & Ind., 1946, 24, 298.
40. Mason, J.A.C.S., 1943, 70, 133.
44. Michaelis & Manten, Biochem. Z., 1913, 42, 333.
49. Perkin & Yoshitake, J.C.S., 1902, 1160.
107. Reid, "The Chemistry of Vegetable Tannins",


109. Roberts, "The Chemistry of Vegetable Tannins",


116. Rottsieper, "Vegetable Tannins", The Forestal Land,
    Timber & Railways Company, Ltd.,
    St. Albans, 1946.


120. Schepps, Ber., 1913, 46, 2572.

121. Schmidt, "Modern Methoden der pflanzenanalyse"

122. Schmidt & Mull, Ber., 1947, 80, 509.


129. Swan & Wright, J. C. S., 1954, 381.


139. Whalley, "The Chemistry of the Vegetable Tannins"

    1952, 35, 143.

Autoxidation of Catechin

A mechanism is proposed for the autoxidation of catechin to a polymer (catechin tannin) which has for a long time been recognized to have tanning properties.

Empirical study of the fate of catechin at 100° has revealed two interesting reaction processes. In agreement with Freudenberg's interpretation of phlobaphene formation, at pH < 2, a buff-coloured phlobaphene, which gave the colour reactions of catechol and phloroglucinol, was rapidly formed both in the presence and absence of oxygen. Between pH 4 and pH 8, however, catechin is autoxidized to red catechin tannin. Autoxidation was arrested by sodium hydrogen sulphite, and only epimerization occurred in absence of oxygen. Catechin tannin had phenolic properties, but it lacked the characteristic properties of catechol and phloroglucinol. Under more alkaline conditions another reaction, possibly ring-fission leading to humic acids, intervened.

Catechin in phosphate buffer (pH 8) was shaken at 35° to constant oxygen uptake; when no catechin could be detected in the paper chromatogram of the reaction mixture, the polymer formed was absorbed on alumina and the hydrogen peroxide in the eluate estimated. The results were: total oxygen uptake 1·59, hydrogen peroxide formed 0·38; oxygen balance, 1·4 moles/mole catechin. Oxygen absorption was represented by a sigmoid curve; but as the rate was slow, namely, 0·0006 mole oxygen/mole catechin/min., an induction period was not detected.

When the autoxidation of catechin in phosphate buffer was followed spectrophotometrically, no intermediate was detected, and the resulting catechin tannin had \( \lambda_{\text{max}} \) 400 mp (pH 6) and \( \lambda_{\text{max}} \) 420–430 mp (pH 8). Silver oxide oxidation of dioxan solutions of catechin and catechol gave compounds of \( \lambda_{\text{max}} \) 370 mp (o-benzoquinone \( \text{C}_6\text{H}_4\text{O}_2 \) \( \lambda_{\text{max}} \) 370 mp). Catechin tannin was formed when a solution of the corresponding o-quinone was shaken with oxygen in phosphate buffer, and by silver oxide oxidation of catechin in phosphate buffer.

It is now suggested that the main reaction sequence involves formation of o-quinone followed by oxidative condensation. Formation of o-quinone probably involves a free radical mechanism, since the reaction takes place in non-polar solvent. Hydrogen peroxide is associated with quinone formation, and its accumu-
lation results from the difference in velocity of the radical processes, conveniently summarized as:

\[ \text{H}_2\text{Q} + \text{O}_2 = \text{Q} + \text{H}_2\text{O}_2; \text{H}_2\text{Q} + \text{H}_2\text{O}_2 = \text{Q} + 2\text{H}_2\text{O} \]

The autoxidation of polycyclic hydroquinones has afforded unpublished results (D. E. H.), which confirm formation of hydrogen peroxide in oxidations leading to quinones. Autoxidation of catechin is slow, since the oxidation to quinone is inhibited by phenols. Previous evidence for intermediate formation of α-quinone includes an attempted isolation of a dianilino-derivative (no analysis or melting point) when tea oxidase acts on catechin. The oxidative condensation is an ionic process requiring dissociating medium, in which addition of phenol to quinone occurs, with CC bond formation between one of the electrophilically activated sites (2',5',6') of the quinone and one of the nucleophilically activated sites (6,8) of another quinone molecule or with the (6,8,2',5',6') sites of a catechin molecule. Addition of phenol to quinone involves transfer of two hydrogen atoms to afford diphenol, followed by oxidation to the corresponding quinone by means of oxygen, hydrogen peroxide or another molecule of quinone. The oxygen balance agrees with this reaction sequence, which permits the formation of a highly irregular three-dimensional polymer, although certain CC bonds are excluded on steric considerations. It is significant that since catechol is not bifunctional, it does not undergo a similar polymerization. A number of catechin derivatives are now being synthesized as model substances with which to test these general conclusions. This reaction sequence has also been postulated to explain the formation of melanin and purpurogallin. It is relevant to a suggestion for the formation of condensed tannins in Nature. By the action of an oxidase on catechin there results α-quinone, which is normally reduced by an oxygen acceptor, for example ascorbic acid; but enzymic oxidation of α-quinone to catechin tannin occurs whenever the reduction mechanism is intercepted. This explanation is similar to that proposed for the action of tyrosinase on tyrosine, and the action of plant polyphenolases on catechin is being investigated.

We thank Dr. H. Phillips, director of the British Leather Manufacturers' Research Association, for his advice and encouragement, and the Council of the Association for permission to publish this communication.

D. E. Hathway
J. W. T. Seakins


The formation of polymers and hydrogen peroxide during the autoxidation of catechin and of related 3':4'-dihydroxyflavans has been studied by measurement of oxygen adsorption, and by elementary analyses, absorption spectra, and colour reactions of the dialysed polymers. The evidence obtained, together with that provided by spectroscopic study of intermediates produced by silver oxide oxidation, supports the theory that tannin formation from catechin occurs by polymerisation through quinones (Part II). Whereas polymerisation through quinones of catechin and 5:7:3':4'-tetrahydroxyflavan involves the phloroglucinol residue, oxidative coupling of 5:7-di-O-methylcatechin and 3':4'-dihydroxyflavan resembles that of catechol. The relevance of catechin autoxidation to the biogenesis of phlobatannins is considered.

In Part II, a manometric study of catechin autoxidation, and the spectroscopic study of an intermediate obtained by silver oxide oxidation, supported the theory that tannin formation from catechin occurs by polymerisation through quinones. Consideration of the data accumulated from autoxidation studies on simpler substances (Tables 1 and 2 and Figures 1 and 2) confirms that catechin autoxidation involves polymerisation of quinones, but that constitutional differences modify this process in the case of the flavans.

Manometry of the Polyhydroxyflavans.—Free hydroxyl groups in the 3'- and the 4'-positions (Table I) cause an accumulation of hydrogen peroxide which is associated with quinone formation (cf. Part II). 5:7-Dihydroxy- and 5:7-dihydroxy-3':4'-dimethoxy-flavans lack vicinal hydroxyl groups and can be recovered almost quantitatively after several days' autoxidation. Adsorption of oxygen in excess of the theoretical 1 mol. may be attributed to (1) manometric difficulties associated with protracted experiments (cf. the rapid oxidation of 5:6-dihydroxyindoles to melanins 1), (2) two oxidative couplings per mole of o-quinone, or (3) oxidative degradation of some of the o-quinonoid residues with incorporation of more oxygen in the polymer. The possibility of experimental error in the total adsorption readings is the most likely, for multiple coupling (2) would lead to polymers exhibiting general light absorption like melanin, but some oxidative degradation of o-quinones by peroxide may take place even under the mild conditions of autoxidation.1 Since the maximum rates of oxygen adsorption were determined at an early stage in these experiments, their accuracy is not subject to the same limitations. The similar manometric results for catechin and 5:7:3':4'-tetrahydroxyflavan autoxidation are consistent with a common mechanism. In the present study the suggestion 2 that in acid solution catechin may polymerise as a reactive flavan has been tested by comparison of the behaviour of catechin and 5:7:3':4'-tetrahydroxyflavan. Autoxidation of the bifunctional catechin molecule is slow, since oxidation to quinone is inhibited by phenols,3 and the faster rate of autoxidation of the 5:7-di-O-methyl derivative agrees with this principle. Since the rate of autoxidation of 5:7:3':4'-tetrahydroxyflavan is faster than that of catechin, the secondary alcoholic group in catechin exerts a steric...
factor which lowers the rate of autoxidation, and the relative rates of autoxidation of 5:7-di-O-methylcatechin and 3':4'-dihydroxyflavan are consistent with this view.

*Polymer Analysis.*—To obtain correspondence between the analyses of the dialysed oxidation products (Table 2) and those of true polymers, a calculated addition of 1.7-3.3 moles of water per mole of starting materials must be made, but a similar assumption was made for the melanins. Evidence for hydration is found in the persistence of a strong spectral band at 1627-1635 cm⁻¹ after protracted drying of the polymer at 70° *in vacuo.* Hergert and Kurth using similar evidence found that (+)-catechin retained water after being dried at 115° *in vacuo* for several weeks. Unfortunately retention of water by the polymers prevents an examination of the fundamental carbon-oxygen stretching vibration in the 1650-1720 cm⁻¹ region. Since these oxidation polymers are hydrated and difficult to analyse, no acceptable conclusion may be drawn from the analyses regarding the secondary oxidation of some of the o-quinone units, or the extent of multiple coupling of o-quinone.

*Polymer Properties.*—Since the acid-catalysed polymer from catechin gives a vanillin reaction (Part II), the application of this reaction to the present polymers is justified. Unlike the corresponding monomers, the polymers from catechin and from 5:7:3':4'-

---

**Table 1. Oxygen adsorption by polyhydroxyflavans at 35°.**

<table>
<thead>
<tr>
<th></th>
<th>Oxygen uptake a</th>
<th>Residual peroxide a</th>
<th>Oxygen balance a</th>
<th>Maximum rate b</th>
<th>Reaction period c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>1.59</td>
<td>0.38</td>
<td>1.40</td>
<td>$5.6 \times 10^{-4}$</td>
<td>2</td>
</tr>
<tr>
<td>5:7-Di-O-methylcatechin</td>
<td>2.00</td>
<td>0.39</td>
<td>1.60</td>
<td>$8.4 \times 10^{-3}$</td>
<td>1.25</td>
</tr>
<tr>
<td>5:7-Dihydroxyflavan</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>$1.9 \times 10^{-4}$</td>
<td>&gt;7</td>
</tr>
<tr>
<td>5:7-Dihydroxy-3':4'-dimethoxyflavan</td>
<td>—</td>
<td>0</td>
<td>—</td>
<td>$5.3 \times 10^{-5}$</td>
<td>&gt;7</td>
</tr>
<tr>
<td>3':4'-Dihydroxyflavan</td>
<td>1.80</td>
<td>0.37</td>
<td>1.43</td>
<td>$1.2 \times 10^{-4}$</td>
<td>1.25</td>
</tr>
<tr>
<td>5:7:3':4'-Tetrahydroxyflavan</td>
<td>2.00</td>
<td>0.80</td>
<td>1.20</td>
<td>$3.0 \times 10^{-5}$</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*The corresponding autoxidation of catechol, maximum rate $5.6 \times 10^{-3}$ mole/min., was complete in 1 day. a moles. b mole/min. c days.*

**Table 2. Polymer analyses.**

<table>
<thead>
<tr>
<th>Polymers from:</th>
<th>Found, %</th>
<th>Calc., %</th>
<th>Empirical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Catechin</td>
<td>52.6</td>
<td>5.6</td>
<td>53.0</td>
</tr>
<tr>
<td>5:7-Di-O-methylcatechin</td>
<td>57.7</td>
<td>5.5</td>
<td>57.7</td>
</tr>
<tr>
<td>3':4'-Dihydroxyflavan</td>
<td>67.1</td>
<td>5.4</td>
<td>67.1</td>
</tr>
<tr>
<td>5:7:3':4'-Tetrahydroxyflavan</td>
<td>54.5</td>
<td>5.1</td>
<td>54.6</td>
</tr>
</tbody>
</table>

*Fig. 2. Absorption spectra of polymers from (I) catechin and (II) 5:7:3':4'-tetrahydroxyflavan.*

---

Fig. 1. Absorption spectra of polymers from (A) catechol, (B) 5:7-di-O-methylcatechin, and (C) 3':4'-dihydroxyflavan.
tetrahydroxyflavan do not react with vanillin reagent, whereas that from 5:7-di-O-methyl-catechin gives a strongly positive reaction.

Absorption Spectra.—The absorption spectra of the polymers are of two classes. The polymers from 3':4'-dihydroxyflavan and 5:7-di-O-methylcatechin have spectra almost identical with that of the polymer from catechol (Fig. 1), whereas the polymers from catechin and 5:7:3':4'-tetrahydroxyflavan have spectra compatible with that expected for a polymer with a repeated quinone unit (Fig. 2). Compounds of the first class exhibit phenolic absorption at 280 mμ, and general absorption at 300—600 mμ. The similarity of the spectra of the polymers from 5:7-di-O-methylcatechin and catechol is consistent with a common mechanism of autoxidation. Methoxyl groups in the 5- and the 7-position of 5:7-di-O-methylcatechin therefore inhibit the head-to-tail polymerisation of the quinones. Substances of the second class exhibit a well-defined maximum at 410—430 mμ, and two maxima at shorter wavelengths.

Discussion.—Since polymerisation through quinones of catechol and 3':4'-dihydroxyflavan involves catechol residues, the similarity of the spectra of the polymers obtained with that of the polymer from 5:7-di-O-methylcatechin is consistent with a common autoxidation mechanism. The possibility of some degree of head-to-tail polymerisation involving the phloroglucinol residues cannot be precluded from a consideration of the autoxidations of catechin and 5:7:3':4'-tetrahydroxyflavan, however, and it is now suggested that head-to-tail polymer units of these substances may account for the profound difference in spectrum of the polymers from those of the catechol polymers.

For two decades the Heidelberg school has maintained 6,7 that the phlobatannins from the barks of Acacia mollissima, birch, chestnut (Castanea sativa), Eucalypts, mangrove, oak, spruce, and willow, and from the heartwood of Acacia catechu and quebracho trees are catechins, polymerised post-mortem under the influence of the plant acids. Two mechanisms have been considered. According to the first,6 ring fission gives a secondary benzyl alcohol, and carbon atom 2 then condenses with carbon atom 6 or 8 of another molecule to afford a bifunctional dimer, capable of further polymerisation. More recently, Freudenberg 8 has suggested that catechin may react as a pair of tautomeric diphenylpropenes which are involved in the first stage of a styrene polymerisation.9 On the assumption that phlobatannins are indeed formed by polymerisation of catechin, then the enzymic autoxidation of catechin by polymerisation through quinones is a more attractive hypothesis, especially since the acid-catalysed reaction suggested by Freudenberg requires low pH (<2) and high temperature (>50°).

Polyhydroxyflavans.—The Simonis synthesis of flavanones from phloroglucinol and cinnamoyl chloride derivatives has been extensively studied,10 and despite the customary low yields this reaction has been used for the preparation of eriodictyol and 5:7-dihydroxy-3':4'-dimethoxyflavanone. Improved yields were obtained, however, by the use of ether instead of nitrobenzene as reaction medium. Clemmensen reduction at room temperature 11 of the flavanones gave the 5:7:3':4'-tetrahydroxy- and 5:7-dihydroxy-3':4'-dimethoxy-flavans. Partial methylation of (±)-catechin by means of methyl sulphate and aqueous alkali in the presence of excess of sodium metaborate 12 gave 5:7-di-O-methyl-(±)-catechin. 3':4'-Dihydroxyflavan was prepared from 3:4:2'-trihydroxy-chalcone by catalytic hydrogenation, followed by benzoylation, and lithium aluminium hydride reduction. 3:4:2'-Trihydroxychalcone was prepared by the condensation of 3:4-dihydroxyacetophenone 8 and salicylaldehyde in strongly alkaline solution. Methylation of 3':4'-dihydroxyflavan gave 3':4'-dimethoxyflavan, which was also synthesised from the available 2'-benzoyloxy-aβ-dihydro-3:4-dimethoxychalcone.13 The attempted preparation of 5:7:3':4'-tetrahydroxy-6:8-dimethylflavan by Simonis synthesis from CC-dimethylphloroglucinol 14 and caffeoyl chloride dibenzoate, followed by Clemmensen reduction, afforded crystals of the flavan which immediately resiniﬁed on exposure to air. Similar difficulty in the isolation of certain flavans has already been described.15
**General Procedure.**—Solutions were concentrated in nitrogen under reduced pressure at $<35^\circ$. Ferric chloride colours refer to reaction in ethanol.

**Manometry.**—Use was made of Haldane's constant-pressure respirometer, in which the reaction vessel was connected by small-bore pressure tubing to a gas-burette, and to a constant-pressure manometer, the other limb of which was connected to a compensation vessel. n-Butyl phthalate was used in the manometer and in the gas-burette. Both vessels, immersed in a thermostat at $35^\circ$, were shaken at a speed sufficient to maintain the reaction mixture as a foam. A small tube containing the flavan, and attached to a glass fibre, was placed upright in the reaction vessel, surrounded by phosphate buffer. In this way the system could be equilibrated before the reaction commenced by dislodging the tube and shaking the contents. A solution of 0.1 mmole of flavan in 10 ml of phosphate buffer (pH 8), or phosphate buffer–methanol (1:1) (10 ml) was used. The progress of the reaction was followed by direct reading of the volume of the system on the gas burette, the movable limb of which was first adjusted so as to bring the liquid in both arms of the constant-pressure manometer to the same level. When absorption of oxygen ceased, no flavan could be detected in the paper chromatogram of the reaction mixture. After acidification of the mixture, the polymer was adsorbed on a column ($10 \times 1.2$ cm.) of alumina, prepared in 2$n$-sulphuric acid. Potassium iodide (1 g.) was added to the sulphuric acid eluate which was set aside ($15$ min.) in the dark, and the liberated iodine was estimated with 0.01N-sodium thiosulphate. In another experiment, the oxidising agent was shown to be hydrogen peroxide, as the distillate gave a positive reaction with titanic sulphate and was indistinguishable from distilled water between 300—700 mg.

**Spectrophotometric Experiments and Analytical Samples.**—A solution of the flavan (1 mmole) in phosphate buffer (pH 8; 100 ml) or its equivalent of phosphate buffer–methanol (1:1) was shaken with oxygen until no more was absorbed. Samples were then withdrawn for spectrophotometric examination of the polymer, the solution being diluted where required with more phosphate buffer. For the isolation of the pure polymer phosphate ion was removed by introducing the reaction mixture into the middle compartment of an electrodialysis cell, constructed from "Pyrex" pipe-line, and fitted with a "Permaplex" A-10 anion-exchange membrane, and a "Permaplex" C-10 cation-exchange membrane. The cell was supplied with d.c. at 100 v until the resistance approached a limit (ca. 10 hr.).; the solution was then free from phosphate ion (molybdenum-blue test). Removal of phosphate ion caused precipitation of the polymer which was removed in the centrifuge, and dried ($3$ days at 20$^\circ$/0.01 mm.). All the polymers were ashless on combustion.

**Vanillin reaction.**—A 10% (w/v) ethanolic solution of vanillin was mixed with 12$n$-hydrochloric acid ($2:1$ v/v) and the freshly prepared reagent was employed at room temperature.

**5 : 7-Dihydroxyflavan.**—This had m. p. 196° (Found: C, 74.2; H, 5.9. Calc. for $C_{15}H_{12}O_6$: C, 74.4; H, 5.8%). When prepared by the method of Robertson, Whalley, and Yates.

**5 : 7-Di-O-methyl-(-)-catechin.**—Aqueous 4$n$-sodium metaborate (10 ml), methyl sulphate (2 ml), and 2$n$-n-sodium hydroxide (8 ml) were shaken with an ethanolic solution of (-)-catechin (2.9 g) for 15 min.; the reaction mixture was then poured into ice-water and extracted with ether. The acidified aqueous phase was extracted with ether ($4 \times 100$ ml), and the ether phase was washed free from mineral acid and dried. Evaporation of the solvent (MgSO$_4$) left 5 : 7-di-O-methyl-(-)-catechin (2.6 g), forming needles (from aqueous ethanol), m. p. 218—219°, $[\alpha]$_D$^219 = -1.0$ (c, 1.1 in acetone; l, 1){(+)-catechin, $[\alpha]$_D$^219 = +12$ (c, 1.7); 5 : 7 : 3' : 4'-tetra-O-methyl-(-)-catechin $[\alpha]$_D$^219 = -2$ (c, 1.0) (Found: C, 64.0; H, 5.6; OMe, 19.1%). 5 : 7-Di-O-methyl(-)-catechin gave a green ferric reaction, and a positive vanillin reaction.

**5 : 7-Dihydroxy-3' : 4'-dimethoxyflavan.**—5 : 7-Dihydroxy-3' : 4'-dimethoxyflavanone, m. p. 202° (Found: C, 64.9; H, 5.2; OMe, 19.7. Calc. for $C_{14}H_{14}O_5$: C, 64.6; H, 5.1; OMe, 19.5%), was prepared in 25% yield by an improvement of Shinoda and Sato's method. Reduction of the flavanone by Robertson, Whalley, and Yates's method gave 5 : 7-dihydroxy-3' : 4'-dimethoxyflavan, nodules (from xylene), m. p. 260° (Found: C, 67.8; H, 5.9. $C_{14}H_{14}O_5$ requires C, 67.5; H, 5.9%), which gave a positive vanillin reaction but no ferric reaction.

**5 : 7 : 3' : 4'-Tetramethoxyflavan.**—Eriodictyol, m. p. 267° (Found: C, 62.0; H, 4.0. Calc. for $C_{14}H_{14}O_5$: C, 62.5; H, 4.2%). was prepared in 20% yield (cf. Shinoda and Sato) by the method used for its 3' : 4'-dimethyl ether. Reduction of eriodictyol by the method used for the 3' : 4'-dimethyl ether gave the flavan which formed felted needles, m. p. 185° (from
water) (Found: C, 62.1; H, 5.4. C₁₅H₁₄O₅ requires C, 61.8; H, 5.5%). which were dried (P₂O₅) at 70°/0.1 mm. for 3 hr., and gave a green ferric reaction and a vanillin reaction.

3':4'-Dihydroxyflavan (with Dr. W. E. Elstow).—A mixture of an ethanolic solution (60 ml.) of 3':4'-dihydroxyacetophenone 6 (19 g.) and salicylaldehyde (16 g.) and 40% (w/v) potassium hydroxide solution (150 ml.) was kept under nitrogen for 1 week. 3':4':2'-Trihydroxy chalcone formed brown needles (30 g.) (from aqueous ethanol), m. p. 189° (Found: C, 70.3; H, 4.8. C₁₅H₁₂O₄ requires C, 70.3; H, 4.7%). An ethanolic solution of the chalcone (25.6 g.) was hydrogenated at 20°/l atm. in the presence of Adams’s platinic oxide catalyst (200 mg.), until hydrogen absorption (Found: 2.35 l.; Calc.: 2.30 l.) was complete. Evaporation of the filtrate left crude a(3-dihydro-3':4':2'-trihydroxychalcone which was benzoylated by the Schotten–Baumann method. 3':4':2'-Tribenzoyloxy-a(3-dihydro-3':4'-2'-trihydroxychalcone formed needles (55 g.) (from ethanol), m. p. 113° (Found: C, 75.6; H, 4.7%. C₃₆H₂₆O₇ requires C, 75.8; H, 4.6%). Lithium aluminium hydride reduction of the dihydrochalcone (5.7 g.) gave 3':4'-dihydroxyflavan which formed needles (2.6 g.) (from light petroleum [b. p. 60—80°]), m. p. 132° (Found: C, 74.4; H, 5.9. C₁₅H₁₄O₃ requires C, 74.4; H, 5.8%). The flavan gave a green ferric reaction.

3':4'-Dimethoxyflavan.—Treatment at 0° of an ethereal solution of 3':4'-dihydroxyflavan (2.6 g.) with ethereal diazomethane (ca. 2.8 g.) gave 3':4'-dimethoxyflavan as thick lamellae (2.6 g.), m. p. 99—100° (Found: C, 75.9; H, 6.5. C₁₇H₁₈O₃ requires C, 75.7; H, 6.7%), undepressed by another specimen, m. p. 99—100°, prepared by lithium aluminium hydride reduction of the corresponding 2'-benzoyloxy-a(3-dihydro-3':4'-dimethoxychalcone.13

The authors thank Dr. H. Phillips for his interest, and the Council of the British Leather Manufacturers’ Research Association for permission to publish this paper. They are also indebted to Professor R. D. Haworth, F.R.S., for the infrared spectrum of the polymer from catechin.

British Leather Manufacturers’ Research Association,
Milton Park, Egham, Surrey.

[Received, October 8th, 1956.]

2 Freudenberg and Weinges, Annalen, 1955, 590, 140.
3 James, Snell, and Weissberger, J. Amer. Chem. Soc., 1938, 60, 2984.
4 Du'Lock and Harley-Mason, J., 1951, 709.
6 Freudenberg and Maitland, Annalen, 1934, 510, 193; Collegium, 1934, 776, 656.
7 Mayer and Bauni, Das Leder, 1931, 7, 55; Schmidt and Mayer, Angew. Chem., 1956, 68, 103.
9 Freudenberg and Ahlhaus, Monatsh., 1956, 87, 1.
(b) 1928, 49, No. 563, 5; (c) 1931, 51, 78; Shinoeda and Kamagoye, 1928, 48, No. 560, 119; Shinoeda, Kamagoye, and Sato, 1928, 49, No. 571, 124; Fujise and Mitui, Bull. Chem. Soc. Japan, 1934, 7, 24; Huzise and Tatsita, Ber., 1941, 74, 275.
13 Robertson, Annalen, 1925, 441, 179.
14 Robertson and Whalley, J., 1951, 3355.
17 Lovering and Smith, Chem. and Ind., 1946, 24, 298.
Enzymic Oxidation of Catechin to a Polymer Structurally Related to some Phlobatannins

BY D. E. HATHWAY AND J. W. T. SEAKINS

The British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey

(Received 15 January 1957)

The formation of polymers and hydrogen peroxide during the autoxidation at 35° and pH 6-8 of catechin and of related 3:4'-dihydroxyflavans has been studied by measurement of oxygen uptake, and by the elementary analyses, absorption spectra and reactions of the dialysed polymers (Hathway & Seakins, 1957a). The evidence obtained, together with that provided by the spectroscopic study of intermediates produced by silver oxide oxidation (Hathway & Seakins, 1955), supports the quinone polymerization mechanism for catechin autoxidation. Whereas oxidative coupling of 5,7-di-O-methylcatechin resembles that of catechol, quinone polymerization of catechin and of 5:7:3':4'-tetrahydroxyflvan involves the phloroglucinol residue.

The mechanism for the autooxidation of catechin leading to a recognizable polymer has now been reported (Hathway & Seakins, 1957a), but as there was little information relating to the enzymic oxidation of catechin and no information concerning the product (Roberts & Wood, 1950), the aerobic oxidation of catechin to a polymer precisely similar to the product (Roberts & Wood, 1952). The plants with which this work is concerned are valued for their phlobatannin extractives.

EXPERIMENTAL

General. Evaporations were carried out in N₂ under reduced pressure at <35°. Paper chromatography was carried out at 23 ± 2° in all-glass apparatus. Chromatograms were dried at room temperature, unless otherwise stated. A Hanovia mercury-arc lamp fitted with a Wood's-glass filter was used to examine chromatograms for fluorescent zones. Hide-powder tests were made by the method of Grassmann, Endisch & Kuntara (1951).

Acetone-dried powders of Psalliota campestris and Nicotiana tabacum. Freshly picked mushrooms (350 g.) were immediately washed five times with 500 ml. portions of ice-cold acetone, and dried at 0° in vacuo. This powder (2-8 enzyme units) had a polyphenoloxidase activity of purpurogallin number 0-14. The purpurogallin number (p.n.) was determined by the method of Keilin & Mann (1938) but purpurogallin was estimated in ethanol solution at 375 and 430 mμ by means of a standard u.v.-light spectrophotometer. One enzyme unit (e.u.) corresponds to the quantity of enzyme which produces 1 g. of purpurogallin in 5 min. at 20°.

The acetone-dried powder of Psalliota campestris was used without further treatment for enzyme-catalysed oxidations, whereas the acetone-dried powder of Nicotiana tabacum, p.n. 0-0024, was first eluted with ice-water (10 ml./200 mg.) until free from amino acids.

Potato juice. A chilled potato was minced and pulverized, and the juice expressed was filtered, chilled, and centrifuged to remove starch (Raper & Wormall, 1925). Freshly prepared juice had polyphenoloxidase activity of p.n. 0-0022, and was used immediately or after preliminary dialysis in Visking 18/32 seamless cellulose tubing.

Manometry. Use was made of Haldane's (1921) constant-pressure respirometer, in which the reaction vessel was connected by small-bore pressure tubing to a gas burette and to a constant-pressure manometer, the other limb of which was connected to a compensation vessel. Both the reaction and the compensation vessels were shaken at 20° at a speed sufficient to maintain the reaction mixture as a foam. n-Butyl phthalate was used in the constant-pressure manometer and gas-burette.

Isolation of tannins from Acacia catechu and Uncaria gambir. A 5% (w/v) aqueous solution (400 ml.) of extract (containing 43% of the total solids as tannin, by hide-powder test) prepared from Acacia catechu heartwood was
extracted for 2 days with ether and then for 5 days with ethyl acetate. Progress of solvent extraction was followed by means of two-dimensional paper chromatography. For this purpose, samples (6 µl) of the test solutions were spotted at a distance of 2 cm from both edges of the lower left-hand corner of Whatman no. 1 filter papers, 25-5 cm. square, and chromatographed by the ascending method with x-acetic acid as first-way solvent and x-butanol-acetic acid-water (6:1:2, by vol.) as second-way solvent.

Extraction with ether was continued until catechin, Bp 0-54, 0-74, and epicatechin, Bp 0-49, 0-62, had been removed. The ether residue (6 g.) constituted 45% (by wt.) of the whole extract. In addition to catechin epimers this fraction contained flavonoids and substances of unknown composition. Extraction with ethyl acetate was similarly continued until other substances of unknown composition had been removed. The ethyl acetate residue (4-6 g.) constituted 23% (by wt.) of the original extract. The residual aqueous solution contained 58% of the total tannin (determined by hide-powder test), and paper chromatography showed a single phenolic component at the origin. This tannin was precipitated from dilute solution by 1% (w/v) gelatin reagent, or when refluxed with a mixture of formalin (40% formaldehyde) and 12% HCl. In order to recover an analytical sample of this tannin from the residual aqueous solution, deionization was first carried out by electrodialysis. The aqueous solution was accordingly transferred to the middle compartment of a cell, constructed from Pyrex pipe-line components (Lovering & Smith, 1946), but fitted with a Permaplex A-10 anion-exchange membrane (The Permutit Co. Ltd., London, W. 4) and a Permaplex C-10 cation-exchange membrane. The apparatus was supplied with 100 v.d.c. until after 30 hr. the resistance approached a limiting value. Precipitation of tannin occurred deionization. The supernatant was centrifuged off, and the sediment was washed with five successive 50 ml. vol. of water, and centrifuged after each addition and the supernatants were discarded. The phlobatannin, which was recovered after deionization and elution as described above, was dried at 70° in vacuo, and did not give the ferric and vanillin reactions.

A 5% (w/v) aqueous solution (400 ml.) of extract (containing 40% of the total solids as tannin, by hide-powder test), derived from Uncaria gambir leaves by water percolation, was similarly extracted with solvents. The ether residue (9 g.) and the ethyl acetate residue (2-5 g.) constituted 45 and 12-5% (by wt.) respectively of the original extract. Catechin (8-6 g.) crystallized from a solution of formalin (40% formaldehyde) and pH 1.2-HC1. In order to recover an analytical sample of this catechin from the residual aqueous solution, deionization was first carried out by electrodialysis. The aqueous solution was accordingly transferred to the middle compartment of a cell, constructed from Pyrex pipe-line components (Lovering & Smith, 1946), but fitted with a Permaplex A-10 anion-exchange membrane (The Permutit Co. Ltd., London, W. 4) and a Permaplex C-10 cation-exchange membrane. The apparatus was supplied with 100 v.d.c. until after 30 hr. the resistance approached a limiting value. Precipitation of catechin occurred deionization. The supernatant was centrifuged off, and the sediment was washed with five successive 50 ml. vol. of water, and centrifuged after each addition and the supernatants were discarded. The phlobatannin, which was recovered after deionization and elution as described above, was dried at 70° in vacuo, and did not give the ferric and vanillin reactions.

5-Methoxy-4:-methylresorcinol. A specimen, m.p. 120° (Found: C, 62-1; H, 6-7. Calc. for C8H9O2: C, 62-4; H, 6-5%), was prepared by the method of Robertson & Whalley (1951).

Separation of the flavonoids from Acacia catechu by paper chromatography. Spots (10 µl) of a 2% (w/v) solution of the ether residue in ethanol were applied to base lines, 4 cm. from the lower edge of five sheets of Whatman no. 3 filter paper 57 cm. long. Single-way ascending chromatography was effected with x-acetic acid. After the papers had been irrigated for 4 hr. they were dried and given a second development with x-acetic acid to ensure complete migration of the catechins. Transverse zones, containing the flavonoids, were then located in u.v. light, cut out, and extracted with boiling ethanol. Spots (5 µl) of a 0-5% solution of the ethanol residue (3 mg.) and marker spots (5 µl) of 0-5% solutions of fisetin, myricetin, quercetagetin, quercetin and robinetin respectively were applied to Whatman no. 1 filter papers, which were irrigated for 24 hr. by the ascending method with the following solvent systems: m-cresol–acetic acid–water (25:1:24; by vol.; upper phase) (Bate-Smith, 1949), and butan-2-ol-acetic acid–water (14:1:5, by vol.). Papers which had been chromatographed with the solvent system containing m-cresol were dried at 60°. Individual flavonoids were detected by their fluorescence in u.v. light.

Isolation of quercetagetin from Tagetes erecta flowers. The occurrence of quercetagetin in Tagetes erecta flowers was observed by Mahal (1938), and the flavonol was therefore isolated from this source by the following simple procedure. Petals (40 g.) from the orange flowers were disintegrated in a top-drive macerator in 250 ml. of 95% ethanol, and the homogenate obtained was refluxed for 1 hr. The filtrate was concentrated to 20 ml. and refluxed with 100 ml. of 2% H2SO4 for 2 hr., when a trace of insoluble material was removed. When residual solvent was evaporated from the aqueous phase, precipitation occurred, and the solid recovered was extracted consecutively with light petroleum (b.p. 40–60°, 800 ml.) and boiling ether (500 ml.). The petroleum extract was discarded. Dropwise addition of water to a solution of the ether residue (350 mg.) in 10 ml. of ethanol deposited traces of tar, which were removed. Further dilution of the filtrate precipitated quercetagetin, which was twice crystallized from ethanol, forming yellow needles (50 mg.), m.p. 310° (decomp.), λmax 259 (log e 4-28); 362 (log e 4-31) µm; inflexion at 372 (log e 4-15) µm. (Found: C, 56-9; H, 3-4. Calc. for C16H14O3: C, 56-6; H, 3-2%).

RESULTS

The present work consists of an exploratory study of the enzymic oxidation of catechin to a polymer, precisely similar to a polymer produced by autoxidation (Hathway & Seakins, 1957a) and comparable with phlobatannins which have now been isolated in high yield from amongst the tannin extractives of two plants (Hathway & Seakins, 1957b).

Crude enzyme preparations were used, since in many cases the polyphenoloxidase activity has been found to be identical with that for the more purified enzyme (Raper, 1932), and our early results also showed that greater difficulty in measuring the initial rate of oxidation was encountered with purified than with crude enzyme preparations.

During the aerobic oxidation of catechin by mushroom polyphenoloxidase at 20° and pH 8, the rate of O2 uptake diminished rapidly from the time
the reaction commenced (Fig. 1). The oxidation stopped before two equivalents of \( \text{O}_2 \) had been taken up, unless a relatively large quantity of enzyme was initially present. Maximum \( \text{O}_2 \) uptake with the lowest enzyme concentration represented incomplete oxidation of catechin; and a second addition of enzyme resulted in an immediate acceleration of \( \text{O}_2 \) uptake (Fig. 1). Owing to the rapid inactivation of the enzyme during the reaction, it was difficult to obtain reliable measurements for the initial rate of oxidation, particularly if purified enzyme was used, and this experience is in agreement with the results obtained by earlier workers on the oxidation of catechol by mushroom polyphenoloxidase. To overcome this difficulty, Graubard & Nelson (1935) used the \( \text{O}_2 \) uptake at the end of 1 hr. as a measure of polyphenoloxidase activity, and the activity of the enzyme thus measured agreed with its activity similarly measured with catechol as substrate. Alternatively, by measuring \( \text{O}_2 \) uptake at 1 min.

---

**Fig. 1.** Progress curves for the oxidation of catechin at 20° by mushroom polyphenoloxidase. In addition to 20 ml. of 0-07M-phosphate buffer, pH 8, and 0-2 m-mole of catechin (○) the reaction flask contained 16 (△), 8 (△) and two separate additions of 4 x 10^-3 e.u. (□). A compensatory flask contained phosphate buffer.

**Fig. 2.** Relationship between rate of \( \text{O}_2 \) uptake and enzyme concentration (mushroom enzyme).

**Fig. 3.** Progress curves for the oxidation of catechin at 20° by potato polyphenoloxidase. Reaction flask contained 20 ml. of 0-07M-phosphate buffer, pH 8, 0-2 m-mole of catechin and 8 (○) or 4 x 10^-3 e.u. (△). A compensatory flask contained phosphate buffer.

**Fig. 4.** Progress curves for the oxidation of catechin at 35° by tobacco polyphenoloxidase. Reaction flask contained 20 ml. of 0-07M-phosphate buffer, pH 6, 0-2 m-mole of catechin and 2 (○), 0-7 (△) and 0-3 x 10^-3 e.u. (□). A compensatory flask contained phosphate buffer.
intervals for the first 2 or 3 min. (Keilin & Mann, 1938) and plotting the initial rate of oxidation observed against enzyme concentration, the usual linear relationship resulted (Fig. 2). The oxidation of catechin by potato polyphenoloxidase under identical temperature and pH conditions was also studied (Fig. 3), but the rate of enzyme inactivation was higher. The progress curves obtained for oxidation by tobacco polyphenoloxidase (Fig. 4) were similar to those for oxidation by mushroom enzyme, and O₂ uptake ceased before oxidation was complete unless a large amount of enzyme was present. Under the different optimum conditions of temperature (35°) and pH (6) for tobacco polyphenoloxidase (Reid, 1956), O₂ uptake at the end of 1 hr. was greater, at comparable levels of activity, for this enzyme than for the other enzymes studied.

The autoxidation and polyphenoloxidase oxidation of catechin gave polymers which had the same elementary analysis, remained at the origin in two-dimensional paper chromatography, were retained by hide-powder, and were precipitated by gelatin or on refluxing with a formalin-HCl mixture. The absorption spectra exhibited bands at 270 and 310 my and a shoulder at 500 my, but the shoulder at 500 my was slightly less intense with the autoxidation polymer (Fig. 5). The intensity of this shoulder decreases during the course of autoxidation, and this may be due to slight oxidative degradation by hydrogen peroxide, which is not formed during enzymic oxidation (cf. Beer, Broadhurst & Robertson, 1954). Figs. 5-7 were drawn from measurements made at 5 mmy intervals. Oxidation of catechin by hydrogen peroxide and horse-radish peroxidase gave a different polymer, which exhibited a single absorption band at 350 mmy.

Percolation of *Acacia catechu* heartwood and freshly harvested *Uncaria gambir* leaves with water gave extracts, the residues of which contained 40% of tannin; precautions were taken to prevent autoxidation during extraction. The residual aqueous solutions were examined after exhaustive solvent extraction, and found to contain approximately 50% of the total tannin. Two-dimensional paper chromatograms in both cases revealed a single phenolic component at the origin. Electrodialysis of the residual solutions, in a cell fitted with ion-exchange membranes, precipitated the tannins, which were separated in the centrifuge after elution. Controlled drying furnished analytical specimens with elementary analyses (Table 1) similar to those for the polymer of catechin oxidation, of empirical formula C₁₅H₁₀O₆,3H₂O. The i.r. spectra provided evidence for hydration, for even after protracted drying at 70° in vacuo a strong band persisted at 1627-1635 cm⁻¹. The absorption spectra of these phlobatannins at pH 8 (Fig. 6) were similar to those of the polymers of catechin oxidation, exhibiting maxima at 270, 410 and 500 mmy. These substances are retained by hide-powder, they do not give the ferric and vanillin reactions and they are precipitated by gelatin or on boiling with a formalin-HCl mixture.

The aerobic oxidation of an equimolecular mixture of homocatechol and phloroglucinol by mushroom polyphenoloxidase gave a polymer which showed absorption at 460 mmy (Fig. 7), in contrast to the single absorption band at 250 mmy of catechol autoxidation polymer (Hathway & Seakins 1957a). homocatechol and 5-methoxy-4-methylresorcinol (Robertson & Whalley, 1951) substrates afforded a polymer which showed a strong absorption maximum at 480 mmy.

A flavonoid fraction derived from *Acacia catechu* heartwood was found to contain in addition to quercetin (Perkin, 1897) two flavonols, chromato graphically indistinguishable in two different

<table>
<thead>
<tr>
<th>Table 1. Phlobatannin analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><em>Acacia catechu</em> tannin</td>
</tr>
<tr>
<td>Catechin autoxidation polymer</td>
</tr>
<tr>
<td><em>Uncaria gambir</em> tannin</td>
</tr>
</tbody>
</table>
solvents (Bate-Smith, 1949) from fisetin and quercetagetin (Table 2). Authentic quercetagetin was isolated from *Tagetes erecta* flowers for the purpose of comparison.

**DISCUSSION**

A study of the course of aerobic oxidation of catechin by different plant polyphenoloxidases suggests that this substance functions as a substrate for these enzymes, which are widely distributed throughout plants. Both those polyphenoloxidases which are associated with the mitochondria, such as mushroom enzyme (Mason, 1955), and those which are not localized within the cell, such as tobacco enzyme (McClendon, 1953), have a common pattern of behaviour.

The products of polyphenoloxidase oxidation have been shown to be precisely similar to the polymer of catechin autoxidation which was formed by the quinone-polymerization mechanism. Additional evidence for the head-to-tail polymerization of catechin was also obtained from the aerobic oxidation of a mixture of *homocatechol* and 5-methoxy-4-methylresorcinol by polyphenoloxidase to a polymer which showed the characteristic absorption of the polymers of catechin oxidation. 4-Methyl-o-benzoquinone and 5-methoxy-4-methylresorcinol therefore undergo oxidative coupling.

Aerobic oxidation of catechin by plant polyphenoloxidases proceeds by a quinone-polymerization mechanism and affords a product, the head-to-tail polymer units of which account for the profound difference in spectrum of the polymers from those of catechol polymers (Hathway & Seakins, 1957a). The fact that 4:5-dimethylcatechol does not undergo oxidative coupling with 5-methoxy-4-methylresorcinol therefore undergoing oxidative coupling.

**Table 2. Chromatographic behaviour of flavonols**

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fisetin</td>
<td>0.72 (0.75)</td>
<td>0.72 (0.75)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.72 (0.75)</td>
<td>0.52 (0.83)</td>
</tr>
<tr>
<td>Robinetin</td>
<td>0 (0.41)</td>
<td>0 (0.41)</td>
</tr>
<tr>
<td>Quercetagetin</td>
<td>0.32 (0.57)</td>
<td>0.42 (0.44)</td>
</tr>
<tr>
<td>Myricetin</td>
<td>0 (0.09)</td>
<td>0 (0.09)</td>
</tr>
</tbody>
</table>

![Fig. 8](image)
resorcinol suggests that in these head-to-tail polymer units the 6'-position of one catechin-o-quinone residue is linked by a CC bond to the 6- or 8-position of another catechin-o-quinone residue. A partial type formula is shown in Fig. 8.

The polymer obtained by the enzymic oxidation of catechin has the analytical properties of a tannin (Gnann, 1949; Schmidt, 1955). The polymer is precipitated from solution by gelatin or on refluxing with formalin-hydrochloric acid mixture, and it is retained by hide-powder (Grassmann et al. 1951). The failure of this polymer to give the vanillin reaction (Procter & Paessler, 1901) provides further evidence that polymerization involves the phloroglucinol residue of the monomer. The work of the Heidelberg School for 20 years has maintained (Freudenberg, 1956; Freudenberg & Maitland, 1934a, b; Freudenberg & Weinges, 1955) that phlobatannins from the barks of Acacia mollissima, birch, chestnut (Castanea sativa), eucalypts, mangrove, oak, spruce and willow and from the heartwood of Acacia catechu and quebracho trees are catechins, polymerized post mortem by plant acids. Two mechanisms have been considered. According to the first (Freudenberg & Maitland, 1934a, b), ring fission gives a secondary benzyl alcohol, and carbon atom C_{86} then condenses either with carbon atom C_{86} or with C_{86} of another molecule to afford a bifunctional dimer, capable of further polymerization. More recently, Freudenberg (1956) has suggested that catechin may react as a pair of tautomeric diphenylpropenes which are involved in the first stage of a styrene polymerization (Freudenberg & Ahlhaus, 1956).

Phlobatannins have now been isolated in high yield from the tannin extractives of Uncaria gambir leaves and Acacia catechu heartwood, which are known to contain large quantities of catechin and its epimeride respectively. Since these substances have identical tannin properties, similar absorption spectra and elementary analyses to the polymer produced by polyphenoloxidase oxidation of catechin, a close structural relationship exists, and it is probable that these phlobatannins result from similar aerobic oxidation of catechin precursors. Formation of phlobatannins from catechin epimers by quinone polymerization recalls the similar formation of artificial melanins from 5,6-dihydroxyindoles (Beer et al. 1954; Bu’Lock & Harley-Mason, 1951), but it was not known whether the products resembled melanins of animal or plant origin (A. Robertson, personal communication). The fact that these new phlobatannins may be accounted for by catechin polymerization is in agreement with Freudenberg & Maitland’s (1934a, b) suggestion, but no evidence has been found for the acid-catalysed reaction, which requires low pH (<2) and high temperature (>50°C). The remaining phlobatannins from Acacia catechu and Uncaria gambir were shown by two-dimensional paper chromatography to contain mixtures of mobile substances. The absorption spectra of the crude mixtures were similar to those of the catechin oxidation polymers, but solubility in solvents and mobility in chromatographic-solvent systems implied a lower degree of polymerization.

Unlike the Acacia catechu and Uncaria gambir phlobatannins, a phlobatanin was isolated during the course of this work from a commercial extract derived from Acacia mollissima bark, which gave a vanillin reaction, a strong-violet ferric reaction, and a different absorption spectrum. It is therefore inadmissible to regard the phlobatannins at present as members of a closely related chemical family, but the presence of catechin derivatives in the cacao bean (Forsyth, 1952a) and tea leaf (Roberts, 1952), and of leucoanthocyanins in the bark of various eucalypts (Hillis, 1954, 1956) may indicate that the phlobatannins arise through quinone polymerization.

The present conclusion that enzymic formation of Uncaria gambir and Acacia catechu phlobatannins occurs in the detached leaves and heartwood respectively connects the formation of these compounds with the plant-browning reaction (Szent-Györgi & Víctorisz, 1931).

**SUMMARY**

1. Catechin was oxidized aerobically by mushroom, potato and tobacco polyphenoloxidases.

2. Catechin autoxidation and polyphenoloxidase oxidation polymers analyse as C_{15}H_{10}O_{6.3}H_{2}O, exhibit absorption bands at 270 and 410 μm, and a shoulder at 500 μm, and have identical analytical properties.

3. Aerobic oxidation of mixed synthetic substrates, such as homocatechol and 5-methoxy-4-methylresorcinol by polyphenoloxidase, gave a polymer showing the characteristic absorption of the polymers of catechin oxidation.

4. Aerobic oxidation of catechin by polyphenoloxidase therefore involves quinone polymerization.

5. Phlobatannins have been isolated in high yield from the extractives of harvested Uncaria gambir leaves and Acacia catechu heartwood, which have the same analytical properties as and similar elementary analyses and absorption spectra to the polymers of catechin oxidation.

6. The conclusion is drawn that these phlobatannins are formed by aerobic oxidation of catechin epimers by polyphenoloxidases in the detached leaves and heartwood respectively.

7. A flavonoid fraction from A. catechu contained three flavonols, chromatographically indistinguishable from fisetin, quercetatin and quercetin.
The authors wish to thank Dr H. Phillips for his interest, and the Council of the British Leather Manufacturers' Research Association for their permission to publish this paper. *Acacia catechu* heartwood and harvested leaves of *Uncaria gambir* were obtained through the good offices of the State Silviculturist, Ootacamund Post, The Nilgiris, Madras State, India. We are also indebted to Dr E. C. Bate-Smith, Low Temperature Research Station, Cambridge, for a specimen of fisetin, to Professor D. Keilin, F.R.S., The Molteno Institute of Biology and Parasitology, Cambridge, for purified horse-radish peroxidase, and to Mr W. W. Reid, F.R.I.C., of Carreras Ltd., London, N.W. 1, for the acetone-dried powder of *Nicotiana tabacum*.

REFERENCES


