" STUDIES ON TANNINS IN PLANTS "

by

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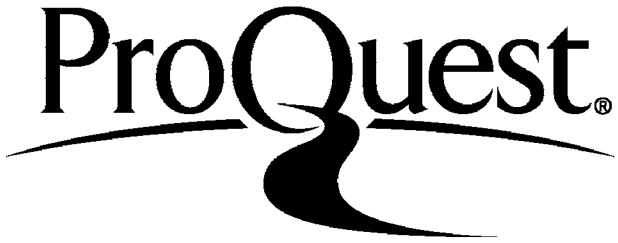
A thesis presented for the degree of M.Sc. in
the University of London



Abstract

Tannins have been defined as phenolic hydroxyl compounds of relatively high molecular weight which precipitate proteins. Chemically, they can be divided into two classes (hydrolysable and condensed tannins), depending on their reaction with hot mineral acid. The structure of both classes has been discussed and a number of theories enumerated regarding possible linkages involved in the polymerisation of condensed tannins. The biosynthesis of tannins has been discussed.

The widespread distribution of tannins in the plant kingdom and the simultaneous occurrence of leuco-anthocyanins and "botanical tannins" is mentioned. The loss of both astringency and tannins in fruits on ripening is discussed. The effect of mineral nutrition, light and season on the concentration of tannins in plants is referred to.

Chemical and physical methods for the analysis of tannins have been discussed, and five analytical methods have been investigated for characterising the phenolic nuclei and applied to model compounds. These methods have been extended and used to study the changes in tannins in bananas, peaches and plums on ripening, with particular reference to the changes in the leuco-anthocyanins. The results with the banana confirm the hypothesis of Swain (1956) that the reduction in astringency on ripening is due to polymerisation of the tannins; somewhat ambiguous results were obtained with the juicy fruits.

In order to develop a different method for measuring astringency, the inhibition of enzymes by tannins was investigated. A number of reagents were shown which could reverse this inhibition, and the use of some of these reactivating agents, both for extracting tannins and for protecting enzymes from inhibition, is suggested.

Lastly, the synthesis of leuco-anthocyanins in sycamore tissue culture was studied, and it was found that both restricted aeration and "strain" differences affect the production of these compounds.

PREFACE

The work for this thesis was carried out by the author at the Low Temperature Research Station under the supervision of Dr. T. Swain. She would like to express her gratitude to him for his help and for access to his unpublished work.

She is indebted to Dr. E.C. Bate-Smith and the Agricultural Research Council for permission to submit this thesis.

She would also like to thank Dr. D.H. Northcote and Mr. K.H. Tjhio for advice on tissue culture; Mr. A.S. Mead for help with approximately one-fifth of the analysis in Chapter 4 and with the preparation of the photo-micrographs in Chapter 6; Dr. F.A. Isherwood for lignin analysis; and various chemical firms listed in Chapter 5 for gifts of chemicals.

This thesis is not substantially the same as one which has been submitted to any other university.

Judith L. goldstein.

Judith L. Goldstein

Cambridge, July 1962.

Chapter 1.

The chemistry of tannins.

Chapter 1

"The term tannin has been employed by different writers, sometimes to denote a particular substance better described as tannic acid, sometimes as a collective term for a whole group of substances having certain characteristics."

Haas & Hill (1928)

1. INTRODUCTION

There is a great deal of confusion in the botanical literature about the term tannin. Thus Vines (1886) defined tannins as aromatic glucosides. Sorauer (1895) also commented that tannins are the same as glucosides and that these compounds "are split by acids and ferments to yield a sugar and an indifferent substance". Pfeiffer (1900) stated that the term tannin was a technical term which had no precise chemical or physiological meaning; for various other phenolic compounds give the same microchemical tests with iron salts and potassium bichromate, whereas others such as phloroglucinol, which have a similar physiological importance, do not. In their classical book on plant physiology, Mayer and Anderson (1939) define tannins as a heterogeneous group of complex compounds of common occurrence in plants. More recently Bonner and Galston (1952) have defined the term tannin as covering a wide range of naturally occurring compounds of varying chemical constitution scattered through the plant kingdom. They mention that tannins are aromatic compounds with numerous phenolic hydroxyl groups, astringent to the taste and capable of precipitating protein.

It can be seen from these quotations that, even today, little recognition has been accorded by botanists to the chemical work which has been carried out over the past 50 years on this

heterogeneous class of substances. The classical experiments of Emil Fischer from 1908 to 1918 on tannic acid stimulated his brilliant research student Freudenberg to make a study of these substances part of his life work, and in the last decade or so the chemical structures of several of these substances have been elucidated, and a sure foundation has been laid for a closer understanding of their role in plants. Before describing the recent work, we should return to the methods by which the presence of tannins has been recognised in plants since much of the ambiguity which has arisen comes from the lack of understanding of the chemical and physical bases of such tests.

Most workers have identified those compounds as tannins which give the following tests. Form a dark blue or green colour with neutral ferric chloride solution, and a red colour with solutions of potassium ferricyanide and ammonia; form precipitates when heated with solutions of gelatine, alkaloids, uranyl and lead acetates and lime water; and reduce Fehlings solution, alkaline potassium permanganate and potassium dichromate.

Although it is true that most tannins which have been isolated give most, if not all, of these tests, all tannins do not react to the same extent. More importantly a number of compounds, which are not tannins at all but which are chemically related to them, give some of these tests. For example, the colorimetric tests, which are the most widely used,are given by many phenolic compounds of the flavone class, and Bate-Smith (1962) has shown such substances are especially widespread in the higher plants. The formation of precipitates by calcium, lead and uranyl salts is also non-specific, as is the susceptibility to oxidation with Fehlings solution and potassium permanganate and dichromate. In fact, the only specific tests for tannins are the formation of precipitates with gelatine and alkaloids. The formation of precipitates between tannins and gelatine parallels the behaviour of natural tannin extracts (oak bark and the like) to convert hides into leather. Many chemists have stressed this as an essential basis for defining tannins.

Thus Rottsieper, in a privately published review (1946), defined tannins as "organic substances which are capable of converting animal fibrous tissue into decay resisting leather". He then lists a number of common properties of different tannins which re-inforce this view and indicates the properties a molecule requires in order to be capable of such action. Thus tannins have a high molecular weight, giving rise to colloidal properties, and a great number of free phenolic hydroxyl groups which are mainly responsible for their solubility in water. He further states that tannins are astringent to the taste and as in older definitions give an intense colouration in the presence of iron salts, and are precipitated from aqueous solution by alkaloids. The Shorter Oxford Dictionary is in agreement with this definition. Tannins are defined as "any member of a group of astringent vegetable substances. The tannins possess the property of combining with and converting animal hide into leather". White (1958) also subscribes to this view and considers that only substances which have been actually shown to convert animal hide into leather can be considered as tannins; he considers that the references to tannins seen on microscope sections of plant tissue should be referred to only as phenols. This is probably going too far, since any simple phenolic compound would undoubtedly be washed out of the tissue during fixing and staining. This is exemplified by the work of Bate-Smith and Metcalf (1957) who found a good correlation between the presence of leuco-anthocyanins (vide infra) in fresh tissue of leaves and the so-called botanical tannins in the collection of slides which had been prepared for the illustrations in the book "Anatomy of the Dicotyledons" by Metcalf and Clark. They found that in over 90% of the cases where tannins of the leuco-anthocyanin type existed in the fresh tissue, the slide of the sectioned material showed a dark staining reaction. They attributed the stain to the fact that the material, which prior to sectioning had been fixed in formalin and cleared with acetic acid and ethanol, was sectioned using a steel microtome knife. As the fixative had been incompletely removed, the acetic acid presumably reacted with the microtome knife to form a ferric salt, which in turn reacted with the tannin to give either

a blue or green colour. As the sections had been treated with alcohol, only the true tanning material would have been left, presumably fixed by acid formalin.

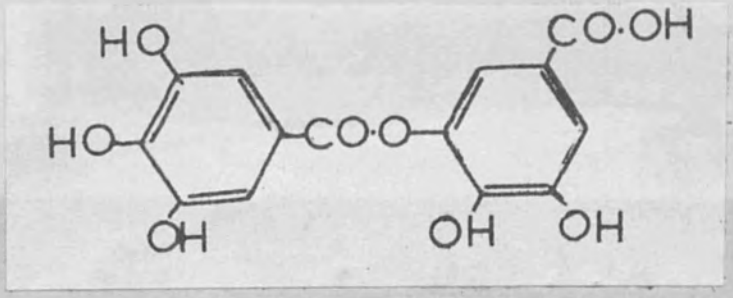
We may therefore define tannins as a class of polymeric phenolic substances which have the property of cross-linking protein in a way analogous to the tanning of leather. This definition means that the compounds would give all the usual chemical and physical reactions of the simpler phenols but would be distinguished from them by their high molecular weight, which imparts to them the property of being able to hydrogen bond to protein to yield stable, usually insoluble, co-polymers. Another property of the tannins which is closely allied to the tanning of protein is the astringency they impart to unripe fruit. Astringency is probably caused by the tannins cross-linking both the protein and the muco-polysaccharides of the tongue and the mouth, causing a puckery taste.

2. THE CHEMISTRY OF THE TANNINS

Chemically, the tannins may be divided into two broad classes on the basis of their reaction to hot dilute mineral acid. Freudenberg (1920) called these the hydrolysable tannins, which are split by such treatment to yield a sugar, usually glucose, and gallic acid or its congeners, and the condensed tannins, which on heating in acid are further polymerised or condensed to yield finally cream or red insoluble precipitates.

As stated previously, the hydrolysable tannins which are produced in oak galls were the first to be examined chemically. Scheele obtained gallic acid from them as early as 1786 and Proust (1798) isolated a specimen of oak gall tannin pure enough for him specifically to recognise it as a distinct entity and free from gallic acid. It was not until later, however, that it was recognised that glucose was also present. The first serious chemical work on these compounds was done by Emil Fischer starting in the 1900's and he proposed a structure for tannic acid, which has not been overthrown up to this day. Since Fischer's time, the work on these compounds has been mainly carried out by Freudenberg and his school at

Heidelberg, and this has been summarised by Mayer (1958) and Schmidt (1961). Most of the hydrolysable tannins which have been isolated in the crystalline state and whose structures have been determined are shown in Figure 1 which indicates the structure and relationship of one with another. In tannins, like tannic acid, gallic acid may be linked to itself to give depsides such as m-digallic acid (I).



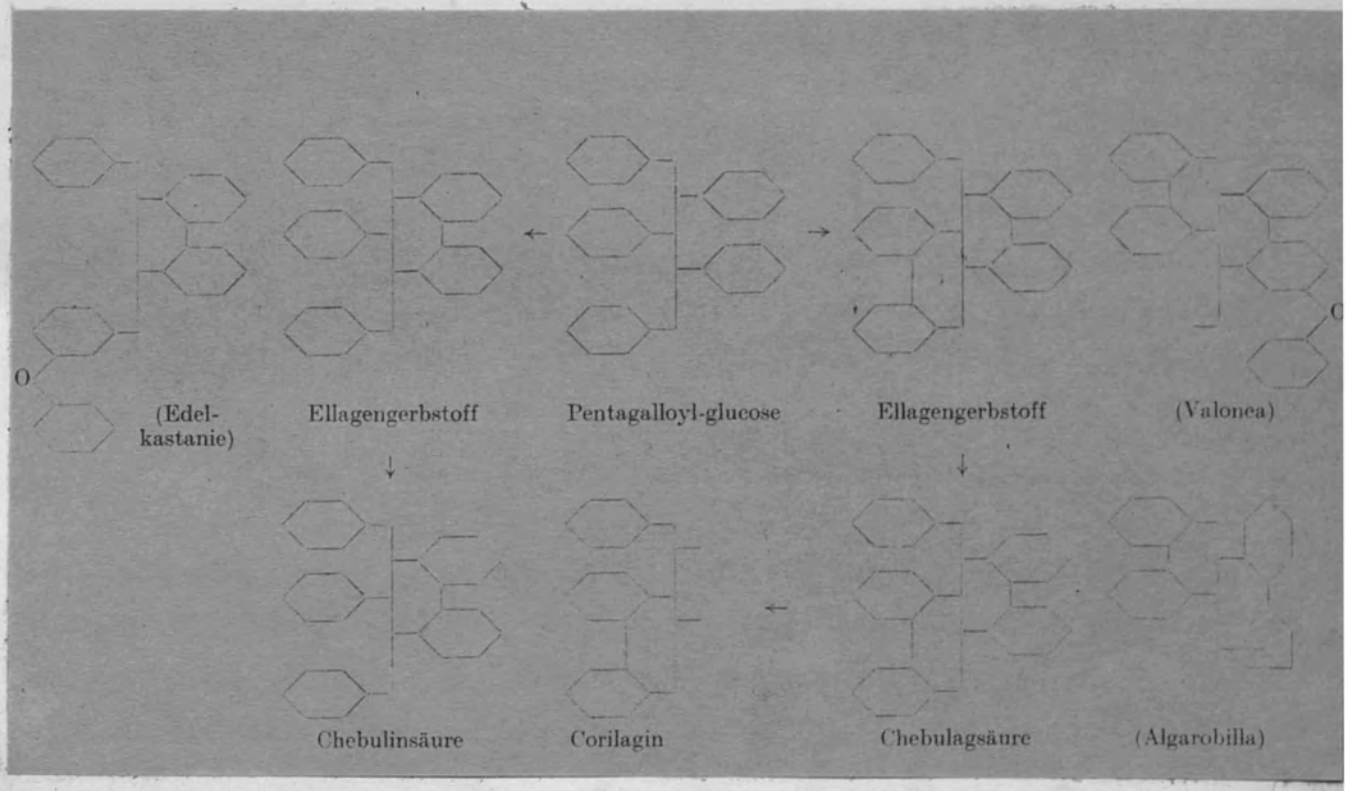
I

A depside is a compound formed by the esterification of a phenolic carboxylic acid with a phenol. The union is analogous to the formation of a peptide, one difference being that only the dimer or trimer is formed with the depside bond.

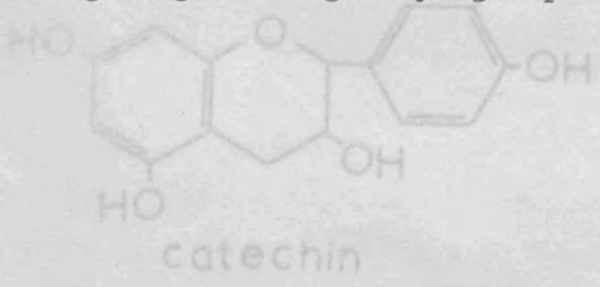
-R.CO.NH.R.- Peptide linkage
-R.CO.O.R.- Depside linkage

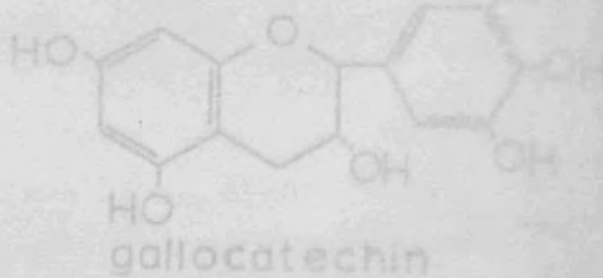
FIGURE 1[1]

The hydrolysable tannins, Figure taken from W. Mayer, (1958).

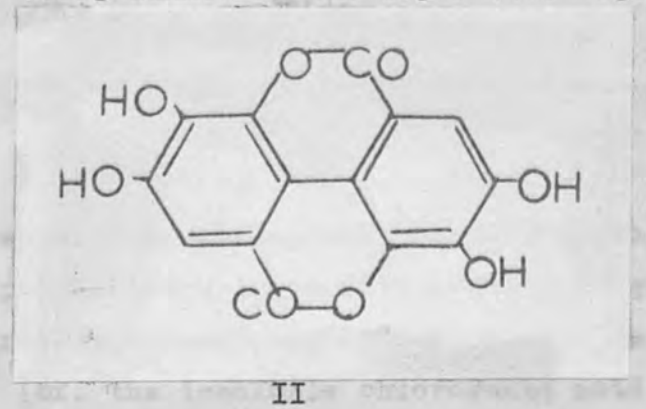


Rings represent galloyl groups





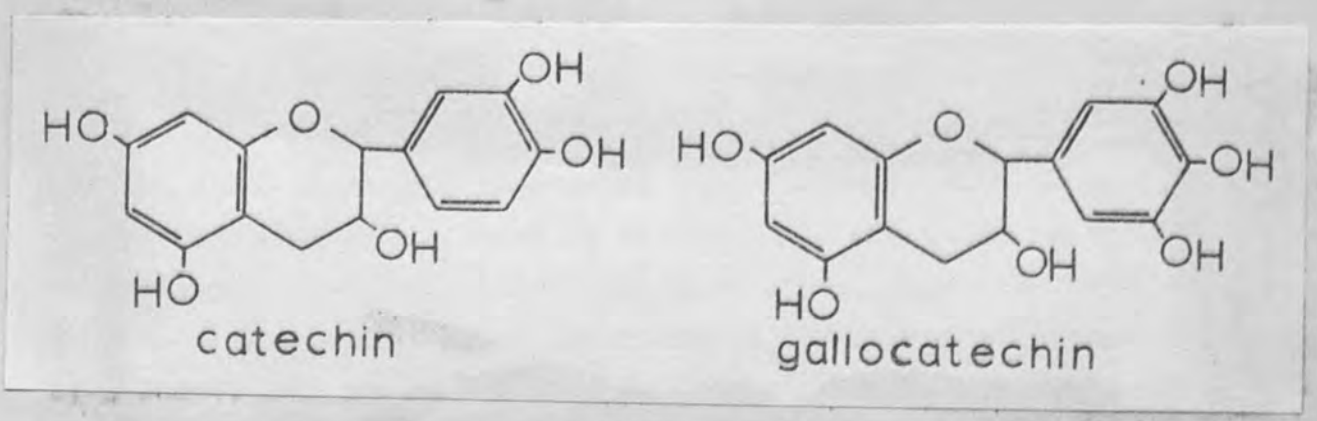
Ellagic acid (II) is an internal double lactone and is readily formed from gallic acid by oxidation which gives the C-C bond. In tannins, ellagic acid exists as the ring opened form, the carboxyl groups being linked to the sugar (see Fig. I ~~carrialagen~~ Corilagin).



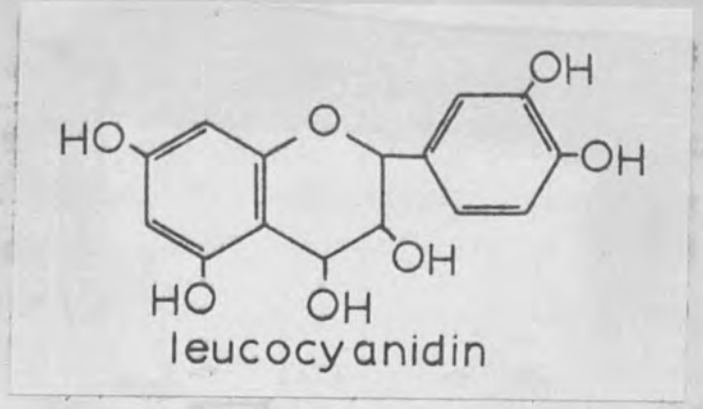
II

The hydrolysable tannins can be isolated from oak galls and other plant products and they are not as widely distributed as the condensed tannins. As they are distinct chemical entities, it seems unlikely that once formed they change during growth of the plant. Of course they are susceptible to oxidation which may lead to polymerisation, but the nature of the products of such reactions have not been studied and, in any case, probably are not formed under normal physiological conditions.

The condensed tannins are recognised by the fact that they form precipitates, the phlobaphenes, when heated with hot dilute mineral acid. The simple (that is monomeric) flavan-3-ols (the catechins III) and the flavan-3, 4-diols (the leuco-anthocyanins IV) also give precipitates under these conditions.



III

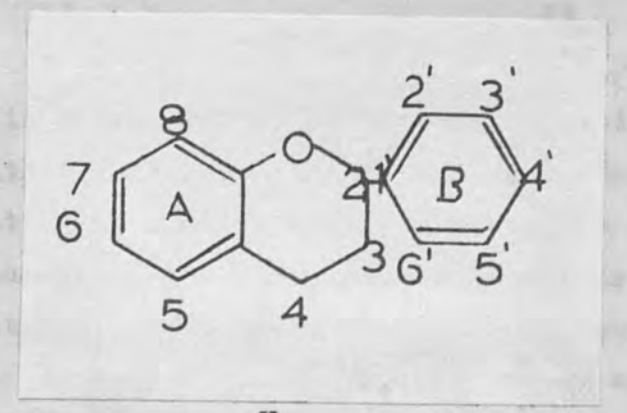


leucocyanidin

IV

However, these compounds themselves do not behave as tannins in the precipitation tests with protein or gelatin, although they can form insoluble precipitates with alkaloids at a high concentration. (cf. the insoluble chlorogenic acid caffeine complex obtained from fresh coffee beans, Gortner, 1908). The condensed tannins proper are polymeric compounds and are formed by linkage of monomeric flavan-3-ols (the catechins) and of flavan-3, 4-diols (the leuco-anthocyanins), either separately or together, and they may also contain other phenolic nuclei depending on their mode of formation.

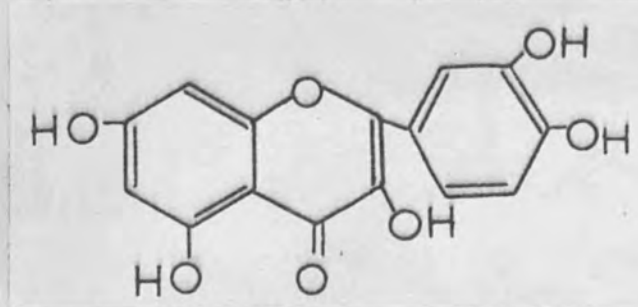
The flavan-3-ols and 3,4-diols are based on the parent compound flavan. See below for the numbering of the flavonoid nucleus, V.



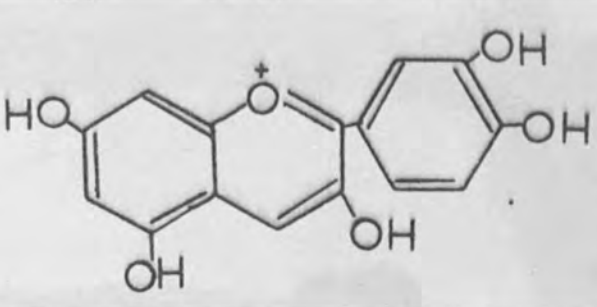
V

All the other flavonoid compounds, e.g. flavonols such as quercetin VI, and anthocyanidins, such as cyanidin VII are based on this general structure, and differ from each other, depending on the state of oxidation of the C_3 portions of the molecule (carbon atoms 2, 3 and 4) and the number and position of the phenolic hydroxyl groups in the benzene rings A and B in V.

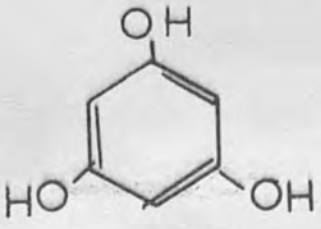
The A ring usually has a phloroglucinol, VIII, or a resorcinol IX pattern of hydroxylation; whereas the B ring has either a monophenol, (4') catechol (3'4') or pyrogallol (3'4'5') hydroxylation pattern.



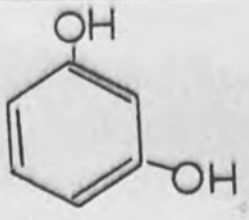
VI



VII



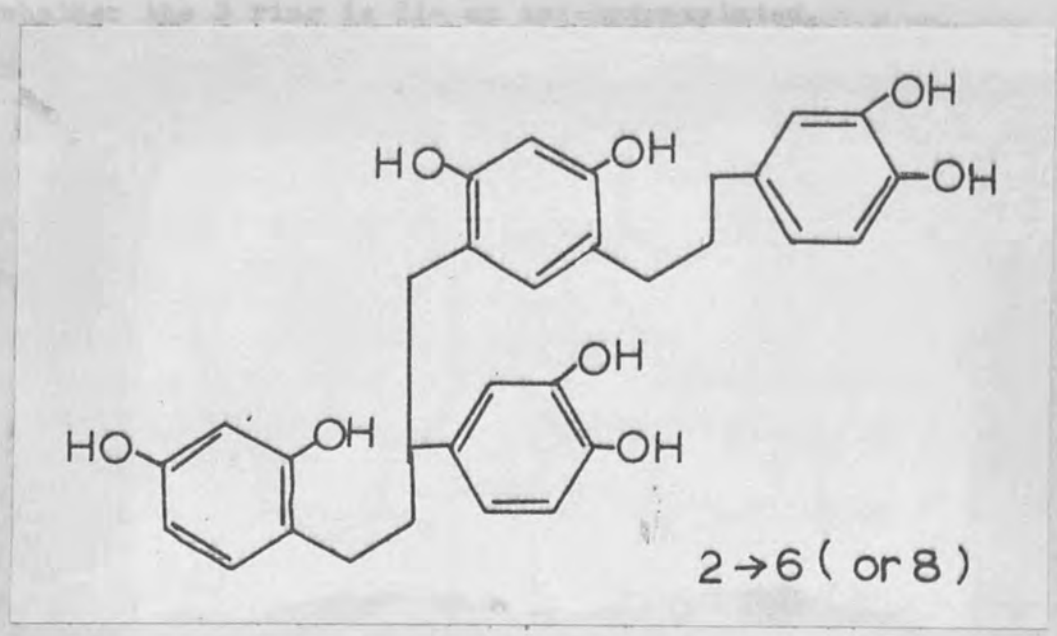
VIII



IX

This difference in hydroxylation of the two rings is due to their different biosynthetic origin. The A ring is derived from a head to tail condensation of acetate units, whereas the B ring, together with the 9C precursor of the phenylalanine type, is formed via the shikimic acid pathway. Both these biosynthetic routes will be discussed later. Condensed tannins usually occur as both oligomers and polymers, and these compounds, together with the monomers (III and IV), are very widely distributed in the plant kingdom.

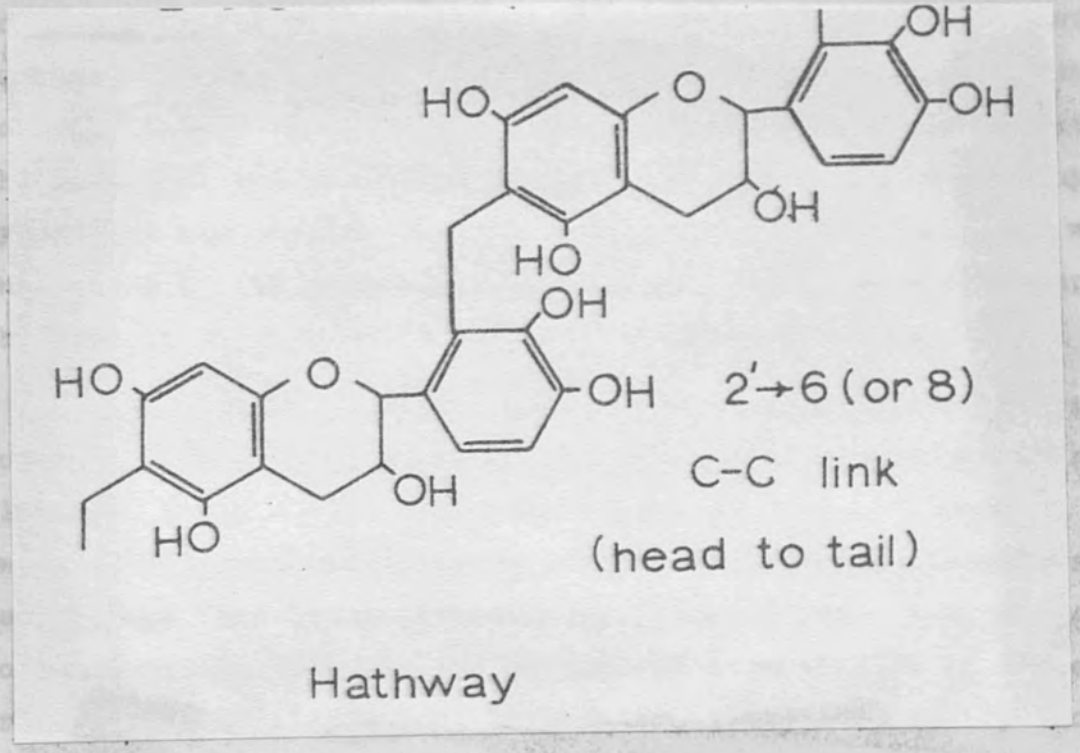
To return to the actual structure of the condensed tannins. There have been a number of theories put forward to explain the way in which the flavonoid units may be linked together. Freudenberg (1958) suggested a repeating unit based on ring opening of the pyran ring with a 2→6, or 2→8, carbon to carbon linkage.



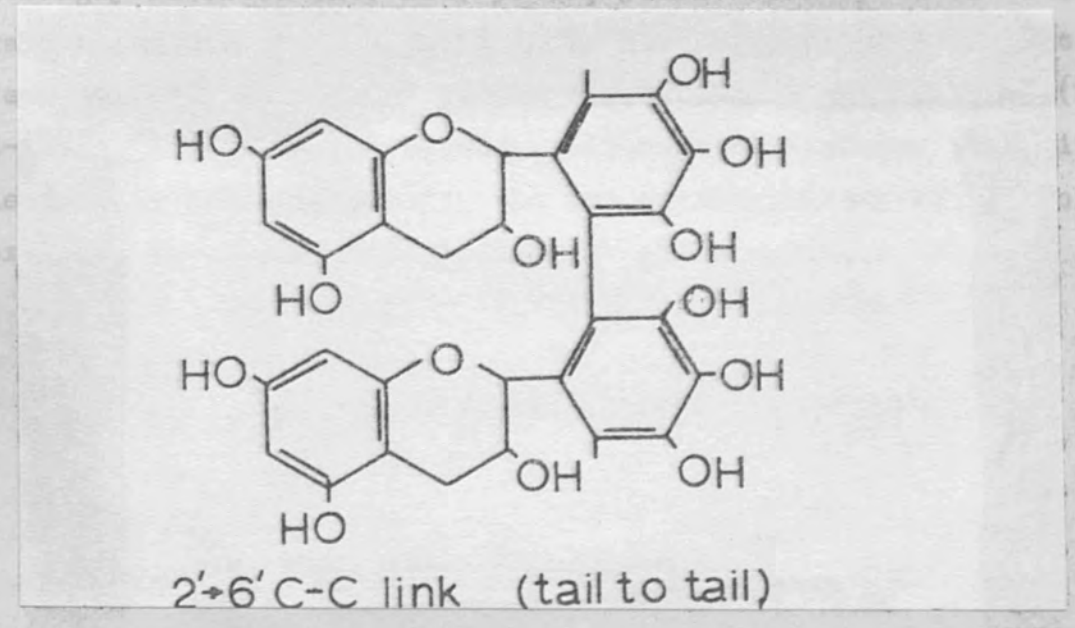
X

This suggestion was based on experiments with the acid catalysed polymerisation of catechins. He showed that simple flavans, such as 7.4'-dihydroxy flavan, can, like catechin, be polymerised on heating in acid and, therefore, the 3,5, and 3' hydroxyl groups of catechin are not obligatory for the process. In other experiments, he isolated a dimer of catchin which on analysis was shown to have structure X. He has pointed out that tannin formation in heartwoods, where condensed tannins can form over 30% of the extractives, is a slow process and since enzymes are not present that it must be due solely to non-enzymic reactions. He showed that the condensation of catechin can take place slowly by boiling in water alone and suggested that this supports his proposed mode of polymerisation. Hathaway and Seakins (1958) have criticised this hypothesis because acid catalysed reactions require a low pH (less than 2) and a high temperature (greater than 50°C) and point out that <u>in vivo</u> the pH is rarely as low as 4 or the temperature much greater than 30°C.

Hathway (1958) favours an oxidative polymerisation to link either 2'→6 or 8, XI, or 2'→6', XII, position depending on whether the B ring is di- or tri-hydroxylated.



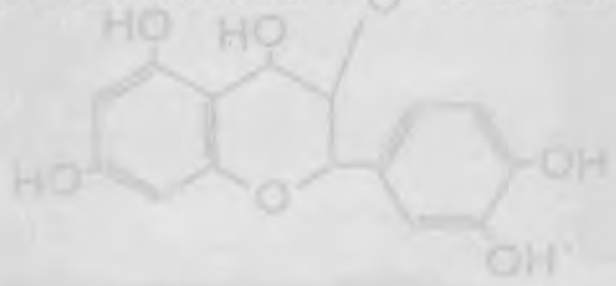
XI

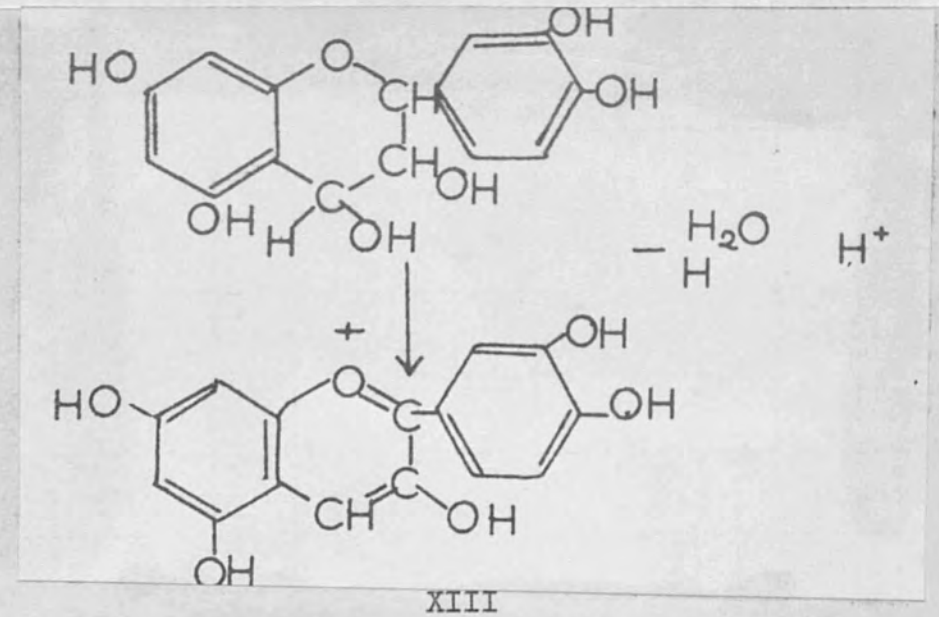


XII

Both these suggestions were supported by experiments with model compounds. He showed that a solution of homocatechol in the presence of methyl phloroglucinol, either when treated with phenolase or when allowed to autoxidise at pH 7.8, yielded a polymer similar to that obtained with catechin. The self-condensation product of homocatechol was quite different. This led him to suggest the head to tail linkage for catechin, (2'→6 or 8), XI. On the other hand, the nature of the oxidative polymerisation product of 5-methyl pyrogallol was similar to that from gallocatechin (XII) and was unaffected by the presence of methyl phloroglucinol, thus supporting the tail to tail (2'→6') linkage for gallocatechins, XII.

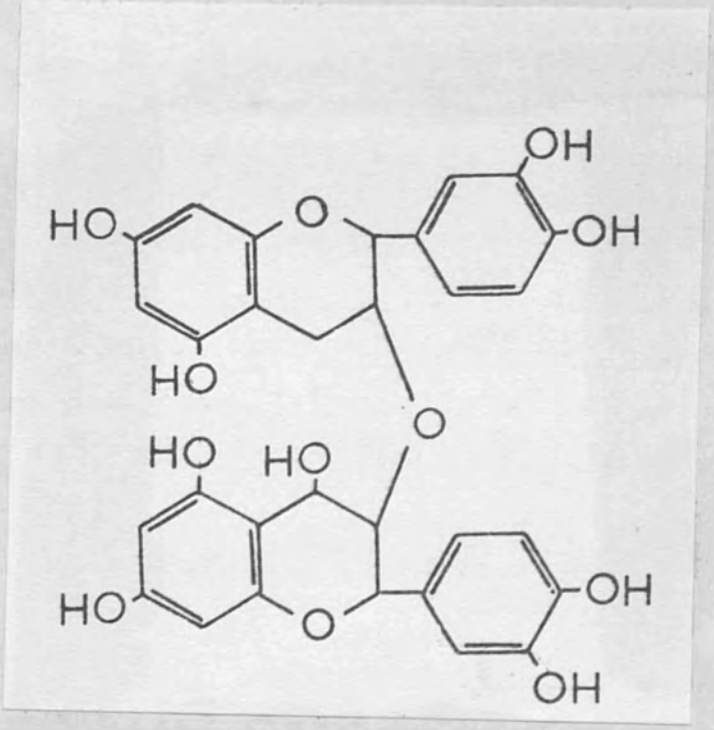
These linkages can occur with all types of flavan molecule. There are, however, some special linkages involving flavan-3, 4-diols (the leuco-anthocyanins) only. Bate-Smith and Swain (1953) were the first to suggest that many condensed tannins were formed from leuco-anthocyanins. The evidence they brought forward to substantiate this was the widespread distribution of leuco-anthocyanins in plants (cf. Bate-Smith, 1962), especially those of woody habit which are the type from which most commercial tannins are obtained, and the fact that isolated leuco-anthocyanins gave the standard tests for tannins. For example, the leuco-anthocyanins were strongly adsorbed onto hide power, yielded precipitates with alkaloids, gave a red colour with vanillin in the presence of strong acids and were markedly astringent to the taste. Leuco-anthocyanins (flavan-3, 4-diols) are so called because, although colourless, they give, on heating in hot dilute acid, the red anthocyanidin, XIII. For example, the leuco-anthocyanidin IV gives cyanidin VII.





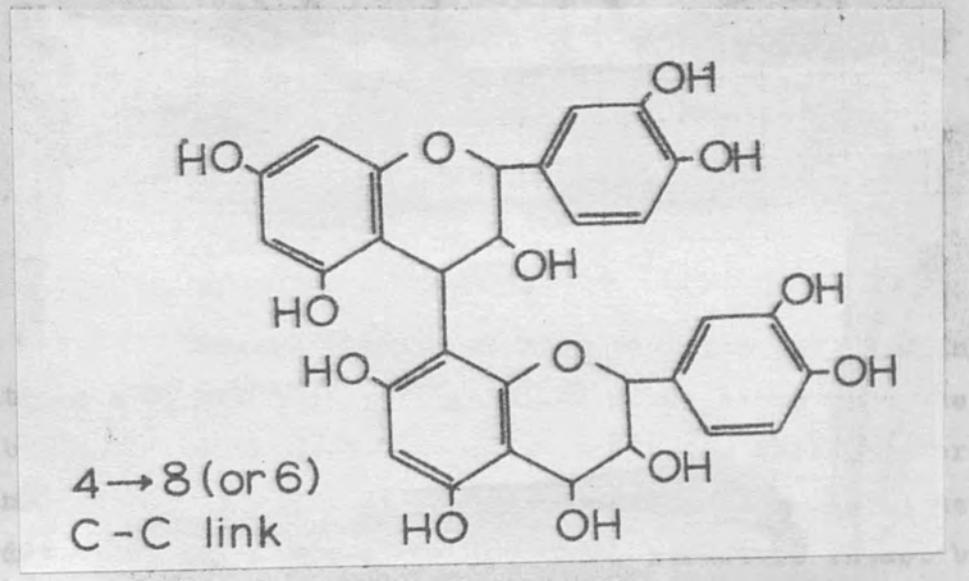
XIII

Even the molecules which are obviously polymers yielded anthocyanidins under these conditions and it is obvious that part of the macro-molecule at least must be joined by linkages other than C-C bonds. This was borne out by the proposed structure of cocoa leuco-anthocyanin (Forsyth and Roberts, 1960) in which two flavan units (epicatechin and leuco-cyanidin) are joined by an ether link (XIV).



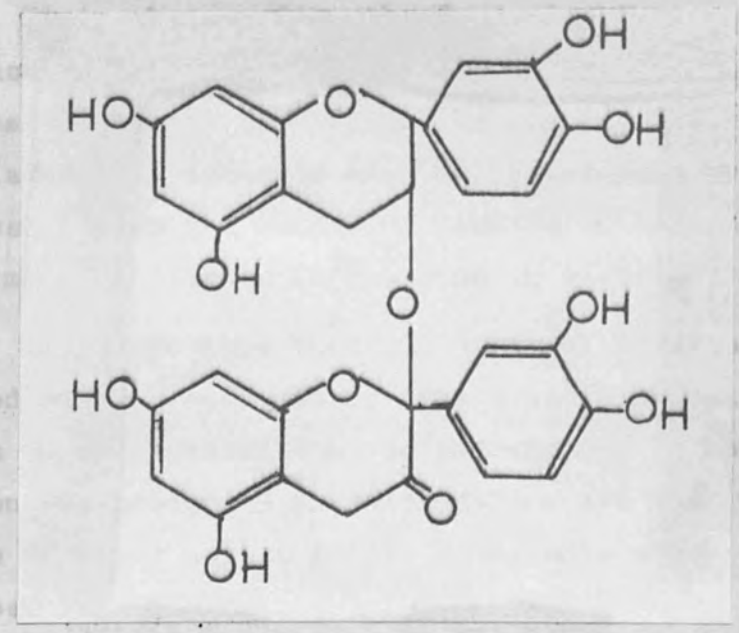
XIV

Freudenberg (1960) suggested a structure such as (XV) to explain the facile cleavage of anthocyanidins from leuco-anthocyanin polymers.



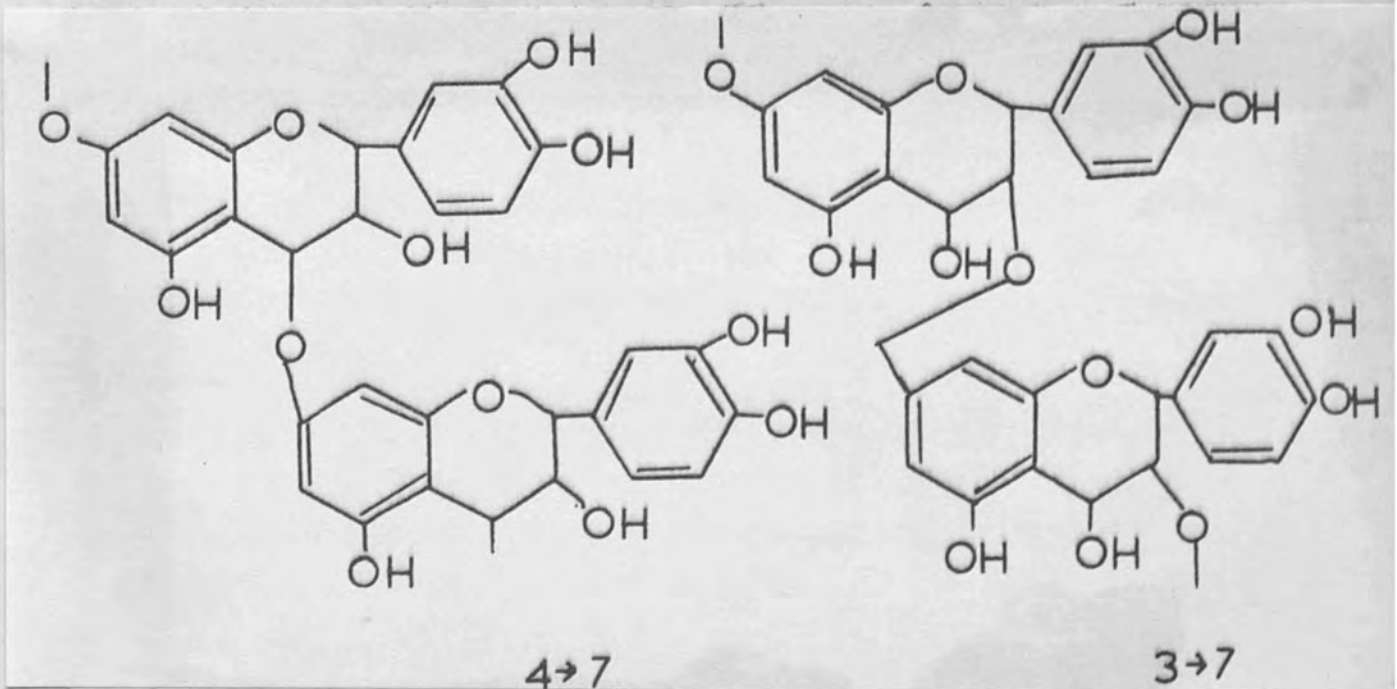
XV

More recently, Freudenberg (1962) has isolated a leuco-anthocyanin from Crataegus oxyacantha with a 3→2 ether link, (XVI).



XVI

Hergert (1962), working with pine bark tannins, thought that the 4→7 and 3→7 (XVII, XVIII) ether links were the most probable way in which the leuco-anthocyanins were polymerised or joined to the polymers in the condensed tannins. This method of condensation would leave the 3, 4-diol structure intact but would mean a decrease in the number of hydroxyl groups.



XVII XVIII

It is obvious from the foregoing that we are not able to write a structure for condensed tannins and it is probable that we will never be able to do so. For, like lignin, the linkage of the monomeric flavans may occur in many different ways to yield a heterogeneous polymer and much work remains to be done to determine whether the mode of linkage is the same in different organs of plants.

It is probable that all types of linkages may occur in the condensed tannins and that no true structure will be found for them. It is to be expected that polymerisation is more facilely induced by enzyme oxidation or autoxidation and this would explain the presence of other nuclei in the condensate which had reacted with quinones.

Biosynthesis

Tannins have been linked with carbohydrate metabolism in the minds of many workers. As early as 1886 Vines stated that as starch disappeared so aromatic glucosides were formed. This correlation between carbohydrate and secondary plant products does not, of course, show that a direct relationship exists. Rather it must be assumed that results showing increases in, for example, anthocyanin production with increased sugar in cress seedlings, (Eddy & Mapson, 1951), may only indicate that an excess of energy is required for their synthesis. In any case, it is obvious that carbohydrates being the first products of photosynthesis are, in fact, the biosynthetic precursors of all other compounds found in plant cells.

In recent years as a result, on the one hand, of biochemical work on mutants of micro-organisms (Davis) and, on the other, of organic chemical speculations (Birch), the biosynthesis of flavonoid compounds and related phenolic substances is now reasonably understood. ~~Previously~~ Actually, two main pathways have been found by which aromatic compounds are synthesised in plants and these explain the different types of hydroxylation pattern found in both the A and B rings of the flavonoid nucleus. These two pathways are respectively the shikimic acid pathway which gives rise to the B ring of the flavonoids and from which gallic acid and its congeners also arise, and the acetate pathway which leads to the formation of the A ring in flavonoid compounds and to the synthesis of many other aromatic substances such as the stilbenes and certain other mould metabolites.

In a series of outstanding papers, Davis and his co-workers (1955) have shown in the bacterium Escherichia coli the aromatic ring of the amino acids, phenyl alanine and tyrosine, are formed from the hydroxy carbocyclic acid, shikimic acid. This compound is formed from the condensation of tetrose and triose

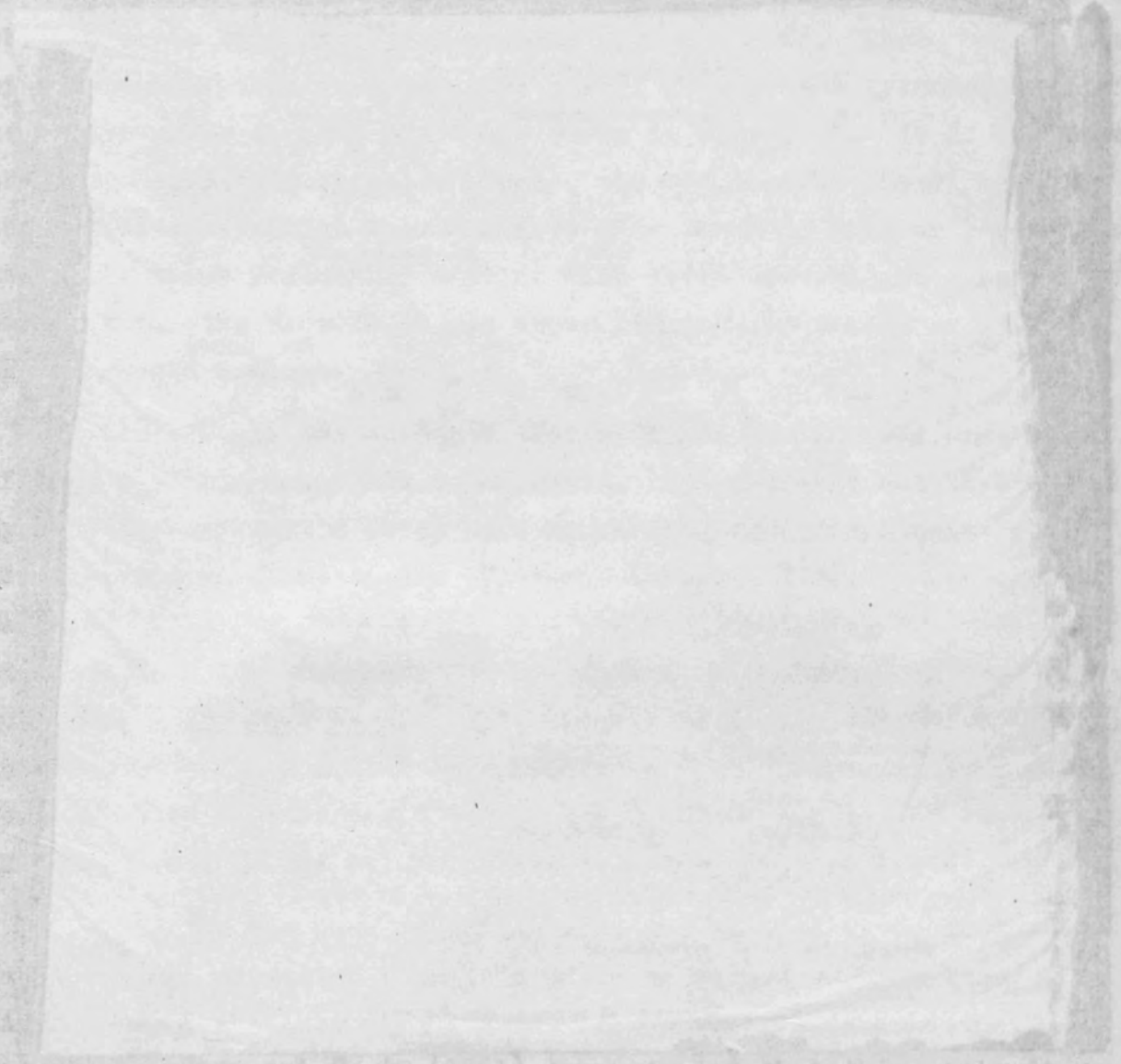


FIGURE 1[2]

The shikimic acid pathway.

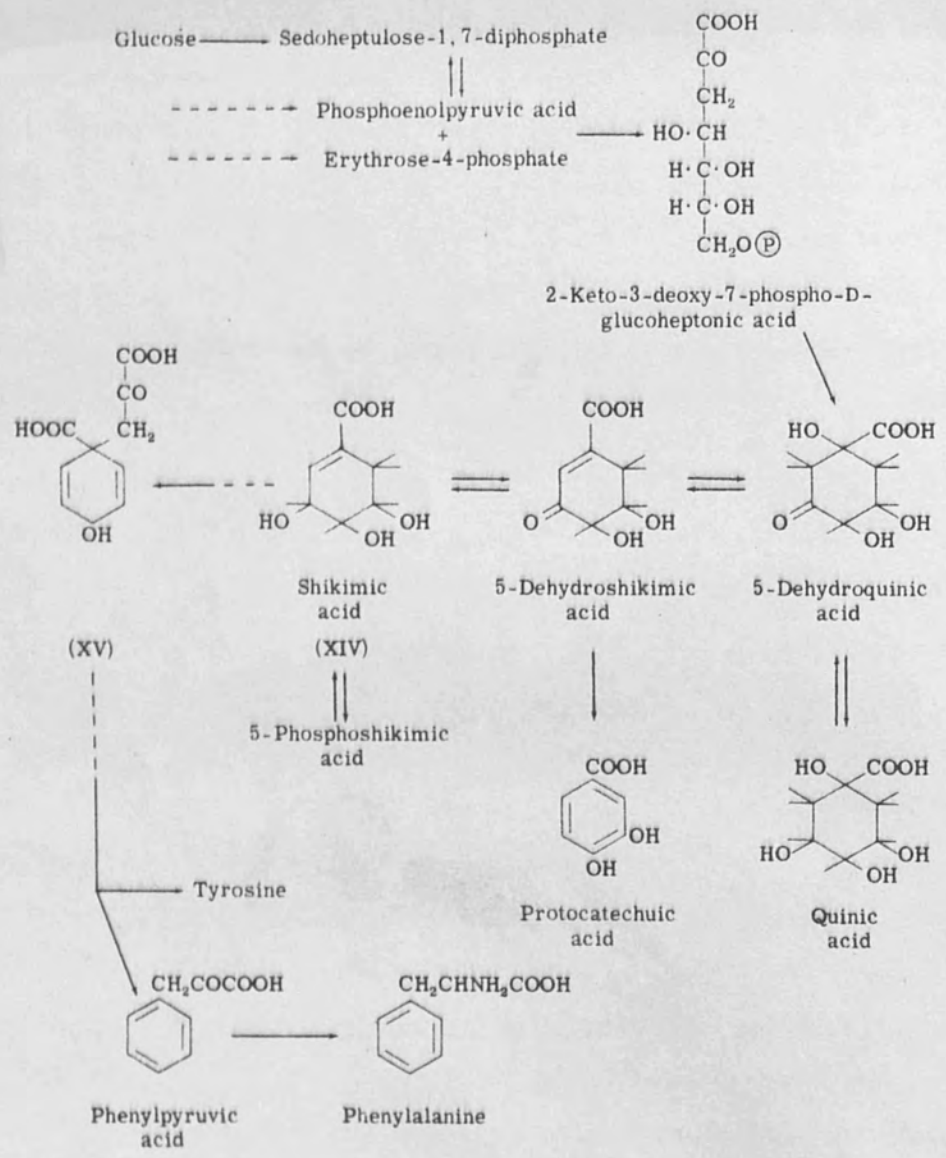


FIG. 1. The route of aromatic biosynthesis.

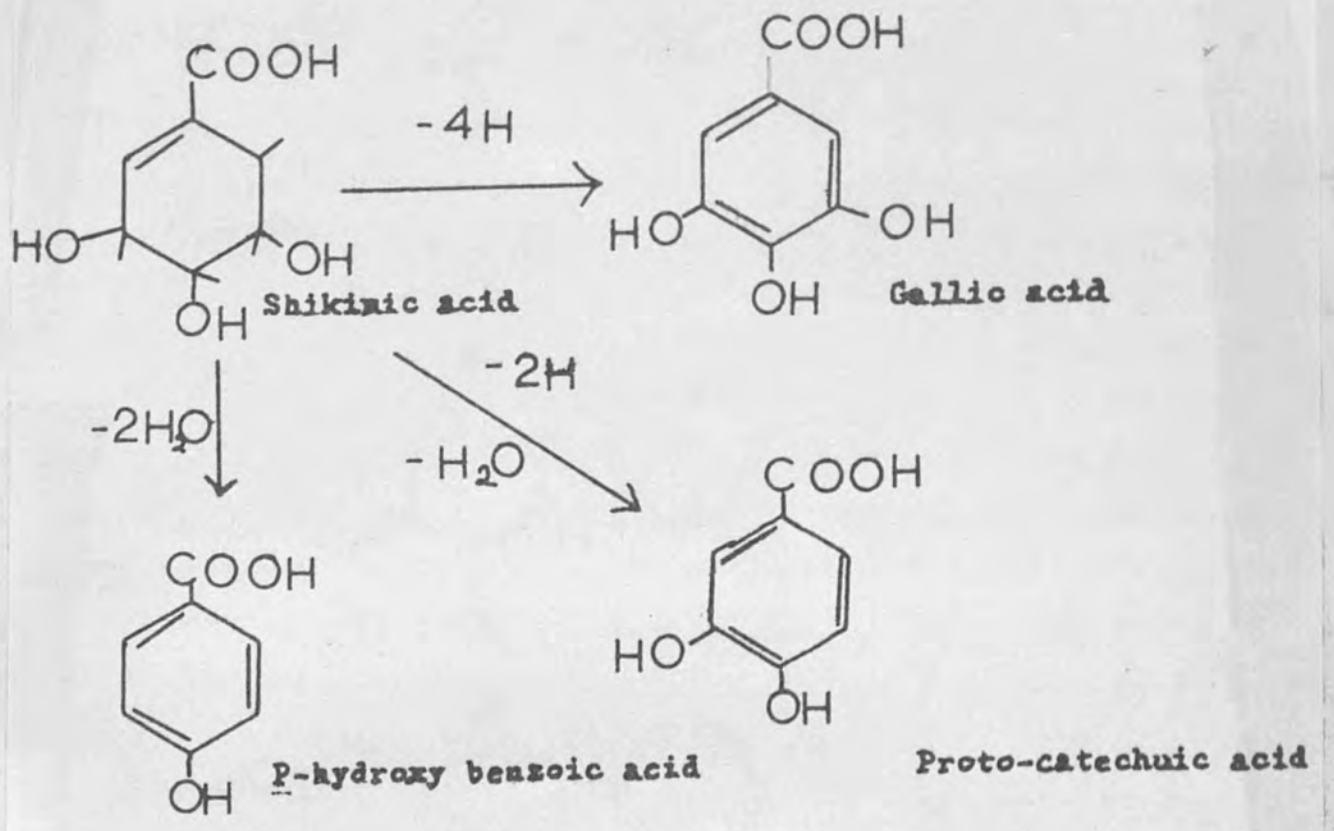
From Swain and Bate-Smith, 1962.

carbohydrate intermediates via 7-phospho-D-glucoheptonic acid. This compound is cyclised to give 5-dehydroquinic acid and this by the loss of water gives 5-dehydroshikimic acid. The latter compound is reduced by a ~~TPN~~ NADP dependant dehydrogenase to give shikimic acid. A C_3 side chain arising from pyruvic acid is added to an activated phosphate derivative of shikimic acid to give prephenic acid. Prephenic acid is unstable and can be readily non-enzymically decarboxylated to give phenylpyruvic acid or p-hydroxyphenyl pyruvic acid. These keto acids by a transamination reaction give phenyl alanine and tyrosine. The essential steps in this route are shown in Figure 1[2]. It is probable that from recent work, Neish (1960), the amino acids phenyl alanine and ~~tyr~~osine are first deaminated to give cinnamic acid or p-coumaric acid, and these presumably combine with three acetate units (see below) with ring closure at the ether link in position 1 to give the flavonoid nucleus.

It is now accepted that shikimic acid is the precursor of many phenolic compounds. In plants, shikimic acid and related cyclohexane carboxylic acids were shown to have a widespread distribution in plant tissue (Hattori, Yoshida, Hasegawa (1954), Hasegawa, Nakagawa, Yoshida (1957)) and has been shown in isotopic studies to be the precursor for the B ring of quercetin in the buckwheat (Fagopyrum esculentum), Underhill, Watkin and Neish (1957). At the same time, a number of radioactive C_6-C_3 compounds have also been shown to be incorporated into the C_6(B)-C_3 portion of flavonoid compounds, and in the related cinnamic acids, Bogorod (1958).

In the case of gallic acid and related C_6-C_1 compounds, Haslam (1961) suggested that this could be formed by oxidation directly from shikimic acid without loss of hydroxide function, Figure 1[3]. Conn and Swain (1961) demonstrated that this route is more probable for the formation of gallic acid in Geranium pyranacium than one involving the loss of a C_2 unit from the side chain of phenyl alanine.

FIGURE 1[3]



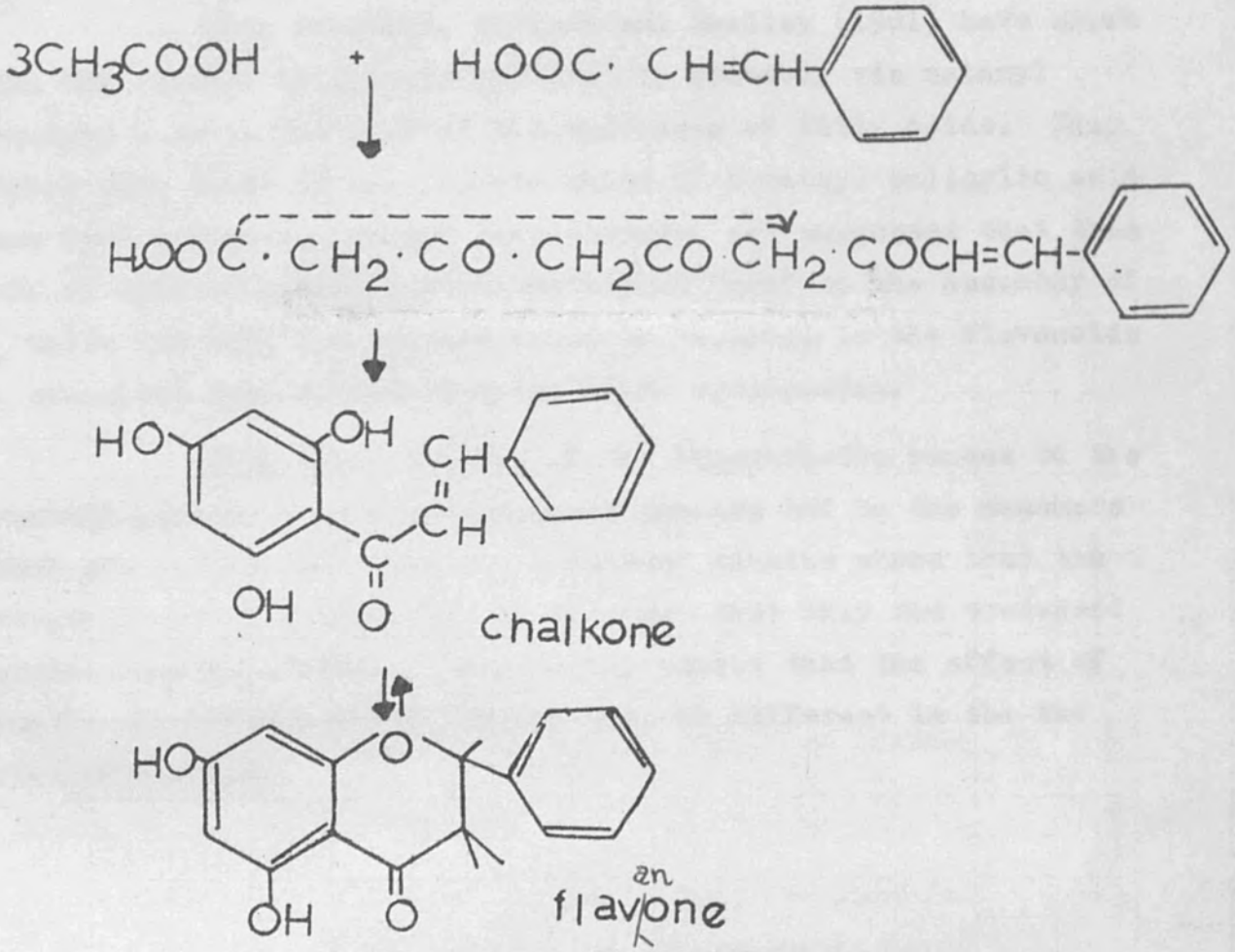
The Acetate Pathway

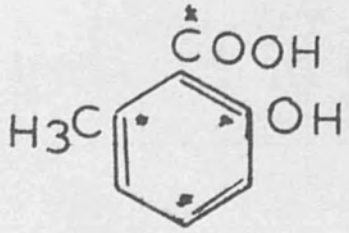
Collie (1893, 1907) suggested that certain aromatics could be formed by head to tail condensation of acetate units. This hypothesis was forgotten until Birch (1957) observed that the hydroxyl groups in the aromatic rings in a large number of natural products of plant and fungal origin were on alternate carbon atoms, e.g. the A ring of flavonoid compounds such as quercetin, ~~antho~~cyanin, etc., see VI and VII, and he suggested that these compounds were formed by head to tail condensation of acetate units. Thus addition of three acetate units to a cinnamic acid would yield a flavanone, see Figure 1[4]. Indeed, using micro-organisms, Birch and his co-workers have shown that this theory is substantially correct in the case of many hydroxy aromatic and oxygen heterocyclic compounds ranging from 6-methyl salicyclic acid ~~XVII~~ XIX to mycophenolic acid ~~XVIII~~ XX.

See overpage.

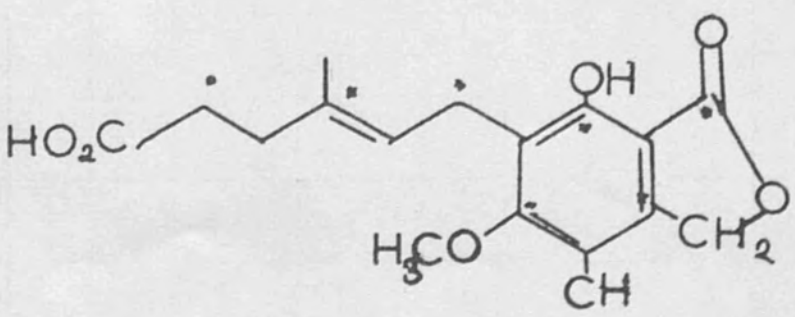
FIGURE 1[4]

the acetate pathway





XIX



XX

More recently, Bu'loch and Smalley (1961) have shown that the linkage of acetate moieties is probably via malonyl coenzyme A as in the case of the synthesis of fatty acids. They showed that three of the acetate units of 6-methyl salicylic acid came from malonate, and one from acetate, and suggested that this mode of synthesis gave a distinguishable "end" to the assembly of C_2 units and that the acetate would be replaced in the flavonoids by cinnamate thus orientating the final cyclisation.

This brief outline of the biosynthetic routes to the aromatic portion of the hydrolysable tannins and to the monomers which are polymerised into the condensed tannins shows that the two groups are distinguished by the fact that only the condensed tannins require acetate. Thus we may expect that the effect of genetic and environmental factors will be different in the two types of tannins.

Summary of Chapter 1

1. Tannins may be defined as polymeric phenolic compounds which have the property of cross-linking protein.

2. Tannins are divided into two classes, depending on their reaction to hot dilute mineral acid. The hydrolysable tannins which are split by this treatment and the condensed tannins which are further polymerised.

3. Whereas the hydrolysable tannins have discrete chemical structure, the condensed tannins are variable depending on the mode of linkage of their flavan monomers.

4. Most of the condensed tannins are polymeric leuco-anthocyanins.

5. Carbohydrate precursors are converted into phenolic compounds via two pathways: (i) the shikimic acid pathway to form the phenolic part of the hydrolysable tannins and the B ring in the flavonoid moiety of the condensed tannins; (ii) the acetate pathway to form the A ring of the flavonoid compounds in the condensed tannins.

Chapter 2.

The metabolism of tannins.

Chapter 2

1. THE DISTRIBUTION OF THE TANNINS IN PLANTS

Tannins are distributed throughout most families of higher plants. In certain parts of tannin containing plants they occur in especially high concentrations (e.g. as much as 58% of the dry weight, Rottiesper (1946)), and it is from these plants that commercial tannin extracts are obtained. A list of the species from which the main commercial tannins are obtained is given in Table 2[1]. It can be seen from this that tannins are present in leaves, fruits, bark and wood. It is probable, however, that the tannins are actually present in all the organs of most tanniferous plants. Thus Williams (1960) has shown that leuco-anthocyanins and catechins occur in the leaf, bark, wood and root bark of all the various members of the Pomoideae that he examined. Similarly, Griffiths (1958) showed all parts of the cocoa tree (Theobroma cacao) contained leuco-anthocyanins. An exception to these findings is the fact that Bate-Smith and Ribereau-Gayon (1959) showed that leuco-anthocyanins were present in many seed coats of plants which otherwise did not contain them (e.g. Vicia faba).

As mentioned in Chapter 1, most, if not all, of the condensed tannins are derived by the polymerisation of flavans. Furthermore, on examination of a number of isolated tannins, Bate-Smith and Swain (1953) showed that they yielded anthocyanidin on treatment with hot mineral acid and may thus be regarded as polymeric leuco-anthocyanins.

Table 2[1]

Family	Plant	Organ	Common name of tannin
Fagaceae	Quercus infectoria Oliver	Galls caused by Cynips tinctoria on twigs.	Turkish tannin
Fagaceae	Quercus aegilops L.	Galls caused by Cynips calicis	Valonia
Leguminosae	Caesalpinia digyna Rottl.	Pods	Tara
Leguminosae	C.cortinaria (Jacq) Willd.	Pods	Divi-Divi
Leguminosae	Acacia catechu (L.f.) Willd.	Wood	Catechu
Leguminosae	A.mollissima Willd.	Bark	Wattle
Anacardiaceae	Schinopsis balansae Engl. S. quebracho colorado Barkley and Meyer	Wood	Quebracho
Anacardiaceae	Rhus chinensis	Lvs. caused by Aphis chinensis	Chinese tannin
Combretaceae	Termidia catappa L.	Fruit	Myrobalan
Rubiaceae	Uncaria gambia Roxb.	Lvs and twigs	Gambia
Rubiaceae	Rhizsphordieae spp.	Bark	Mangrove
Pinaceae	Tsuga canadensis Carr	Bark	Hemlock
Pinaceae	Picea abies L Karst.	Bark	Spruce
Pinaceae	Larix decidua Mill. Coste	Bark	Larch

Following this observation, Bate-Smith (1954) and Bate-Smith and Lerner (1954) examined the occurrence of leuco-anthocyanins in the leaves of various plant species. The leaves were crushed in dilute hydrochloric acid, heated to boiling for 20 minutes, and the resulting solution extracted with a small quantity of amyl alcohol, which served to concentrate any anthocyanidins formed into a smaller volume of solution suitable for paper chromatography, and, at the same time, free them from interfering substances. The amyl alcohol extracts were examined chromatographically, which permitted the identification of the anthocyanidins and any other flavonoid aglycones and related substance which would be formed from their glycosides and other derivatives by hydrolysis. In later work (see Bate-Smith J.Lin.Soc. 1962), Bate-Smith recorded the presence of the anthocyanidins, cyanidin, pelargonidin and delphinidin (formed from leuco-anthocyanins), the flavones, kaempherol and quercetin and myricetin; and the cinnamic acids, p-coumaric, caffeic, ferulic and sinapic acids in about 2,000 species of angiosperms. He showed that the monocotyledons as a group are characterised by having fewer species containing flavonoid components than the dicotyledons and many more species which have the methoxy cinnamic acids, ferulic and sinapic acid. The other significant fact which has emerged from this survey is that the presence of leuco-anthocyanins in the dicotyledons is highly correlated with woodiness. Table 2^2 is a summary of Bate-Smith's work.

Table 2[2]

Distribution of various classes of Flavonoid and Related Compounds in the Angiospermae. (From Swain and Bate-Smith, 1962)

Class of Compound	Percent of Species examined from			
	Monocotyledons	Dicotyledons		
		Woody	Herbaceous	Total
Leucoanthocyanins	35	61	19	40
Flavonols	41	90	58	72
Caffeic acid	65	93	78	83
Methoxycinnamic acids	51	20	46	34
Null	6			3

It can be seen that the leuco-anthocyanins are extremely widespread, especially in "woody" families and, as mentioned earlier, his results for tannin distribution tallied almost exactly with those found for "botanical" tannins, Bate-Smith and Metcalf (1957). One other important point is that leuco-anthocyanins, which yielded cyanidin, are greatly in excess of those which yield the other two anthocyanidins; indeed, very few species yielded pelargonidin. The distribution of this type of leuco-anthocyanin is paralleled by that of the flavonols. In a separate more limited survey on tropical fruits, Forsyth and Simmons (1954) have shown that the anthocyanidins also conform to this pattern. It also confirmed the pioneering work of Sir R. Robinson (1939) and his co-workers made 30 years ago on the occurrence of the anthocyanidin glycosides in flower petals. No survey of the distribution of hydrolysable tannins appears to have been carried out.

2. THE DISTRIBUTION OF TANNINS WITHIN THE PLANT

It is not known in what form tannins occur in relatively dead tissue, such as heart wood and old bark, but it is generally

assumed that these compounds are present in the vacuole in most living tissue, although there is little direct evidence for this (vide infra.). However, some plant tannins have been shown to occur exclusively in specialised cells. For example, in the banana fruit (Musa sapientum) there are two types of tannin cells; single cells scattered in the skin and the latex ducts which are present in both skin and pulp. In the testa of the bean (Phaseolus vulgaris) Feenstra (1958) found that the leuco-anthocyanins were entirely confined to the sub-epidermal layer of cells, although he did not investigate whether all or only a few of such cells contained tannins. In the cocoa bean also tannins are restricted to special cells in both the testa and cotyledons. About 10% of the cells in the cotyledons are tannin cells; these are readily distinguishable since not only are they larger than the surrounding parenchyma cells but they are deeply coloured as they contain the anthocyanin pigments. Brown (1954) was able to separate these tannin cells from the others in disintegrated cotyledons by taking advantage of their higher density and showed they contained besides leuco-anthocyanins, anthocyanins, catechins and theobromine. He also demonstrated that these cells did not contain any enzymes.

In other plants where special tannin cells do not occur, it has been generally presumed that the tannins are separated spatially from the cytoplasm which contains enzymes capable of oxidising them. This presumption is based on the fact that although the cut surface of many plants darkens on exposure to air, due to the oxidation of phenolic compounds catalysed by oxidative enzymes, especially phenolases and peroxidases, no sign of darkening occurs in intact plants. As Onslow (1925) demonstrated, most plants which contain phenolic compounds also contain phenolases; it is natural to presume that these components must occur in separate parts of the cell.

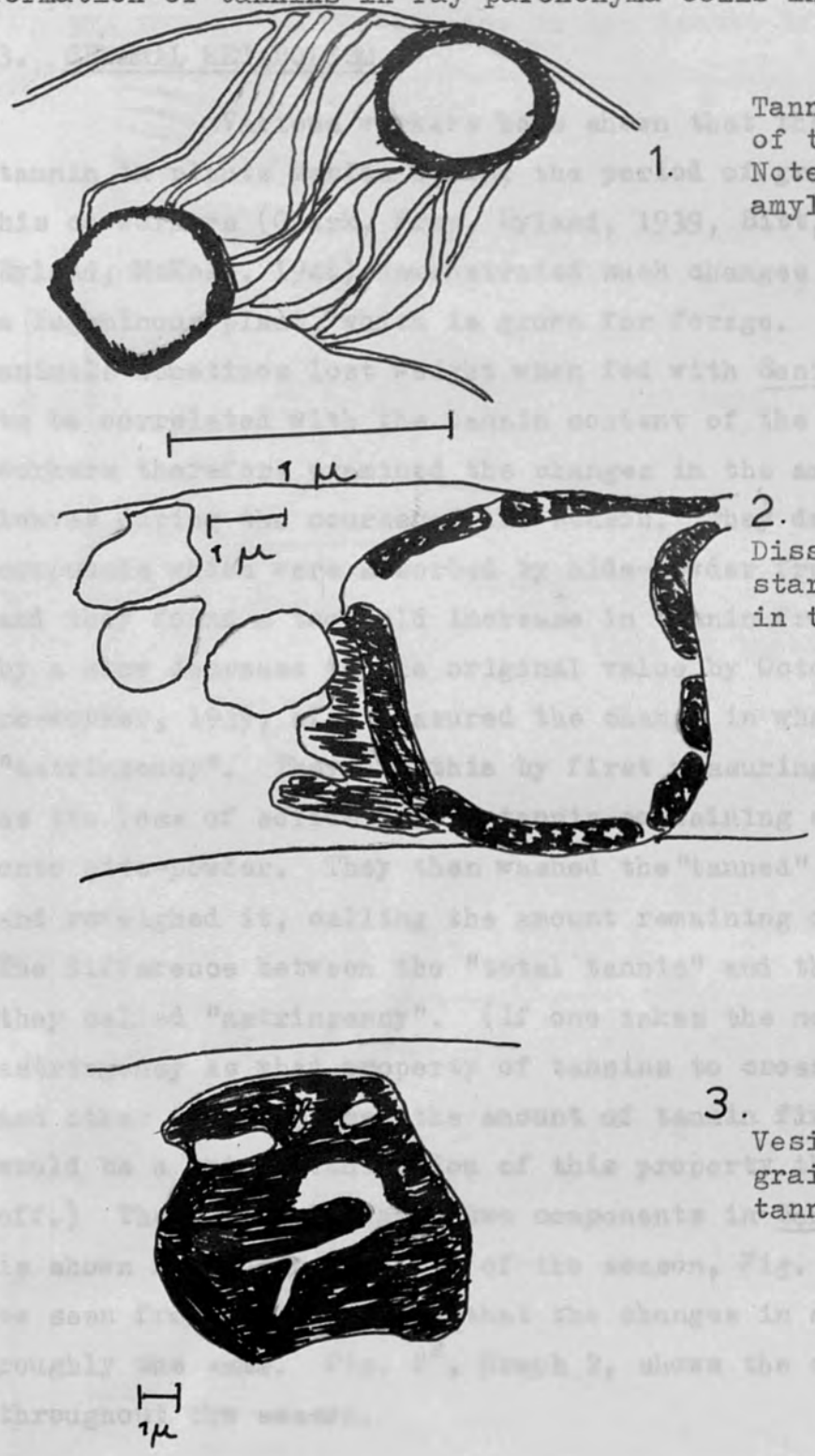
Tannins have been shown to occur in vacuoles by staining techniques which probably accounts for the number of references in the botanical literature to tannins as waste products. However,

the results obtained by staining techniques are somewhat equivocal since the process of fixing etc. allows diffusion of both the tannins and the protein of the cytoplasm which can then form insoluble precipitates in a part of the cell where normally they do not occur.

More recently, Wardrop and Cronshaw (1962) have demonstrated that tannins are formed in vesicles in the cytoplasm rather than in the vacuole itself. They examined the formation of tannins in Eucalyptus elaesphore in the xylem ray parenchyma cells in a region near the cambium. They used an elegant technique to demonstrate this by first fixing the sections in potassium permanganate and then staining them with ferrous sulphate/formaldehyde solution. This showed the tannins as a dark stain in the electron-micrograph of the material. They showed that the tannins were formed in starch grains inside a chloroplast-like organelle, which they called an amyloplast. At first, tannins were formed at the edge of these starch grains and, on dissolution of the starch, the tannins filled the resulting vesicle. At the same time, the characteristic lamellae-like structure of the amyloplast became lost. Figure 2[1] shows this process and is redrawn from Wardrop and Cronshaw's paper. This work shows that in woody tissues, at least, these phenolic compounds are contained in special 'vacuoles' (vesicles) separated from the cytoplasm. It is possible that the vesicles may unite at some stage with the vacuole proper but there seems no reason why this should happen. This mode of distribution may probably be the case for other tannin cells. In any living cell it seems improbable that tannins are formed, or are present free, in the cytoplasm where they could react with any polymeric material such as protein or cellulose, or be oxidised.

FIGURE 2[1]

The formation of tannins in ray parenchyma cells in Eucalyptus elaesphora.



1. Tannins forming at the edge of the starch grains. Note the lamellae of the amyloplast.

2. Dissolution of both the starch grain and the lamellae in the amyloplast.

3. Vesicle left by the starch grain becoming filled with tannins.

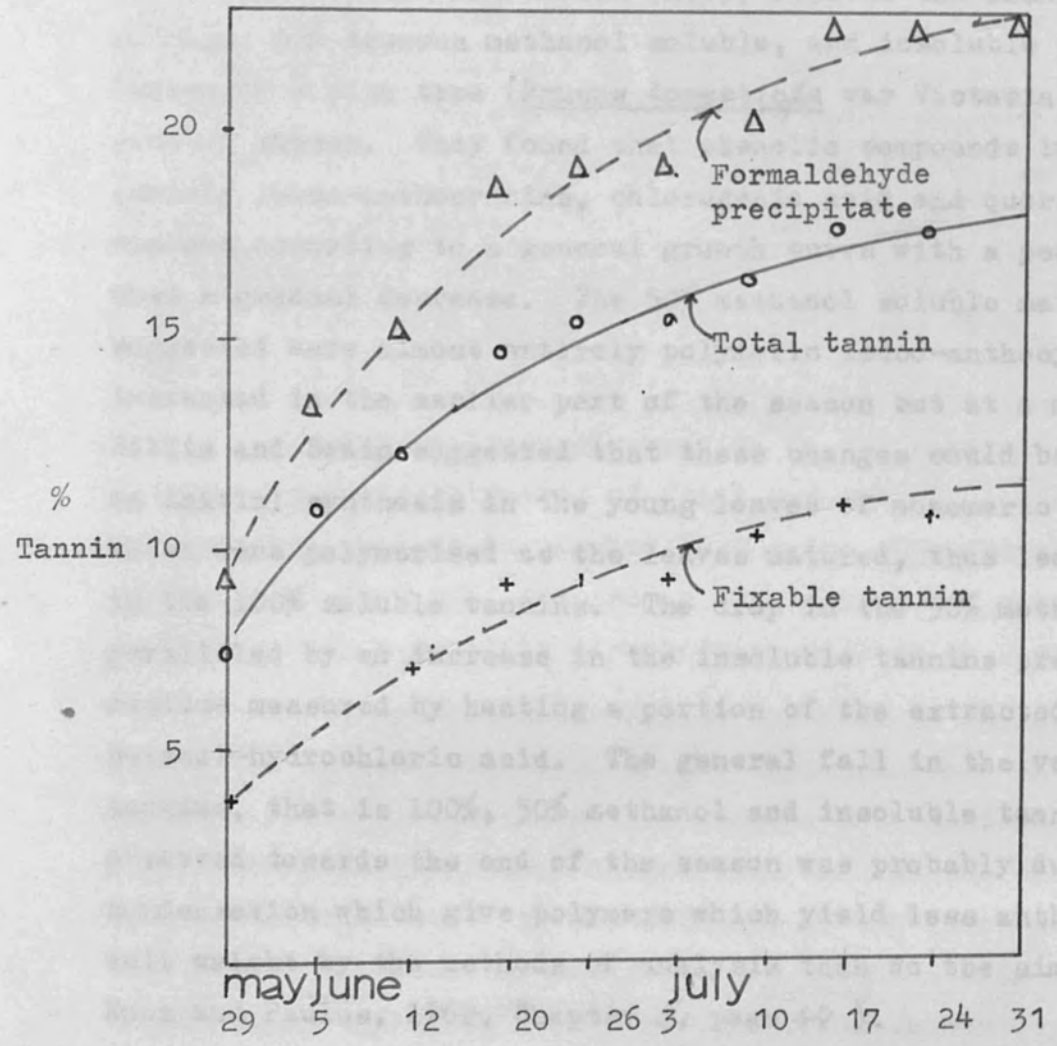
Drawing from an electron micrograph by Wardrop and Cronshaw 1962.

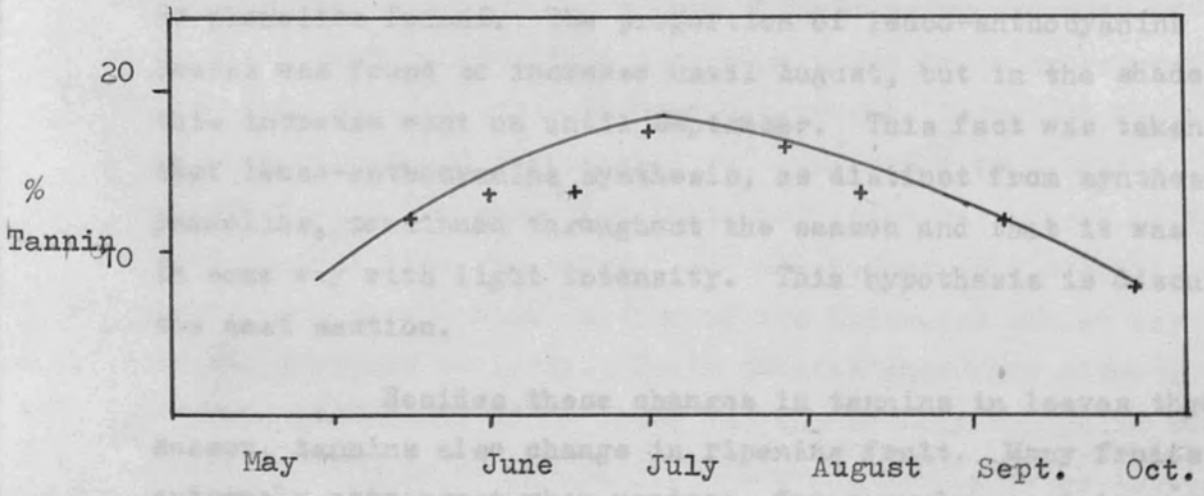
3. GENERAL METABOLISM

Various workers have shown that the concentration of tannin in plants varies during the period of growth. Hyland and his co-workers (Clark, Frey, Hyland, 1939, Sitt, Clark, 1941, Sitt, Hyland, McKnee, 1946) demonstrated such changes in Senicea lespedeza, a leguminous plant, which is grown for forage. It was observed that animals sometimes lost weight when fed with Senicea and this appeared to be correlated with the tannin content of the leaves. The American workers therefore examined the changes in the amount of tannin in the leaves during the course of the season. They defined tannins as those compounds which were adsorbed by hide-powder from an aqueous extract, and they found a two-fold increase in tannin from May to June, followed by a slow decrease to the original value by October. Clark and his co-worker, 1939, also measured the change in what they called "astringency". They did this by first measuring the "total tannin" as the loss of solids from a tannin containing extract by adsorption onto hide-powder. They then washed the "tanned" hide-powder, dried and reweighed it, calling the amount remaining on "fixed tannin". The difference between the "total tannin" and the "fixed tannin" they called "astringency". (If one takes the normal definition of astringency as that property of tannins to cross-link the collagen and other protein, then the amount of tannin fixed onto the hide-powder would be a better indication of this property than the amount washed off.) The change in these two components in Senecia lepidezia leaves is shown for the early part of the season, Fig. 2^2, Graph 1. It can be seen from their results that the changes in each fraction is roughly the same. Fig. 2^2, Graph 2, shows the change in total tannin throughout the season.

FIGURE 2[2]

The change in the tannins in the leaves of Senecia lepidezia.





Swain and Hillis (1959) studied the change in 100% methanol soluble, 50% aqueous methanol soluble, and insoluble phenolics in the leaves of a plum tree (Prunus domestica var Victoria) through the growing season. They found that phenolic compounds in the 100% extract (mainly leuco-anthocyanins, chlorogenic acid and quercetin glycosides) changed according to a general growth curve with a peak about July, then a gradual decrease. The 50% methanol soluble materials which they suggested were almost entirely polymeric leuco-anthocyanins also increased in the earlier part of the season but at a much slower rate. Hillis and Swain suggested that these changes could be interpreted as an initial synthesis in the young leaves of monomeric leuco-anthocyanins which were polymerised as the leaves matured, thus leading to a drop in the 100% soluble tannins. The drop in the 50% methanol solubles was paralleled by an increase in the insoluble tannins present in the residue measured by heating a portion of the extracted residue in butanol-hydrochloric acid. The general fall in the values of total tannins, that is 100%, 50% methanol and insoluble tannins that was observed towards the end of the season was probably due to further condensation which give polymers which yield less anthocyanidin per unit weight by the methods of analysis than do the simple monomers (see Roux and Paulus, 1962, Chapter 3, page 49).

In leaves which were on the shady (i.e. north) side of the tree, they found that the production of the maximum amount of tannins was delayed and there was also an overall decrease in the total amount of phenolics formed. The proportion of leuco-anthocyanins in the sunny leaves was found to increase until August, but in the shaded leaves this increase went on until September. This fact was taken to indicate that leuco-anthocyanins synthesis, as distinct from synthesis of other phenolics, continued throughout the season and that it was connected in some way with light intensity. This hypothesis is discussed in the next section.

Besides these changes in tannins in leaves through the season, tannins also change in ripening fruit. Many fruits are extremely astringent when unripe; for example, certain apple varieties,

plums, quinces, banana and persimmon. This astringency is lost when most fruits ripen, but not in others, for example crab-apple. Weurman and Swain (1955) found a rapid drop in total phenols per unit weight in apples from 50 days after petal fall until full maturity, 150 days later. Here, the main phenolic compounds are leuco-anthocyanins and chlorogenic acid; these results have been confirmed by Hulme (1958). Similar changes were found by Hillis and Swain (1959) for the leuco-anthocyanins in the plum fruit.

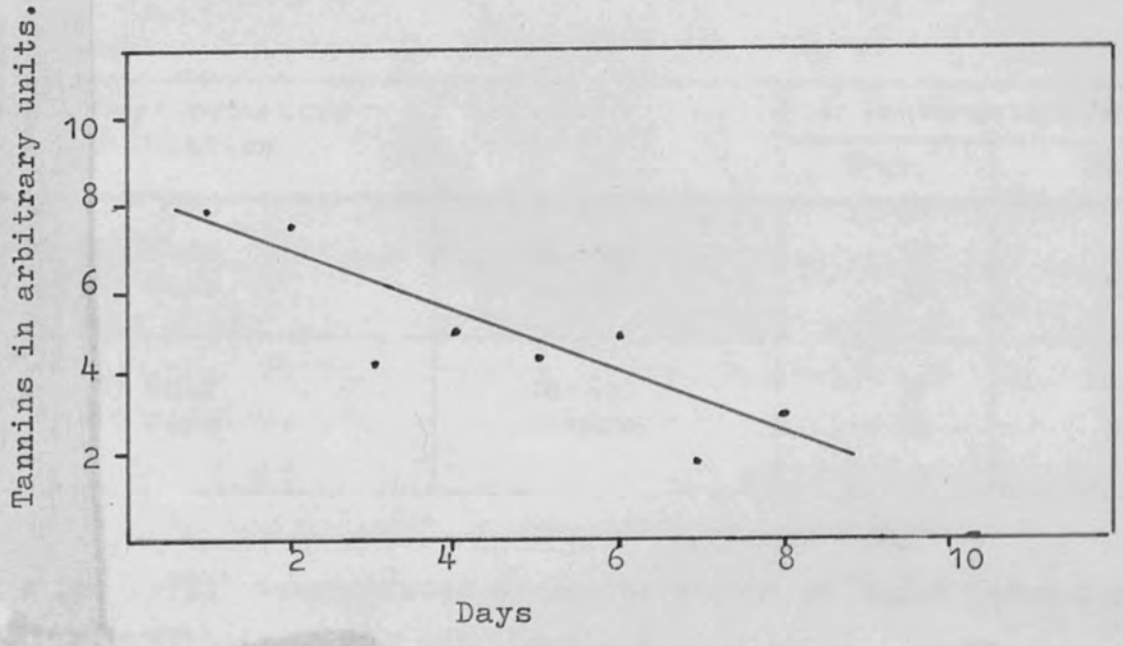
The earliest observation on the general loss of astringency during ripening of fruit was made by Lloyd (1911, 1912) who examined changes in tannins during the ripening of persimmon. He suggested that the decrease in astringency was caused by the tannins combining with a specific carbohydrate colloid to form insoluble material.

A notable contribution was made by Barnell and Barnell (1945) who investigated the changes in astringency in the banana during ripening. They investigated the problem both anatomically and chemically. As mentioned earlier, they showed that tannins are confined to scattered tannin cells in the skin and in the latex-containing cells. They showed there was no change in the appearance of the tannins in the scattered cells on ripening; the latex in the ripe fruit, however, instead of being free flowing, congealed and solidified and the tannin in the latex cells partly disappeared and partly seeped into adjacent cells. They also measured changes in the tannins of the fruit on ripening by measuring the effect of cold water extracts of the fruit on the time for a standard starch solution to be hydrolysed by diastase. They demonstrated that the time was greatly increased in the presence of tannic acid and the same effect was caused by extracts from the unripe fruit. To show that this inhibition was caused by tannin, they treated both the extract and the tannic acid solution with hide-powder and showed that neither of the filtrates caused any reduction of the diastase activity. Their results show that after 10 days in a store at 53° and 68°F. there was an enormous change in this tannin fraction, Figure 2^3 (Graph 3). However, they give no indication as

to the fate of those tannins which disappear. It is probable that, as in the leaves of the plum, the tannins are polymerised and therefore are less extractable. This change would also cause a decrease in astringency as judged by taste.

FIGURE 2[3]

The change in the "active tannins" in the pulp of the banana on ripening, from Barnell and Barnell (1945).



4. THE EFFECT OF ENVIRONMENTAL FACTORS

(a) Light

Various environmental factors have been shown to affect synthesis of tannins and related phenols. In the Swain and Hillis experiment described previously it was shown that light was an important factor. In leaves which were totally shaded at a time when monomeric (i.e. 100% methanol soluble) leuco-anthocyanins were increasing rapidly (end of June) the amount of leuco-anthocyanins in the shaded leaves did not alter and this indicates that leuco-

anthocyanin synthesis is either catalysed by light or was perhaps dependant on a supply of photosynthetic assimilates. When leaves were shaded in the latter part of the season (mid-July) when the amount of 100% methanol soluble tannins was decreasing and the leuco-anthocyanins in the 50% methanol was still increasing (that is polymerisation was taking place), shading again delayed these changes indicating that light may catalyse the polymerisation process. See Table 2^3.

Table 2^3

Date test commenced and Duration	Sample	% Leuco-anthocyanin	
		Start	End
20 June 13 days	Shaded Control	32 32	32 50
23 July 17 days	Shaded Control	36 36	36 32

Philips (1954) demonstrated a similar effect of light intensity on lignin synthesis in the ash (Fraxinus elcelsior)

(b) Mineral Nutrition

Mineral nutrition is also important; for example, Davis Coulson and Lewis (1960) compared the tannins which were capable of precipitating gelatin in oak and beech leaves from trees grown on both mull and mor sites. They found that there was three times as much tannin in the leaves from the tree grown on the mor site as compared with the mull site; that is, more tannin was formed on the base poor site.

Handley (1954) had previously suggested that the difference in humus on mull and mor sites was due to the stability of the tanned protein of the leaves. Jenkenson (1958) had shown that potassium deficiency in sitka spruce seedlings caused an increase in anthocyanin in the leaves. Stitt, Hyland and McKee (1946) had demonstrated that soil type was important in determining the amount of tannin in the leaves of Senecia lepidezia. These results demonstrate the importance of mineral nutrition on the production of plant secondary products.

(c). Genetic Effects

A most important factor affecting the production of tannins is, of course, genetic. Feenstra investigating the pigment present in the seed coat of Phaseolus vulgaris found that the presence or absence of leuco-anthocyanins was controlled by a single gene Sh (recessive sh). In the presence of Sh, both the mono and dihydroxylated leuco-anthocyanins (leuco-pelargonidin and leuco-cyanidin) were formed. Another gene V controlled the formation of the trihydroxy compounds and when present with Sh leuco-delphinidin was formed; V had no effect on leuco-anthocyanin formation in the presence of the recessive gene sh.

Summary of Chapter 2

1. A general distribution of tannins in plants has been discussed; leuco-anthocyanins are present in 40% of the angiosperms and were shown to be associated with "woodiness". It is probable that these compounds are present in all organs of tanniniferous plants.

2. Tannins are sometimes located in plants in special tannin cells but more often are confined to the vacuole or to special vesicles in most cells of the tannin-bearing organ.

3. The concentration of tannins changes in leaves during the growing season, and in fruits during ripening. This latter change causes a diminution in astringency.

4. Both environmental factors, light and mineral nutrition, and genetic factors have been shown to influence tannin synthesis.

Chapter 3.

Chemical methods of analysis and separation of tannins.

Chapter 3

METHODS FOR THE ANALYSIS OF TANNINS

1. INTRODUCTION

It was pointed out in the previous chapter that one of the most remarkable changes which take place in many fruits on ripening is the reduction in the amount of tannins, and this is accompanied by a reduction in the astringency of the fruit. The question "what is meant by a reduction in the amount of tannins?" does not seem to have been satisfactorily answered by previous workers in this field. In fact, it generally seems to have been assumed that tannins actually disappear (that is, are metabolised) during the ripening process. This, of course, might be true and the total quantity of tannin may be lessened; but it is equally possible that there is no actual loss but, instead, a change in the chemical reactivity of the tannins, that is, the same amount of tannin may give less reaction with the reagent used for the analysis. Alternatively, the amount of extractable tannin might change due either to polymerisation or to binding onto cellular protein or polysaccharide material. The work described in this and the next two chapters attempts to obtain some insight into the different ways in which a reduction in tannin might be experienced. In this chapter, methods have been developed or extended for the chemical analysis of tannins which may yield information about the degree of polymerisation, and other methods by which various tannin polymers might be separated by simple means. The next chapter (Chapter 4) describes the application of these methods to the changes of tannins which occur during the ripening of fruits. Finally, in Chapter 5 experiments are described for determining changes in astringency by measuring the binding power of tannins for enzyme protein and ways in which tannins can be recovered from such complexes for further study.

2. PRACTICAL METHODS OF DETERMINATION

Most of the classical methods for the determination of tannins in plant extracts have been developed for use with commercial tannin extracts (see Mitchell 1936, Schmidt 1955). Since such extracts are available in large quantities and also contain large amounts of tannin, it is hardly surprising to find that these methods are unsuitable in their original form for the determination of tannins in plant tissue generally. Thus the classical Lowenthal permanganate titration (1877) is carried out on the tannin extract in the presence of indigo carmine as an oxidation reduction indicator on a litre scale. The classical hide-powder method of analysis (see Schmidt 1955) in which the tannins are adsorbed from solution is a gravimetric procedure. This is also true of the Stiasny method, (Duthie 1938), in which an insoluble polymer is formed by reacting the tannin with a boiling solution of formaldehyde in hydrochloric acid solution, and the Duthie method (Duthie 1938) which relies on precipitation of the tannin with cinchonine sulphate. These methods, besides being time consuming, cannot be applied for the determination of milligram amounts of material. The last three procedures probably measure tannins only, but the Lowenthal and other methods of this type (for example, the Folin-Denis) which could be used for the determination of smaller amounts of tannin, use reagents which will also react with other phenolic compounds often present in excess of the tannins. It was of interest to investigate more unequivocal methods for the analysis of tannins proper which can be applied on the microgram scale and to use reagents which could measure polymerisation.

Such considerations led to the rejection of the gravimetric and titrimetric methods of analysis, with their possible errors when applied to sub-milligram quantities, and all the methods examined were colorimetric or, more properly, spectrophotometric.

We may divide such methods for analysis of tannins into four types. Those which use reagents which react with all phenolic hydroxyl groups, e.g. Folin-Denis, Lowenthal; those in

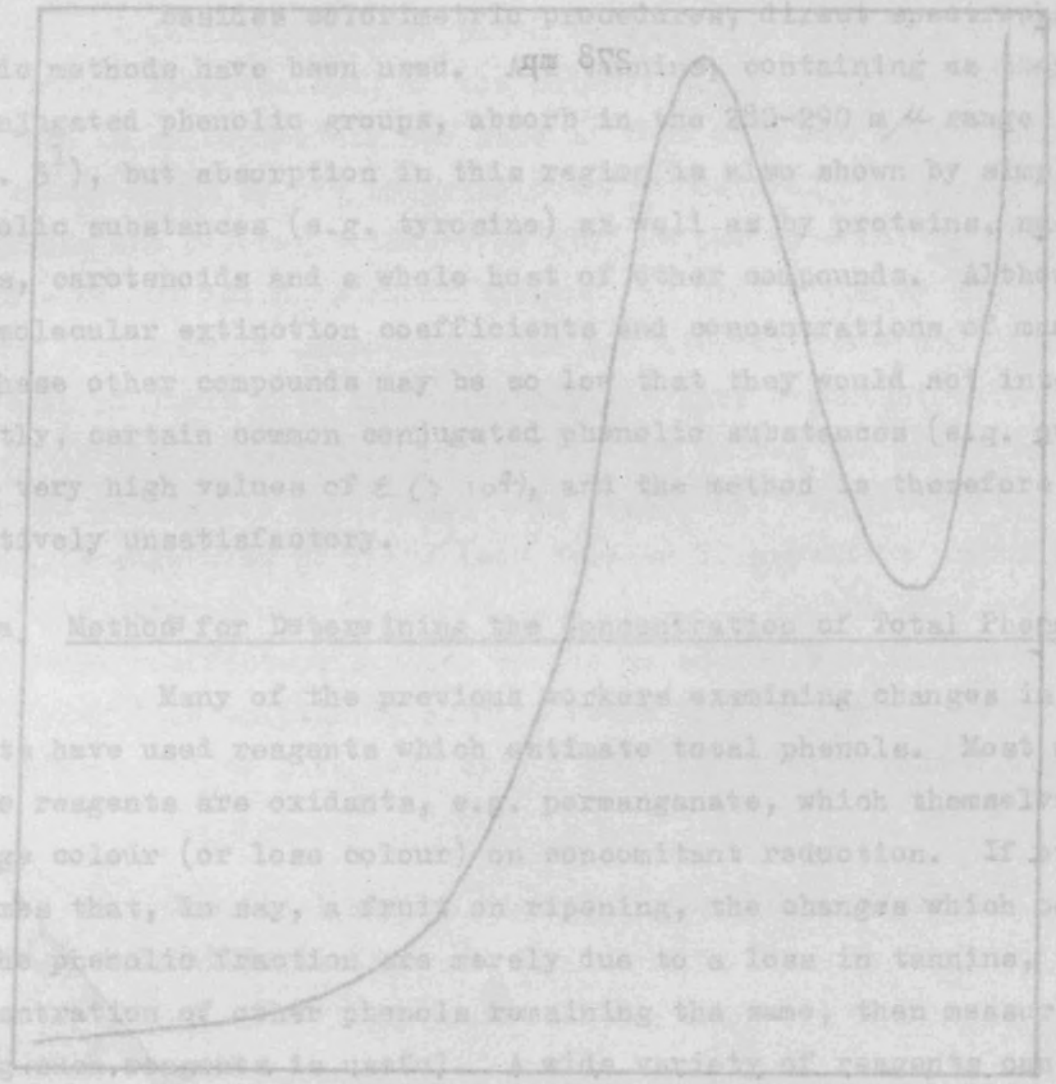
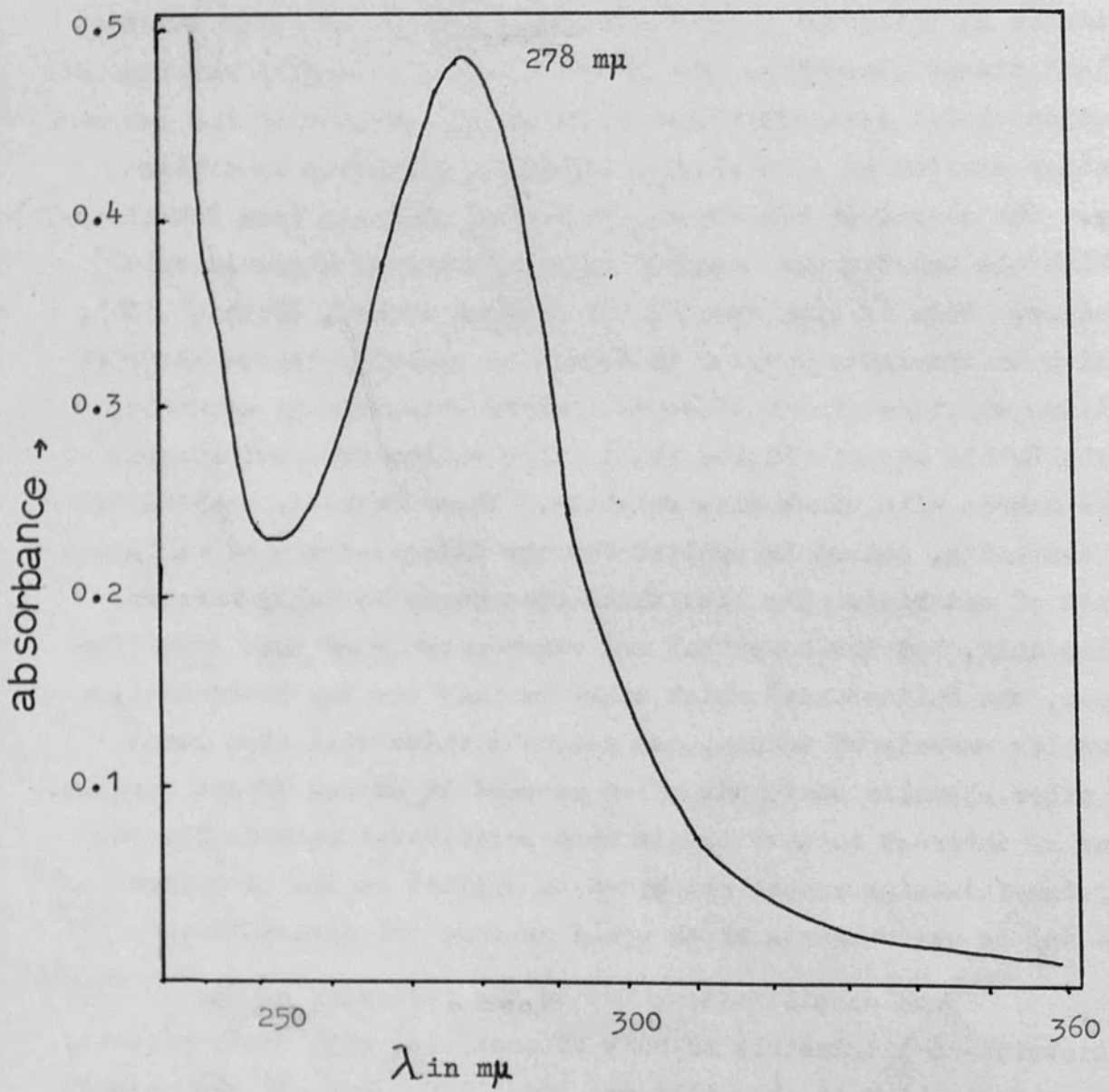


FIGURE 3.

The absorption spectrum of the tannins from avocado vanilla cell cultures.

FIGURE 3[1].

The absorption spectrum of the tannins from sycamore cambial cell cultures.



which various phenolic nuclei, for example the ortho or meta-dihydroxy groups react, e.g. ferrous salts, vanillin hydrochloric acid; those reagents which react with activiated aromatic rings, e.g. diazotised amines, 4-amino antipyrine; and those which measure only flavan units, of which the only type is the leuco-anthocyanin determination.

Besides colorimetric procedures, direct spectrophotometric methods have been used. All tannins, containing as they do ~~un~~conjugated phenolic groups, absorb in the 280-290 mμ range (Fig. 3[1]), but absorption in this region is also shown by simple phenolic substances (e.g. tyrosine) as well as by proteins, nucleic acids, carotenoids and a whole host of other compounds. Although the molecular extinction coefficients and concentrations of many of these other compounds may be so low that they would not interfere greatly, certain common conjugated phenolic substances (e.g. quercetin) have very high values of ε ($> 10^4$), and the method is therefore relatively unsatisfactory.

(a) Methods for Determining the Concentration of Total Phenols

Many of the previous workers examining changes in plants have used reagents which estimate total phenols. Most of these reagents are oxidants, e.g. permanganate, which themselves change colour (or lose colour) on concomitant reduction. If one assumes that, in say, a fruit on ripening, the changes which occur in the phenolic fraction are merely due to a loss in tannins, the concentration of other phenols remaining the same, then measurement using such reagents is useful. A wide variety of reagents can be used. Swain and Hillis (1959) examined many of these and concluded that no advantage was to be gained by using any one and chose the Folin-Denis method which has been used in this investigation. The Folin-Denis reagent is a complex mixture of phospho-molybdic and phospho-tungstic acids which oxidise phenolic compounds and are themselves reduced to give a mixture of molybdenum and tungsten blues (see Fig.3[2]).

The colour produced is stable and has a broad maximum from 620-740 mμ. This reagent does not react in a stoichiometric manner with phenolic hydroxyl groups for it can be seen from Table 3.1, pyrogallol (with three hydroxyl groups) reacts less than catechol (with two such groups) and whereas homocatechol gives less colour than catechol, methyl pyrogallol gives more colour than pyrogallol. This non-stoichiometry is a feature of all such reagents and presumably depends on the relative oxidation-reduction potentials of the various phenolic groupings. Nevertheless, in the catechins the intensity of colour produced is approximately the same as that obtained by addition of the colour given by the separate phenolic nuclei. Thus epicatechin, which has one phloroglucinol-type ring and one catechol-type ring, gives a colour which is about the same as that given by a mixture of the two separate compounds. One may assume, therefore, that catechin polymers give approximately the same amount of colour with the Folin-Denis reagent on polymerisation unless the phenolic hydroxyl groups are involved. This means that Freudenberg's and Hathway's suggested polymers (see Chapter 1, page 10, 11), providing in the latter the ortho-quinones were reduced, should give a molecular extinction coefficient similar to the monomers, but Hergert's type of polymer (see Chapter 1, p. 15), where the 7-OH group is involved, may be expected to give less colour per unit flavan molecule.

Method

The final method used was scaled down from that given by Swain and Hillis (1959).

Reagents

(a) Folin-Denis reagent.

(Recipe for Folin-Denis from the "Official and Tentative Methods of Analysis" of the Association of Official Agricultural Chemists, 8th edn., 1955, p. 144, Washington.)

750 mls. water are refluxed with 100 g. of sodium tungstate ($Na_2WO_4.2H_2O$) 20 g. of phosphomolybdic acid and 50 mls. of phosphoric acid (H_3PO_4) for two hours and made up to 4 litres.

(b) 10% ($^{W}/_{v}$) sodium carbonate.

To 1.0 ml. of the solution under examination contained in a test-tube, 1.0 ml. of the Folin-Denis reagent was added and the tube shaken. Three minutes later, 1.0 ml. sodium carbonate was added and the whole well mixed and allowed to stand for one hour. The absorbence was measured on the Unicam SP 600 at 725 m μ in a 1 cm. glass cell, against a blank which was prepared by using 1.0 ml. of water instead of the solution of the phenol under investigation.

Notes

1. The time between adding the Folin-Denis reagent and the sodium carbonate was found to be critical, and to get repeatable results it was found necessary to keep the time between the addition of these two reagents constant.

2. The absorbence was read after one hour because there was an initial increase in colour for the first 45 minutes.

3. Beer's law is not obeyed for this reagent although there is only a slight deviation from a straight line in the absorbence range 0.1-0.5; above this range the curve falls off more steeply, and solutions were diluted so as to give readings below 0.5.

4. The reagent is suitable for 1-10 μg of phenol. (+)-Catechin was used as a standard, 10 μg giving an absorbence of 0.30.

Results

The results from a number of phenolic compounds are given in Table 3[1]. (The molecular extinction coefficients (ε) were calculated from the amounts giving an optical density 0.2-0.3, due to the deviation from Beer's law.)

(b) Methods for Determining Phenolic Nuclei

(1) Vicinal phenolic hydroxyl groups.

Several methods are available for the estimation of vicinal hydroxyl groups. All depends on the fact that such groups form chelation type compounds with metals or with oxygen (or other electron donating groups). King and White (1956) described modifications of the Mitchell ferrous tartrate method (Mitchell 1936) for distinguishing between and determination of ortho-dihydroxy (catechol) and vicinal tri-hydroxy (pyrogallol) groups in tannins. This method depends on the change in colour of a ferric complex in the presence of borate buffer. Since such complexes also change colour with pH, the differences are measured at constant pH in the presence of borate and acetate (or phosphate) buffers. However, the amounts of tannin required are in the order of milligrams, and also since the extracts to be examined (Chapter 4) were in a methanolic solution, this method was not suitable for modification.

A qualitative spectrophotometric method for ortho-dihydroxy groups was developed by Swain (1954) making use of borate chelation and this was later improved by Jurd (1956). However, as pointed out above, spectrophotometric methods for tannins suffer from interference by other light-absorbing materials and modifications of this method for use with plant extracts were not examined.

A method developed by Rae (1930) for the determination of adrenaline was finally used. This method consists of measuring the intensity of the yellow colour of the chelate formed between o-dihydroxy compounds and ammonium molybdate solutions. This method has been used for the determination of o-dihydroxy compounds in the presence of monohydroxy compounds in the study of hydroxylation of the latter compounds by tyrosinase (Kendal 1949). Thus, although the method is not very sensitive, it does give a measure of vicinal hydroxyl groups only; it was found that sodium molybdate was as effective as ammonium molybdate and the reagent could be obtained in a purer form and it was used in all subsequent experiments.

The absorption spectrum of the reaction product with catechol showed that it had a broad peak with a λ max at 325 mμ and that the complex with pyrogallol gave a sharper peak with a λ max at 350 mμ ; consequently, measurements were made in this region. The reagent gave reasonably stoichiometric results with all catechols tested. (ε = 1.8 $\times 10^3$, see Table 3[1]). With pyrogallol the ε value was higher, 5.3 $\times 10^3$, and this method may have some use in determining one type of group in the presence of another. The reagent reacts with all phenolic compounds containing the correct arrangement of phenolic hydroxyl groups but does not react with compounds containing only meta-dihydroxyl groups (see Table 3[1]). In the condensed tannins, it is possible that quinones present (as in the Hathway model) may give a reduced colour on a molar basis. The complex formed from molybdenum is shown in Fig. 3[2].

Method

Reagent (a) 5% ($^W/_v$) sodium molybdate in water
(b) 0.1 M phosphate buffer pH 6.5

To 1.0 ml. of an aqueous solution under test (concentration of 50 μg/ml or higher of the phenol) in a test-tube, 2.0 ml. phosphate buffer was added, followed by 1.0 ml of the molybdate reagent. The tube was well shaken and readings were taken at 350 mμ after 15 $\pm$ 2 minutes in a 1 cm. quartz cell using a Unicam SP 500 spectrophotometer. The blank was prepared with a 1.0 ml. of water instead of the phenol solution. Using this reagent, the standard curve follows Beer's law and gives a straight line passing through the origin. It can be used for 50 to 500 μg. catechin which was used as a standard (100 μg catechin gives an absorbence of 0.16)

Notes

1. The effect of pH.

At a pH greater than 7, phenolic compounds, especially pyrogallol, are rapidly oxidised, whereas below pH 6.0 the reagent shows a considerable absorption at the wavelength used, therefore the reagent was buffered to pH 6.5.

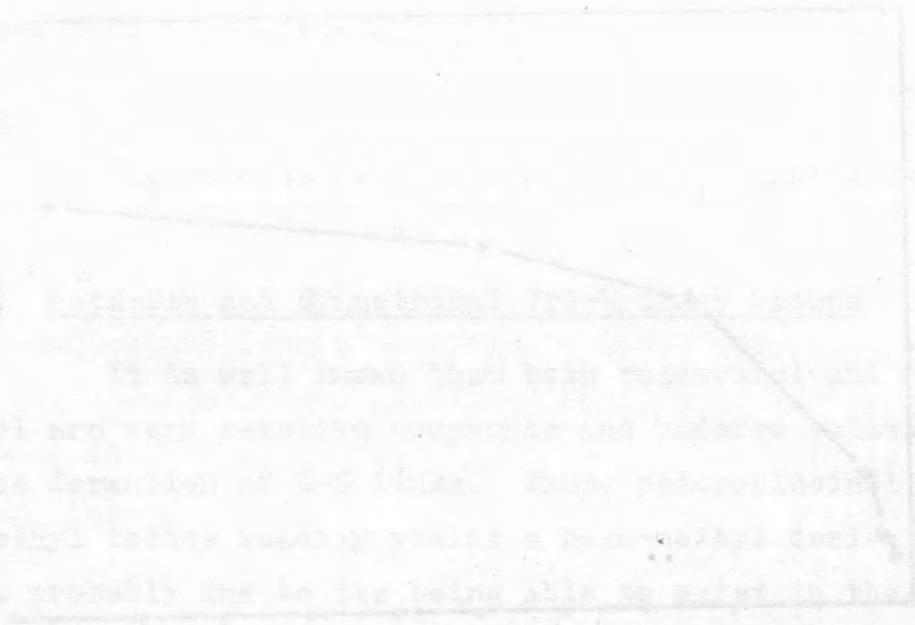
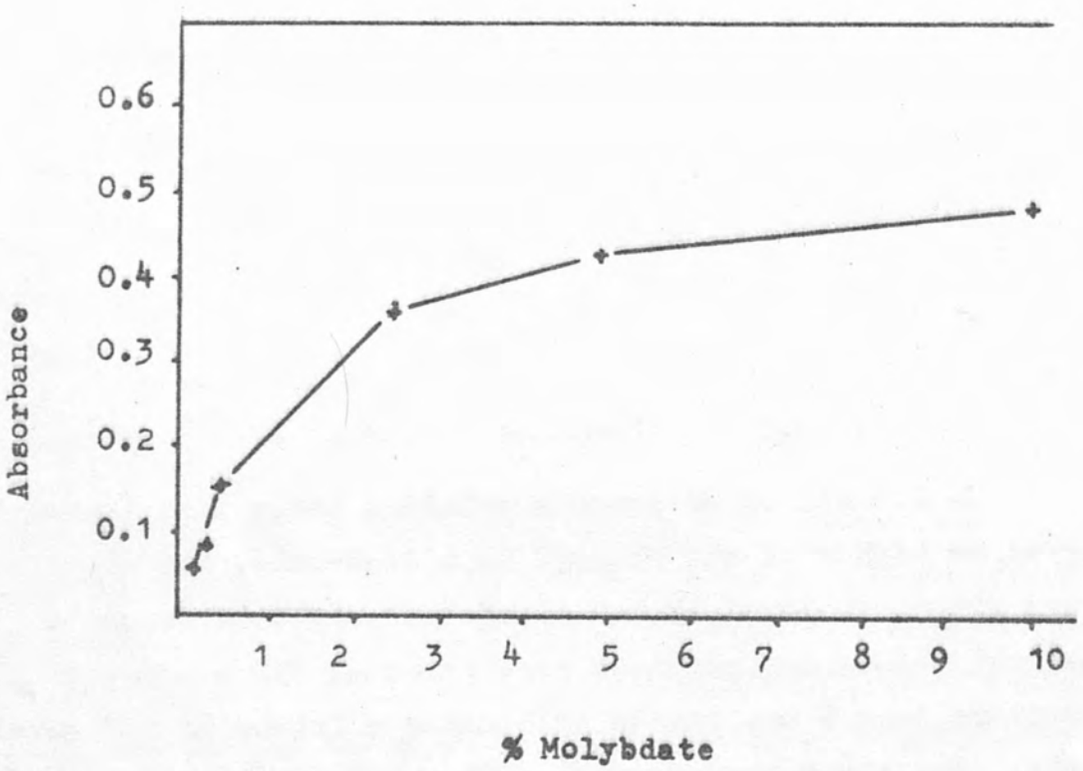


FIGURE 3^3

The effect of increasing the concentration of molybdate on the colour formed with 96μg catechol.



2. The effect of time.

On the addition of the reagent the colour developed fades (cf. Kendal who found that the colour in acid solution increased with time); after 15 minutes it is relatively constant.

3. The effect of concentration of the reagent.

5% ($^{w}/_{v}$) sodium molybdate was found to be the most useful concentration to give full colour development. At lower concentrations the optical density was lower and although higher concentrations did increase the optical density slightly the absorption of the blank was increased much more (see Fig. 3^{3}).

Results

The results for the phenols tested are shown in Table 3^{1}.

(c) Meta-Di- and Symmetrical Tri-Hydroxy Groups

It is well known that both resorcinol and phloroglucinol are very reactive compounds and undergo substitution reactions with the formation of C-C links. Thus, phloroglucinol on methylation with methyl iodide readily yields a hexa-methyl derivative (Mee, 1946). This is probably due to its being able to exist in the keto form. Similar reactivity is shown by resorcinol and pyrogallol both of which contain meta orientated hydroxyl groups. The phloroglucinol-hydrochloric acid test for lignin, in which a red colour is formed, is also evidence of its reactivity. In this case, an adduct is formed between coniferyl aldehyde groups present in the lignin and phloroglucinol. Similarly, other aromatic aldehydes in the presence of strong acid yield coloured adducts with phloroglucinol. Lindt (1881) used vanillin and concentrated hydrochloric acid for the detection of phloroglucinol groups in tannins. Swain and Hillis (1959) developed this reagent and used 1% vanillin in 70% sulphuric acid which they claimed gave more stable colours. On examination of several phenols using the Swain and Hillis reagent, it was shown that these could be divided into three broad classes. Those with

the phloroglucinol groupings which gave ε values of over 30×10^3, λ max. 500 mμ (this includes phloroglucinol, catechin, epigallo-catechin etc.); those with the resorcinol type groups which gave values of around 40×10^3, λ max 520 mμ (resorcinol, pyrogallol, brazilin) and those which had effectively no reaction. In the latter class are gallic acid which, although it has the correct grouping, is presumably less reactive due to the deactivating influence of the carboxyl group. Swain and Hillis (1959) noted that compounds with phloroglucinol or resorcinol groups, which also contained deactivating substituents such as the carbonyl group (e.g. quercetin), also gave no reaction with the reagent.

Thus when dealing with a mixture of condensed tannins based on leuco-cyanidin (or catechin) and other phenols, the colours with the vanillin reagent will represent a measure of the flavans only. Furthermore, we may expect that since polymerisation involves the phloroglucinol ring in almost all cases (see Chapter 1) there may be a reduction in the amount of vanillin reacting groups in the polymers. This is borne out by considering the results for resorcinol and ethyl resorcinol, pyrogallol and methyl pyrogallol, phloroglucinol and methyl phloroglucinol in Table 3[1]. In all cases, substitution of a methyl group causes a reduction in the values of ε. In the case of tannin polymers, this reduction might be expected to be more pronounced, due to the bulky nature of the groups attached (see formulae X, XI, Chapter 1) which would cause steric hindrance as well as enhanced deactivation of the ring.

Method

Reagent 1.0% vanillin in 70% v/v sulphuric acid.

To 1.0 ml of a solution under investigation in a 10 ml conical flask, 2.0 ml of the vanillin reagent was added from a burette, the time of addition taking 5-10 seconds; the flask was well shaken in a bowl of cold water, then left to stand for 15 minutes. The absorbance was measured on the Unicam SP 600 between 480-550 mμ, depending on the λ max of the phenol-vanillin, in a 1 cm. glass cell against a corresponding blank using 1 ml of water instead of the phenol.

With this reagent from 1-50 µg of reactive phenol can be determined, and the standard curve follows Beer's law up to an absorbance of 2.0. Catechin was routinely used as a standard; 10 µg. gives an absorbance of 0.33.

Results

The ε values for various phenols are given in Table 3[1].

(d) Methods for determining Phenols using Reagents which substitute in the Aromatic Ring

Although the vanillin reagent described above is of this general type, it can be seen that it only reacts with meta di- or symtri-substituted phenolic compounds. The reagents described in this section show no such selectivity. Many qualitative tests for phenols depend on the formation of colours with diazotised amines, and quantitative methods have been developed using such reagents.

Thus Bray et al (1952) used diazotised p-nitroaniline for the determination of phenolic compounds in urine. A number of reagents of this type were examined with little success. It was found that the diazotised p-nitroaniline not only produced a deep yellow colour with methanol alone, and indeed with many other organic solvents tested, but that the intensity of the colour produced varied with time, thus making the preparation of suitable blanks almost impossible.

An examination of several commercially available stable diazonium salts gave similar results. The salts were as follows:

Fast blue V.B. salt	4-amino-4 methoxy-diphenylamine
Fast black K salt	p-nitro-aniline azo 2:5 di-methoxyaniline
Fast red A.L. salt	alpha-amino-anthraquinone
Fast red B salt	5-nitro-2-aminoanisole
Fast red R salt	5-nitro-anisidine

Method

Reagents (a) 0.1% solution of the salt
(b) 20% ($^{w}/_{v}$) sodium acetate

To 1.0 ml of a solution under investigation containing the phenol in a test-tube, 1.0 ml of reagent (a) was added followed by 2.0 ml of the sodium acetate solution. In all cases there was a tendency for precipitates to form. Beer's law was obeyed only over a small concentration range, and for most reagents there did not seem to be a clear absorption peak.

Since these reagents could not be used easily, attention was paid to the use of 4-amino-antipyrine (see Fig. 3[2]) which, in the presence of potassium ferricyanide as a mild oxidant, gives coloured complexes with phenols. This reagent, although it had several drawbacks mainly due to the instability of the colour formed, the variation in the λ max with different phenols and the instability of the blank, was better in these respects than the diazonium salts described above. The reagent substitutesin the ortho or para position to the phenolic hydroxyl groups. The results obtained indicate that the molecular extinction coefficients vary greatly (Table 3[1]) and with polymers it might be expected to give less colour, due to the blocking of reactive sites.

The method used was that of Baruah and Swain (1957).

Method

Reagents (a) 0.2% ethanolic 4-amino-antipyrine, dissolved in 0.5Mammonium hydroxide
(b) 0.2% potassium ferricyanide

To 1.0 ml solution containing the phenol in a test-tube, 1.0 ml of the 4-amino-antipyrine was added, followed by 1.0 ml potassium ferricyanide. The optical density was read 1 minute later in a 1 cm. glass cell at 505 mμ against a blank which contained water instead of the phenol.

Discussion

Since all the substitution reagents examined were

unsatisfactory, other types, e.g. Gibbs reagent, (King, King and Manning (1957)), were not examined.

(e) Method for measuring the Flavan Unit

As mentioned previously, the conversion of leuco-anthocyanins into the corresponding anthocyanidin by heating in acid solution is characteristic for this type of molecule. It was mentioned in Chapter 1 that both monomeric and polymeric leuco-anthocyanins give this reaction. However, it must be stressed that this reaction is not quantitative, even for the monomers, and the yield of anthocyanidin produced is also dependant on the nature of the linkage and the size of the polymer. Roux and Paulus (1962) have shown that for leuco-anthocyanins based on 7-3', 4', -trihydroxy flavan -3, 4-diol (leuco-fisetidin), the yield of fisetinidin produced decreased from 24% for the monomer to 7% for the presumed trimer. However, little further decrease in yield was found up to the presumed decamer (5%). Thus, although a reduction in the leuco-anthocyanin value of an extract of a fruit on ripening may represent the conversion of monomeric units to dimers, it may also be due to the actual loss of higher polymers, probably due to reduced solubility.

The method used was that of Swain and Hillis (1959)

Method

Reagent n-Butanol containing 5% v/v concentrated hydrochloric acid

To 1.0 ml of a solution containing the phenol in a stoppered glass tube 10.0 ml butanol-hydrochloric acid was added. The tube was shaken, the stoppers removed and the tubes were put into a constant temperature water bath at 95°C and left for three minutes; the stoppers were replaced and the tubes heated for a further forty minutes. They were removed, cooled in tap water for five minutes and the absorbence of the solution was read in a 1 (or 4) cm. cell on the Unicam SP 600 at 550 mμ, against a reagent blank using

1 ml of water instead of the phenol. A standard curve was constructed using cacao leuco-cyanin, and since Beer's law was not obeyed, results were expressed as cacao leuco-anthocyanin equivalents.

Discussion

The use of all five analytical methods described in detail, Folin-Denis, molybdate, vanillin sulphuric acid, 4-amino-antipyrine and the leuco-anthocyanin reagent would be capable of yielding results of value in the examination of changes in tannins in plants. In actual fact, only three of these reagents have been used; 4-amino antipyrine was found to give poor replicates with plant extracts, and the molybdate method was developed later so that these two reagents were not examined further. The three remaining reagents taken in conjunction with the simple separation method described below gave results of value (see Chapter 4) but it is apparent more work requires to be done before the results can be interpreted correctly.

3. METHODS FOR THE SEPARATION OF TANNINS

The separation of most simple phenolic compounds, including catechins and flavan-3, 4-diols can be readily accomplished by paper chromatography (Harborne 1956). Most of the polymeric tannins cannot be separated by this means, since these substances trail badly and the higher polymers stay on the starting line in all solvents and cannot be removed from the paper by any subsequent treatment. These larger tannin molecules are, of course, absorbed onto many other polymers besides cellulose (e.g. the obvious example of proteins) and Roux (1955) made use of this in his separation procedures. He showed that simple molecules could be removed from tannins proper by filtration through colmns of hide powder and cellulose. Similarly, separation of tannins in beer has been made by adsorption onto powdered nylon (Harris and Ricketts, 1959). Such methods are useful in removing tannin which then may be estimated by difference, but have the drawback that the material which is adsorbed cannot be readily recovered.

Another way of separating tannins from other phenolic compounds is by precipitation of the polymer with protein (e.g. gelatin-salt solutions) or with alkaloids. When the actual work described in this thesis was carried out (Chapter 4), methods for the quantitative precipitation and re-isolation of tannins from such precipitates (Chapter 5) had not been worked out, and this was also true with the use of powdered nylon as an adsorbent.

Another method for the separation of tannins makes use of the differential solubility. Thus Huber (1927) stated that 40% acetone extracts more tannins than water alone, although 100% acetone extracts no tannin at all and this observation was confirmed by Duthie (1938). Unfortunately, acetone cannot be used as an extractant since it gives enhanced colours or variable blanks with several of the reagents used for analysis. This fact was realised by Hillis and Swain (1959) who used methanol instead of acetone. As described earlier (Chapter 2), they found that whereas 100% methanol readily extracted simple phenolic compounds, such as quercetin and chlorogenic acid and (presumably) monomeric leuco-anthocyanins, more condensed material was only extracted by 50% methanol. This separation of leuco-anthocyanins into monomers soluble in absolute methanol and polymers soluble in aqueous methanol has been confirmed by Swain (1960) for many other tissues. He also showed that in each case leuco-anthocyanin reacting material was left in the residue after the dual extraction. This simple method for separating the classes of tannins in fruit has been used in the work described in this thesis, and will be discussed in Chapter 4.

Table 3[1]

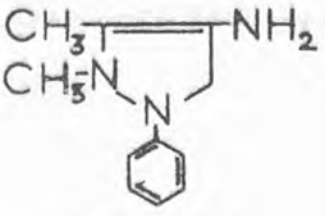
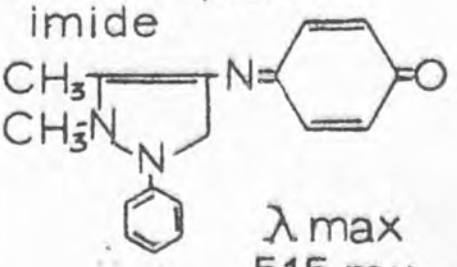
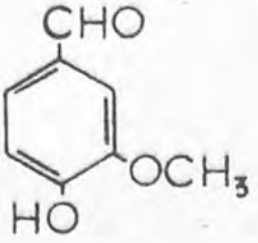
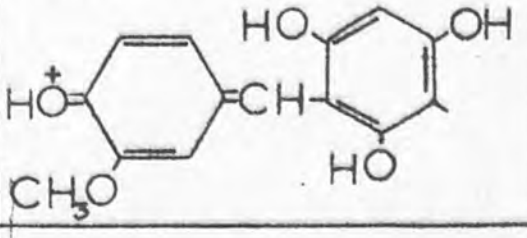
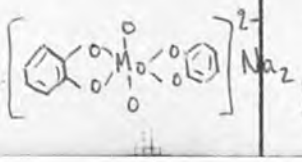
MOLECULAR EXTINCTION COEFFICIENTS OF VARIOUS PHENOLIC COMPOUNDS ANALYSED BY THE FOLIN-DENIS, VANILLIN, 4-AMINO ANTI-PYRINE AND MOLYBDATE METHODS

$\varepsilon \times 10^3$

Compounds	Folin-Denis	Vanillin	4-Amino Anti-Pyrine	Molybdate
Catechol	20.2	0.04	6.1	1.8
Homocatechol	17.6	3.6	4.0	1.7
Tertiary butyl catechol	9.7	0.05	2.0	1.3
Resorcinol	15.8	45.7	6.8	0
4-Ethyl resorcinol	10.4	38.4	2.5	0
2-4-Dimethyl resorcinol	9.3	38.4	1.6	0
Pyrogallol	10.9	44.6	0.50	5.3
Methyl pyrogallol	13.2	21.6	0.20	–
Phloroglucinol	7.7	35.0	2.6	0
Methyl phloroglucinol	5.2	28.0	0.18	0
7-Hydroxy flavan	8.7	42.1	0	0
5-7 Dihydroxy flavan	13.3	30.5	0	0
Catechin	30.7	32.9	3.3	1.8
Epicatechin (cacao)	26.7	24.7	3.2	1.8
Epigallocatechin	16.7	24.0	2.5	2.4
Epigallocatechin gallate	43.5	32.1	4.0	10.7
Brazilin	36.8	39.1	–	–
Gallic acid	18.5	0	–	–
Chlorogenic acid	21.8	0	–	–

These results are the mean of six independent determinations

FIGURE 3^2
List of phenolic reagents

Reagent	Formula	Compound formed
Folin-Denis reagent	Mixture of complex phosphotungstic & phosphomolybdic acids. $H_3PW_{12}O_{40}$ & $H_3PMo_{12}O_{40}$	Oxidises phenols & is reduced to a mixture of tungsten & molybdenum blues. W_8O_{23} & Mo_8O_{23} broad λmax 750 mμ
4-Amino-antipyrine or 4-Amino-phenazone	 + $K_3Fe(CN)_6$	Yields quinone imide  λmax 515 mμ
Vanillin	 + conc. H_2SO_4	Adduct with resorcinol & phloroglucinol rings. λmax 500 mμ 
Sodium molybdate	Na_2MoO_4 	Chelates with o-di- & vicinal tri hydroxy phenols to give yellow complex of type ~~$Na_3[OMo(O_2C_6H_4)_3]$~~ broad λmax 330 mμ

Summary to Chapter 3

1. Five different methods have been selected for assaying phenolic nuclei.

2. Four of them, the Folin-Denis, vanillin sulphuric acid, 4-amino antipyrine and molybdate method, have been investigated with model compounds and the advantages and disadvantages of these methods are discussed.

3. Methods for separating polymeric tannins from monomeric phenolic compounds have been discussed.

Chapter 4.

The changes in tannins on ripening of fruits.

Chapter 4

AN INVESTIGATION INTO THE CHANGES IN TANNINS ON RIPENING OF FRUITS

1. INTRODUCTION

The chemical methods of analysis described in the previous chapter were applied to a study of the changes in the tannins of fruits on ripening. All the fruit used for these experiments was grown in Africa; bananas (Musa sapientum L.) in West Africa, and plums (Prunus domestica L. var Gaviota) and peaches (Prunus persica Stokes) in South Africa. These fruits present particular problems to the experimenter. The fruit is generally picked at an immature stage to allow for the long sea passage to this country, and during this voyage it is held in cold storage at 5°C. Ordinarily when fruit is shipped commercially, it is ripened in this country in special storage rooms at 68°F (20°C) for a number of days before sale to the public. Under certain circumstances, this conditioning does not produce properly ripened fruit; in the case of bananas, for example, the fruit appears to be ripe on preliminary examination but the skin has a brownish cast instead of being pure yellow, and the edible portion is often astringent. The cause of this condition is not properly understood; it can be induced to some extent by keeping the fruit too long at temperatures of (+5°C) or by low temperature (<2°C) injury. Other factors, such as duration of transport and the time of picking, probably also play a part. It was in order to investigate the reason for these variations that these experiments were undertaken. However, it must be stressed at the outset that the experiments present here were more concerned with development of methods to measure changes in tannin molecular size during ripening than with a solution of the problem of transport of fruit from overseas.

Special arrangements were made with the growers associations in Africa so that the unripe fruit on arrival in this country was delivered direct from the ship to Cambridge. Unfortunately, these arrangements were not perfect, and there were sometimes overlong

delays between arrival of the fruit in Liverpool and delivery. It was unfortunate also that the total sample was very small and that we had no choice of the actual fruit received or more information about the conditions of growth of the crop. If this series of experiments were repeated, it would be essential to have much more control over the sample of fruit dispatched and preferably to have it flown to this country so that the minimum time elapsed between picking and sampling.

2. EXPERIMENTAL PROCEDURE

(a) Method of Sampling of Banana

Three bananas were selected for each sample, two replicates were done for each experiment. A 1.5" ~~cylinder~~ segment was cut from the straight part of the fruit. The skin was removed from this cylinder in longitudinal strips, care being taken to see that the pulp did not adhere to it. Two discs were cut from the skin of each of the bananas with a number 5 cork borer (8mm diameter) and the discs (six in all) were placed in a tared conical flask containing 25 mls of absolute methanol, the flask reweighed. Two longitudinal cores were then taken with a number 5 cork borer from the cylinder of pulp from each banana, one from the outer and one from the inner zone. The sample from the inner pulp contained the sterile ovules and the latex ducts and was usually brown in colour and slightly sticky. 4-5 cm. from each section, fresh weight about 2 g., (3 samples in all) were trimmed at the ends and placed in a tared flask containing 25 mls absolute methanol as before. Dry weight determinations were made in triplicate on separate samples of tissue.

(b) Method of Sampling Peaches and Plums

In both cases the fruit was cut in half lengthwise, the stone being removed. A core was taken with a number 5 cork borer from the depression where the stone had been to the outside of the fruit. This cylinder was divided into two at the non-conformity between the inner and outer mesocarp. The separate pieces were put in a tared

conical flask containing 25 mls absolute methanol, the flask was reweighed. Samples from four peaches were used, the samples being bulked to give a fresh weight of approximately 2 g. Two plums were selected for each sample, and in this case cylinders were taken from each half of the fruit and bulked to again give a sample of 2g. The plum experiment was carried out on duplicate samples and the peach experiment on triplicate samples. Dry weights were determined, as before, for each type of tissue.

(c) Method of Extraction

The flasks containing the samples were heated to boiling on a water-bath for 10 minutes. The soft samples were squashed with a glass rod to ensure good solvent penetration and extraction. (The skin samples from the banana were first heated for 10 minutes in boiling methanol to inactivate the enzymes and then macerated for 3 minutes in a top drive macerator). The flasks were removed from the water-bath, cooled and the extracts decanted through cotton wool filters into clean flasks. The tissue was re-extracted four times more with absolute methanol and the extracts from each sample bulked. The total extract was filtered by suction using Celite as a filter aid, and then reduced in volume to 50 mls on a rotatory evaporator. The residues from each sample after extraction with absolute methanol were then extracted three times with boiling 50% methanol in a similar manner. The bulked extracts from this extraction were treated with Celite, filtered, and reduced to 25 mls. on the rotatory evaporator.

(d) Methods of Analysis

The Folin-Denis and vanillin-reacting phenols were determined on each of the extracts and expressed as (+)-catechin in mg per 1 g. dry weight of tissue. The leuco-anthocyanins were also determined as described in Chapter 3 and the results expressed as cacao leuco-anthocyanin per 1 g. dry weight.

3. DESCRIPTION OF EXPERIMENTS

(a) Bananas

The bananas were hard and dark green when they arrived in Cambridge. On ripening in the dark at 20°C a number of visible changes occurred. The colour of the skin changed from bright green to yellow, water was lost to the pulp from the skin, which became thinner and formed a distinct readily peeled layer. The latex, which was present in special ducts in both skin and pulp, no longer exuded when the ripe fruit was cut. The texture and colour of the pulp changed from hard and white to soft and yellow, and the extreme astringency of the unripe fruit was lost.

Three experiments were carried out with bananas. In two experiments the bananas were sampled initially, then dark ripened for eight and eleven days respectively. In the last experiment the bananas were sampled initially, then they were divided into two lots, one lot was chilled for three days at +1°C, the other for five days, then they were both dark ripened at 20°C for eleven days. The reason for this experiment was to see how chilling affected the changes in the tannins since, as was pointed out earlier, fruit which has been chilled does not ripen normally and is often very astringent to the taste. Only the results of the first of these experiments are given in Table 4^2. The results of the second experiment were similar, and the chilling experiment gave ambiguous results, probably due to the poor initial quality of the sample.

(b) Plums

The plums were originally very hard and green and they ripened to a yellow or a red colour, with the flesh very soft and extremely juicy. In the first experiment the changes in the Folin-Denis, vanillin and leuco-anthocyanin reacting compounds were determined in large and small plums, sampled initially and at intervals over a period of twenty-three days storage at 11.8°C. The second experiment was similar to this but only the small plums were sampled; initially, these were chilled for five days at 0°C before

being ripened at 11.8°C and sampled over a period of 62 days, when the fruit had become eating ripe. The results observed with the large plums in the first experiment are given in Table 4^3. The results from the small plums from this and the other experiments were similar and no real difference was apparent as a result of chilling.

(c) Peaches

Two batches of peaches were analysed, samples from the first batch were sampled initially and the remainder ripened for one week at 19°C in the dark. The second batch was over-ripe when they were received and they were only sampled initially. The interesting point about the second batch was that some were extremely astringent. The results for the first of these experiments are shown in Table 4^4 and a comparison of astringent and non-astringent fruit in the second in Table 4^5.

4. DISCUSSION OF RESULTS

(a) Before discussing the results shown in Tables 4^2, 4^3, 4^4 and 4^5 in detail, it is of interest to consider the changes which may be involved in the tannins on ripening. As mentioned in the introduction to Chapter 3, the observed "disappearance" of tannins as analysed could equally be due to changes in their chemical reactivity and/or their extractibility.

Swain (1960 unpublished) examined the changes in catechins on autoxidation in solution at pH 8.3 (cf. Hathway and Seakins, 1958) and his results are given in Table 4^1.

Table 4[1]

Changes in Vanillin Reacting Compounds and Vanillin/Folin-Denis Ratios on Autoxidation of Certain Phenolic at pH 8.3

	% Change After: 4 Hours		24 Hours	
	V/FD	V	V/FD	V
(+)-Catechin	- 4	-13	-36	-56
(+)-Gallocatechin	+ 7	-15	+15	-45
Phloroglucinol	+ 8	- 4	-35	-38
Phloroglucinol + Catechol	-65	-73	-	-
Phloroglucinol + Pyrogallol	-32	-67	-	-

V/FD. vanillin/Folin-Denis ratio. V = vanillin; V/FD and V at time, 0 ≡ 100 (no precipitate was formed until solutions were acidified.)

It can be seen that all compounds show a reduction in their reaction with vanillin after oxidation and we can conclude, therefore, that under these polymerisation conditions there is a loss in chemical reactivity. Swain (1960 unpublished) also showed that acid polymerisation of catechin gave similar results. Bearing this in mind, it is obviously difficult with a single analytical method to decide what is happening to the tannin fraction. Swain (1956) suggested that use could be made of the relative changes in the reactivity of these molecules as the molecular weight increases. He believed that the unsubstituted phloroglucinol moiety of the leuco-anthocyanins reacted stoichiometrically with vanillin, and the pyran ring (as a whole) was responsible for the development of anthocyanidin colour on heating (cf. Chapter 3). He suggested that as polymerisation proceeds, the ratio of vanillin to leuco-anthocyanin reacting material would decrease, presumably because the formation of head to tail links (cf. Hathway, 1958) reduces the propensity of vanillin to react with the C-C linked phloroglucinol nuclei; the later results he obtained (Swain, 1960)

support this hypothesis. For example, in the case of (+)-Catechin, Table 4[1], there is a marked reduction in the V/FD ratio. (Leuco-anthocyanin reacting material could obviously not be used as a measure of total polymer in this case.) With (+)-gallocatecatechin, where tail to tail polymerisation probably ensues (cf. Hathway) no reduction in the ratio (V/FD) would be expected and this is what was observed (Table 4[1]). If we now consider the further polymerisation of a leuco-anthocyanin trimer with an initial vanillin leuco-anthocyanin ratio (V/LA) of, say, 1.0, then this ratio will be expected to decrease since, as mentioned above, there will be a reduction in the number of vanillin reacting sites available, due to the formation of C-C bonds, whereas the leuco-anthocyanin value will probably remain constant (cf. Roux and Paulus 1962, also Chapter 3 page 49). For a monomeric leuco-anthocyanin, of course, initial dimerisation may give a reduction in anthocyanidin production (Roux and Paulus 1962) which would increase the vanillin/leuco-anthocyanin ratio. In the case of leuco-delphinidin-like polymers, no change in the vanillin/leucoanthocyanin ratio might be expected if the further polymerisation involves tail-to-tail polymers.

These arguments presuppose that polymerisation of the leuco-anthocyanins occurs by the formation of C-C links which interfere (or not) in the vanillin reaction. Although such links are most probably formed when catechins polymerise (see Table 4[1]), the polymerisation of leuco-anthocyanins may well follow a different course involving the formation of ether links (cf. Hergert formulae, Chapter 1, page 15). Such changes, although not involving the formation of C-C bonds, might well reduce the ability of vanillin to react with the phloroglucinol ring by some steric effect. Obviously, more experiments with model compounds are required before any changes which might be observed can be adequately described in chemical terms.

As mentioned in Chapter 3, Hillis and Swain (1959) showed that monomeric, oligomeric and polymeric tannins could be partly separated from each other by their differential solubility in absolute and 50% methanol. Thus, the ratio of compounds soluble in these two solvents (50/100 ratio) should give a measure of polymerisation.

Freudenberg (1958) stated that the dimer of catechin, unlike catechin itself, had true tannin-like properties, and was very astringent to the taste. It seems likely, therefore, that it is such "oligomeric" substances (dimers and trimers) which impart astringency to the unripe fruit. When they polymerise further, they may become too large to be capable of effectively cross-linking protein. Thus an increase in the 50/100 ratio would indicate a decrease in tannins of the right size to cause astringency.

We have, therefore, two measures of increased molecular size and hence reduced astringency. One, a low vanillin/leuco-anthocyanin (V/LA) ratio, and two, a high 50/100 ratio. The application of these measures to the results in Tables 4^2, 4^3, 4^4 and 4^5 will now be discussed.

Table 4^2

The Tannins in Banana

Banana outer mesocarp. stage of ripeness	Extract MeOH%	F.D.	V	L.A.	Ratio V/LA
Unripe	100%	9.3	6.3	9.0	0.70
Unripe	50%	0.7	0.4	1.5	0.27
Total		10.0	6.7	10.5	
Ratio 50/100		0.08	0.06	0.17	
Ripe	100%	3.2	1.4	0.6	2.3
Ripe	50%	2.1	1.2	4.7	0.25
Total		5.3	2.6	5.3	
Ratio 50/100		0.66	0.86	7.8	
Banana skin					
Unripe	100%	58.0	21.0	28.0	0.75
Unripe	50%	3.6	5.3	8.0	0.66
Total		61.6	26.3	36.0	
Ratio 50/100		0.06	0.25	0.29	
Ripe	100%	65.0	16.0	29.0	0.55
Ripe	50%	9.5	5.5	13.0	0.42
Total		74.5	21.5	42.0	
Ratio 50/100		0.15	0.34	0.45	

F.D. Folin-Denis reacting compounds as mg catechin/g. dry weight.

V vanillin reacting compounds as mg catechin/g. dry weight.

L.A. leuco-anthocyanin reacting compounds as mg cacao leuco-anthocyanin/g. dry weight.

Table 4[3]

The Tannins in Large Plums

Plum Outer Mesocarp Stage of Ripeness	Extract MeOH%	F.D.	V	L.A.	Ratio V/LA
Unripe	100%	12	6	9	0.67
Unripe	50%	1	1	3	0.33
Total		13	7	12	
Ratio 50/100		0.08	0.17	0.33	
Ripe	100%	16	10	7	1.43
Ripe	50%	4	2	7	0.29
Total		20	12	14	
Ratio 50/100		0.25	0.20	1.0	

F.D. Folin-Denis reacting compounds as mg catechin/g. dry weight

V Vanillin reacting compounds as mg catechin/g. dry weight.

L.A. Leuco-anthocyanin reacting compounds as mg cacao leuco-anthocyanin/g. dry weight

Table 4^4

The Change in Tannins in the Peach on Ripening

Peach Outer Mesocarp Stage of Ripeness	Extract MeOH%	F.D.	V	L.A.	Ratio V/LA
Unripe	100%	18	7.8	13.1	0.59
Unripe	50%	0.6	0.3	1.4	0.21
Total		18.6	8.1	14.5	
Ratio 50/100		0.03	0.04	0.11	
Ripe	100%	21	10.4	15.3	0.68
Ripe	50%	1.3	0.5	3.6	0.14
Total		22.3	10.9	18.9	
Ratio 50/100		0.06	0.05	0.24	

F.D. Folin-Denis reacting compounds as mg catechin/g. dry weight.
V Vanillin reacting compounds as mg catechin/g. dry weight.
L.A. Leuco-anthocyanin reacting compounds as mg cacao leuco-anthocyanin/g. dry weight

Table 4^5

A Comparison in the Tannins in Ripe Astringent and Non-Astringent Peaches

Non-Astringent Outer Mesocarp	Extract MeOH%	F.D.	V	L.A.	Ratio V/LA
Ripe	100%	20	10	15.2	0.67
	50%	1.4	0.66	2.6	0.25
Total		21.4	10.66	17.8	
Ratio 50/100		0.07	0.07	0.17	
Astringent Outer Mesocarp					
Ripe	100%	20	10.1	8.9	1.14
	50%	1.5	1.1	3.7	0.30
Total		21.5	11.1	12.6	
Ratio 50/100		0.07	0.1	0.42	

F.D. Folin-Denis reacting compounds as mg catechin/g. dry weight.
V Vanillin reacting compounds asmg catechin/g. dry weight.
L.A. Leuco-anthocyanin as mg cacao leuco-anthocyanin/g. dry weight

(b) Discussion of Tables 4^2, 4^3 and 4^4

(i) General Trends.

In each case an increase is observed in the 50/100 ratio in the Folin-Denis, vanillin and leuco-anthocyanin reacting compounds in the ripe fruit compared to the unripe fruit. This is paralleled by an increase in the vanillin/leuco-anthocyanin (V/LA) in the 100% methanol extract (except for banana skin, Table 4^2) and a smaller decrease in this ratio in the 50% methanol extract. It should be also noted that the (V/LA) ratio is higher in the 100% methanol extract than in the 50% extract in each case.

(ii) Changes in Tannins in the Banana on Ripening.

The results of one of these experiments is shown in Table 4^2 and is the most dramatic in this series. The changes in tannins are shown in both the skin and the outer mesocarp. If we consider the actual change in the banana outer mesocarp in detail: First the "total extractable tannins" analysed by Folin-Denis fell by nearly 50% (10.0 to 5.3), and there is a marked difference in the tannins extracted by absolute and aqueous methanol. Tannins in the first class decrease to one-third of the initial value, whereas those of the second class increase threefold. These changes are paralleled by the vanillin and leuco-anthocyanin reacting substances; in the case of the latter, the drop in absolute methanol extractives being over 90%.

The vanillin:leuco-anthocyanin ratio of the absolute methanol extract increases on ripening, which shows that there is a loss of (presumed) oligomeric material (the final ratio is greater than that expected for monomers); the decrease in the ratio in the 50% methanol extracts suggests that some polymerisation may have taken place. The smallness of the change might be due to the fact that since further polymerisation might be presumed to lead finally to complete insolubility, the limit in molecular size has almost been reached. In this fruit the analytical changes are in accord with the hypotheses made above, p.60 The oligomeric tannins, which were present in the unripe fruit, had been polymerised further to give 50% methanol soluble and insoluble polymers

which were no longer astringent; whereas monomeric substances, which were too small to be astringent, have remained unchanged. These changes found in the outer mesocarp on ripening are similar to those found by Barnell and Barnell (1945), (Chapter 2, p. 34).

There is quite a different series of changes in the banana skin (Table 4^2). Here, the total tannins (F.D.) actually increase on ripening and although the general trends discussed above show polymerisation has taken place it is not nearly as marked as in the fruit. This is hardly surprising since even in the ripe banana the skin is markedly astringent.

(iii) Changes in Tannins in Peach and Plum.

The changes in these two fruits were similar and can be discussed together (Tables 4^3 and 4^4 respectively). Unlike the banana, both the peach and plum mesocarp showed an <u>increase</u> in tannins on ripening whether measured by Folin-Denis, vanillin, or leuco-anthocyanin reactions. However, there was a similarity between these fruits and the banana in that the changes in the ratios of vanillin/leuco-anthocyanin reacting compounds and the 50/100% methanol solubles were in the same direction, and this fits in with the hypothesis of an increase in molecular size of the tannins on ripening. The observed overall increase in the amount of tannins could be due to causes other than net synthesis. Both the plum and the peach, when unripe, do not have the same type of astringent taste as the unripe banana, which gives such a puckery taste that it is spat out at once. The astringency of the unripe plum and the peach is less objectionable than their acidity. Thus the actual molecules giving the relatively high tannin analysis are obviously not as undesirable, weight for weight, as those in the banana, and it follows we cannot equate molecular size as shown by analysis to astringency in the mouth; also, on ripening, most of the insoluble polymeric carbohydrates in the peach and plum are converted to sugars and at the same time there is a break-down in cellular structure. This may reduce the amount of polymeric material available to complex with the tannin molecules so they are more easily extracted from the ripe fruit than from the unripe fruit.

A further difficulty lies in obtaining absolute methanolic extracts from the ripe juicy tissue of both peach and plum, and it is certain that these extracts would include some of the aqueous methanol-soluble material which would invalidate the results.

This is brought out by the results for non-astringent and astringent peaches, Table 4[5], where it can be seen that the 50/100 ratio for the Folin-Denis reacting compounds is the same in both cases. These results do show, however, that in the peach at least higher astringency is related to a high vanillin/leuco-anthocyanin ratio in both absolute and aqueous methanol extracts, but it should be noted that the 50/100 ratios for the leuco-anthocyanin reacting material are anomalous. These results point again to the fact that the changes in tannins in each fruit must be considered separately and no all-embracing theory can be set up.

One important feature which has not been mentioned is that the main leuco-anthocyanins in the banana are based on leuco-delphinidin (Simmons, 1959), whereas in the plum and the peach the compounds are based on leuco-cyanidin. One would expect, therefore, that in the banana, polymerisation of the tannin might involve no change in the vanillin/leuco-anthocyanin ratio (cf. (+)-gallocatechin, Table 4[1]) if this takes place by tail-to-tail linkage (Hathway, see Chapter 1, p. 11). Whereas in the plum and peach, polymerisation would lead to much greater changes than those actually observed. (cf. (+)-catechin, Table 4[1]). These anomalies point to our lack of knowledge on polymerisation processes and again stress the need for more experiments with model systems.

5. CONCLUSION

We may conclude from these experiments that the analytical methods described in Chapter 3 are of some use in studying the changes in tannins in the ripening of fruits, but that in juicy fruits, especially the simple method of separation of tannins, are not applicable and this makes an argument based on chemical analysis difficult to sustain. In an effort to overcome these difficulties, it was decided to investigate the changes in the astringency of tannins by measuring their binding power for enzymic protein. These experiments are described in Chapter 5.

Summary to Chapter 4

1. The changes in tannins on the dark ripening of bananas, peaches and plums have been determined in order to gain some insight into the causes of loss in astringency.

2. Loss of astringency has been assumed to be due to polymerisation of the tannins and has been measured by the changes in two ratios, a reduction in the vanillin to leuco-anthocyanin ratio (V/LA) and an increase in the 50% methanol soluble to 100% methanol soluble extractives.

3. The results would seem to confirm in part the hypothesis put forward by Swain (1956) that the loss of astringency in fruits on ripening is due to polymerisation of the tannins with the leuco-anthocyanins playing an important part. However, difficulties in dealing with juicy fruits point to the need for obtaining supporting evidence by other means.

Chapter 5.

The determination of the molecular size of tannins by their effect on enzymes.

Chapter 5

THE DETERMINATION OF MOLECULAR SIZE OF TANNINS BY THEIR EFFECT ON ENZYMES.

1. INTRODUCTION

In Chapter 1, tannins were described as compounds capable of cross-linking protein, presumably by forming hydrogen bonds between the phenolic hydroxyl groups of the tannin and the amino groups of the protein. This reaction is the basis of the conversion of animal hide into decay-resisting leather. The manufacture of leather by tanning animal hides with vegetable extracts is almost as old as the documented history of man. Leather articles have been found in the tombs of the Pharaohs in Egypt some 4000 years old, and pictures of tanneries have been found on Egyptian papyri from the second dynasty. Leather articles have been found dating from Neolithic times in both England and Denmark, and leather of Roman origin has been found in certain tombs in Paris. Like so many traditional crafts very little is known about the reaction between animal hide "collagen" and aromatic phenolic compounds "tannins". The first serious study on this problem was by Sir Humphrey Davy in 1803. He studied the ability of extracts from various plants to precipitate gelatin and to tan calf-skin. In experiments on the precipitation of gelatin he found the relative concentration of the gelatin and the tannin to be important. When the quantities were kept constant but were present in more dilute solution, less precipitate was formed. He also noticed that if excess gelatin was present, the tannin-gelatin precipitate redissolved: allowing for this he determined the quantities of tannins from various plant extracts which combined with gelatin and with calf-skin (Table 5[1]).

Table 5[1]

PERCENTAGE TANNIN ~~ADSORBED BY GELATIN AND CALF-SKIN~~ IN PROTEIN-TANNIN COMPLEXES

Extract from:	Gelatin	Calf-skin
Oak bark	41	26
Leicester willow bark	43	28
Spanish chestnut	39	-
Alpello galls	46	39
Catechu	41	21

These results show that the type of protein rather than the type of tannin seem to be important in determining the amount of tannin in the complex. These results have been confirmed by later workers (Table 5[2]).

Table 5[2]

PERCENTAGE TANNIN ~~ADSORBED BY VARIOUS PROTEINS~~ IN PROTEIN-TANNIN COMPLEXES

Source of tannin	Protein	% Tannin adsorbed	Author
Calluna vulgaris	Casein	26	Handley (1961)
Circacaca lutetiana	"	31	"
Chamaenion angustifolium	"	31	"
	Codfish		Gustavson (1956)
Catechu	Collagen	22	"
Gambia	"	20	"
Tannic acid	"	22	"
Sumach	"	17	"
	Eel-collagen	36	"
Mimosa	"	26	"

The amazing thing about these results is that Gustavson obtained the same result for catechu-tanned cod-skin that Sir Humphrey Davy did for calf-skin 153 years earlier.

Handley (1961) suggested that tannin-protein complexes are important, concerning both the digestibility of certain plants as forage and the formation of vegetable litter. He suggested that the formation of humus on mor sites is due partly to the acidity of the soil which prevents the dissociation of the tannin-protein complex and partly to the type of tannin present in the leaves of plants which grow on such sites. He suggested that these complexes are much more resistant to breakdown with release of nitrogen to the soil than complexes formed from tannins present in plants grown on mull sites.

He mentioned that sheep fed on fresh Calluna leaves compared well with those fed on grass, but if the dried Calluna was used the sheep lost weight, and he suggested that trypsin could not act on tanned-protein present in the Calluna hay.

There has not been much work on the action of tannins on enzymes and most of the published work refers unfortunately to the action of enzymes on substrates which are themselves polymeric and capable of binding tannins. Thus in the work of Barnell and Barnell (1945), which has been referred to previously, the measurement of astringent tannin in banana was carried out by adding the tannin containing extract to starch solution subsequently adding the enzyme diastase. Although they showed that inhibition was not obtained when the tannin containing extract was run down a column of hide-powder prior to the addition of starch, it was not clear whether the enzyme or the substrate had been tanned. We have observed that agar, starch and pectin form complexes with tannins (Goldstein and Swain, 1962 unpublished), which destroy their ability to form gels. A similar criticism can thus be levelled against the work of Hathway and Seakins (1958) who determined the effect of myrobalan and gambia tannins on the hydrolysis of "native" pectin and low methoxypectin by various pectinases by a viscosity method. In one experiment they treated a solution of pectin at pH 3.0 with tannins and measured the decrease in viscosity on adding the enzyme. When their results are expressed as time for loss of half the original viscosity, it is apparent that no true inhibition is found except in the case of myrobalan tannin with low methoxy pectin (Table 53). With pectin itself there is actually an apparent increase in rate.

Table 53

EFFECT OF TANNINS ON PECTINASES

Treatment	Low methoxy pectin	High methoxy pectin
	Time for loss of half viscosity in minutes	
Control	54	60
Myrobalan tannin	210	50
Gambia tannin	53	34

Here again it is undoubtedly the substrate which has been tanned. Hathway and Seakins did demonstrate that treatment of pectinase itself with gallo-tannin gave inhibition, and that a part of the enzyme activity could be recovered by "solvent stripping" which presumably refers to treatment with acetone. (Table 54)

Table 54

TIME IN MINUTES FOR LOSS OF HALF THE ORIGINAL VISCOSITY

Control	115
Gallotannin-pectinase complex	1320
Regenerated complex	263

The inhibition of pectinase by tannins is thought to be one factor responsible for the resistance to fungal attack by certain plants. Many fungi secrete pectinases (macerating enzymes) in order to gain entry into the plant. Thus Byrde (1957), investigating the resistance of some varieties of cider apple to infection by the brown rot fungus (Sclerotinia fructigena), found that the presence of tannins and oxidised tannins caused a reduction in the activity of the "macerating enzyme" from this fungus. Table 55 shows some results obtained using the oxidised juice of the cider apple Yarlington Mill.

Table 55

THE EFFECT OF TANNINS ON THE MACERATING ENZYME

Enzyme treatment	Mean macerating activity
Control (water)	7.5
Gelatin 0.2%	7.5
Yarlington Mill juice	2.9
Gelatin treated juice	6.7
Gallotannin 0.2%	2.0

Again the use of a complex substrate, in this case cucumber slices, does not preclude the possibility that the tannin-protein dissociates and the tannin attaches itself to the substrate which is thereby protected from attack.

It has been shown that protein-tannin complexes can be split, and in some cases the enzyme activity can be completely recovered. The Polish workers Mejbaum-Katzenellenbogen, Dobryszycka, Jaworska and Morawiecka (1959, 1961, 1962) in a series of papers showed that pH and the concentration of the reagents were important in forming complexes between tannins and various proteins. They demonstrated that such complexes could be dissociated by dilution, or by treatment with urea or caffeine, and the enzymic, electrophoretic, and immunological properties of the protein were thereby recovered unimpared. The tobacco-mosaic virus has also been shown to be inactivated and precipitated by a strong solution of tannic acid, and both Thresh (1956) and Cadman (1959) demonstrated that this inactivation could be reversed by dilution, presumably due to the complex being dissociated.

With these experiments in mind it was decided to investigate the inhibition of enzymes by tannins as a measure of their protein-binding power, and hence to have a direct measure of their astringency. It was hoped also that these experiments would indicate what importance such complexes might have on the process of ripening (e.g. the climacteric of banana, Biale and Young (1961)) and on manufacturing processes (e.g. the fermentation of cacao). Other experiments on methods for the dissociation of such complexes were designed to afford ways of obtaining tannin fractions for further chemical study. The enzymes chosen were those which act on a simple substrate, and are as follows:

β-glucoside glucohydrolase (β-glucosidase) I.U.B. No. (3.2.1.21.)
Donor: H_2O_2 oxidoreductase (peroxidase) I.U.B. No. (1.11.1.7.)
H_2O_2:H_2O_2 oxidoreductase (catalase) I.U.B. No. (1.11.1.6.)
Alcohol:NAD oxidoreductase (alcohol dehydrogenase) I.U.B. No. (1.1.1.1.)
L-lactate:NAD oxidoreductase (lactate dehydrogenase) I.U.B. No. (1.1.1.27.)
(I.U.B. No. refers to the number assigned by the Commission on enzymes of the International Union of Biochemistry).

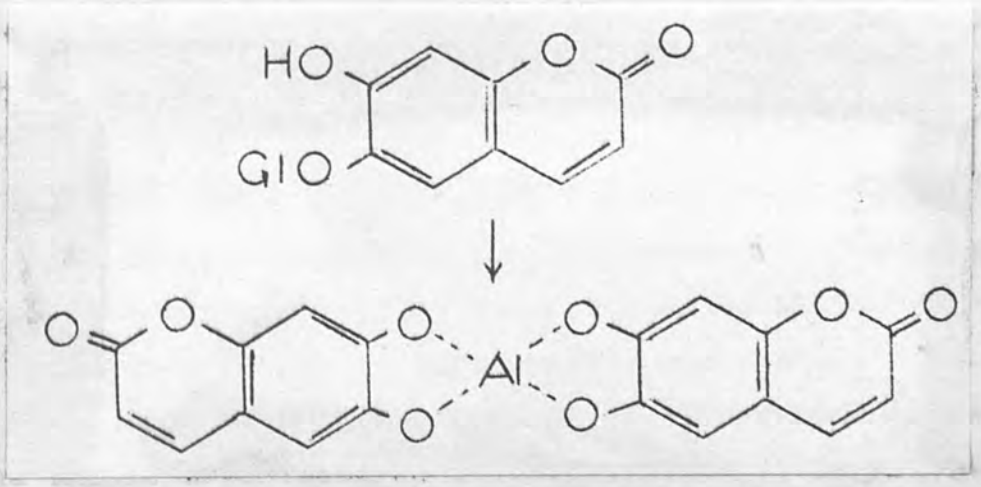
After a series of preliminary experiments, β-glucosidase from sweet almonds was chosen for detailed study. This enzyme was commercially available in a reasonably pure form, easy to assay using the method described below, there was little or no possibility of the substrate (aesculin) forming a complex with the tannins, and this enzyme is known to be precipitated by tannins since this method is used in its purification (Helfrich et al 1932)

2. THE EFFECT OF TANNIC ACID ON SWEET ALMOND β-GLUCOSIDASE

(i) Method of assaying the activity of the enzyme.

Although continuous methods for the measurement of β-glucosidase activity have been described, they make use either of artificial substrates (p-nitro phenyl-β-D-glucoside, Jermyn, 1955) or are measured at wavelengths at which tannin may interfere, (salicylic-acid-β-D-glucoside at 309 mμ Hofstee 1955). Barnet and Swain (1959 unpublished) described a non-continuous method using aesculin as a substrate in which the amount of aesculetin produced was determined by measuring the yellow aluminium chelate at 400 mμ. This method has been modified to give a continuous method for use with a recording spectrophotometer whereby initial rates of reaction may be determined in 2 - 5 minutes.

The basis of the reaction is shown below.



(ii) Experimental

Reagents.

(a) 15 μM/ml aluminium chloride in 0.1M acetate buffer pH 4.8

(b) 4 μM/ml aesculin in 0.1M acetate buffer pH 4.8

(c) 333 μg/ml β-glucosidase (from sweet almonds, Worthington Biochemical Co.) in 0.1M tris pH 6.0

The reagents were kept separately in a constant temperature water-bath maintained at 30°C. 2.0ml of the aluminium chloride solution and 1.0ml aesculin solution were pipetted into both reference and sample 1 cm. quartz cells. The cells were covered with a paraffin wax strip (parafilm), inverted to mix, and placed in the constant temperature cell attachment of the Beckman D.K.2 recording spectrophotometer. The sample and reference were balanced 385 mμ 0.05 ml of enzyme solution was added to the sample cell from a micro-pipette, and the contents of the cell well mixed. The increase in absorbence was recorded automatically which allowed the initial rate to be calculated. The hydrolysis of aesculin follows ~~first~~ zero order kinetics for at least the first ten minutes and a straight line plot is obtained until a great excess of aesculetin is formed. A standard curve for aesculetin was determined over the range under test, (the absorbence of 0.1 M aesculetin /3mls is 0.22). The pH used, 4.8 was chosen as it is near the maxima for this enzyme (5.2) determined by a non-continuous method by Swain 1961 using a series of buffers including phosphate and citrate mixtures. Because these two acids interfere with the chelation of aluminium by aesculetin, buffers containing them were not used. In acetate buffers above pH 5.6 there was a tendency for aluminium to be thrown out of solution (as the hydroxide?) and so pH 4.8 was chosen as a suitable compromise.

(iii) Method for obtaining a tannic acid β- glucosidase complex.

It was found that the formation of a precipitate between tannic acid and β -glucosidase was dependant on pH, ionic strength and concentration of both protein and tannin.

To examine the effect of pH, the enzyme alone, or enzyme and tannic acid (both at a concentration of 1mg/ml) were allowed to stand for 30 minutes at various pH values and the supernatant

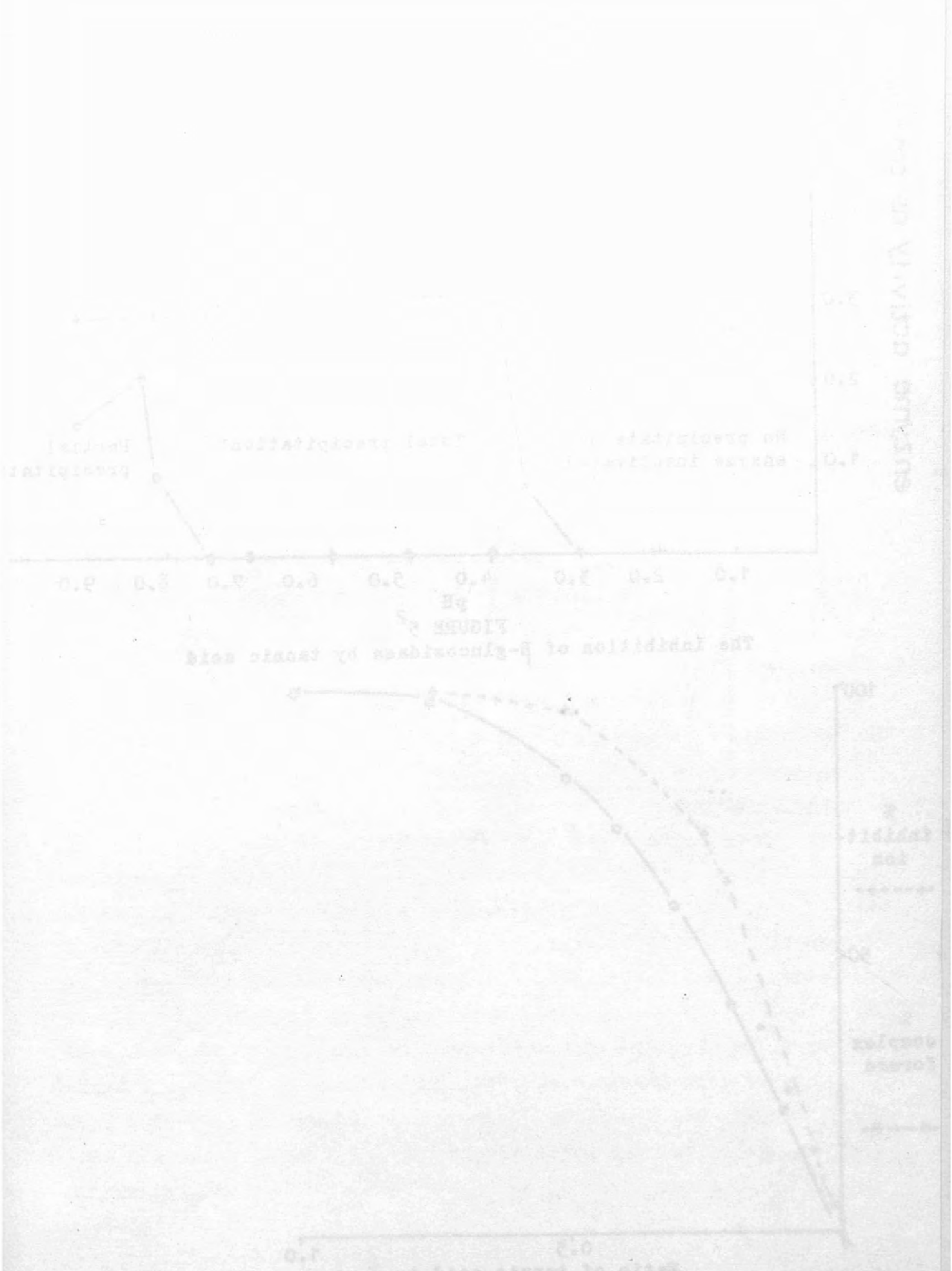


FIGURE 5[1]

The Effect of Precipitating an β-glucosidase-tannic acid complex at different pH

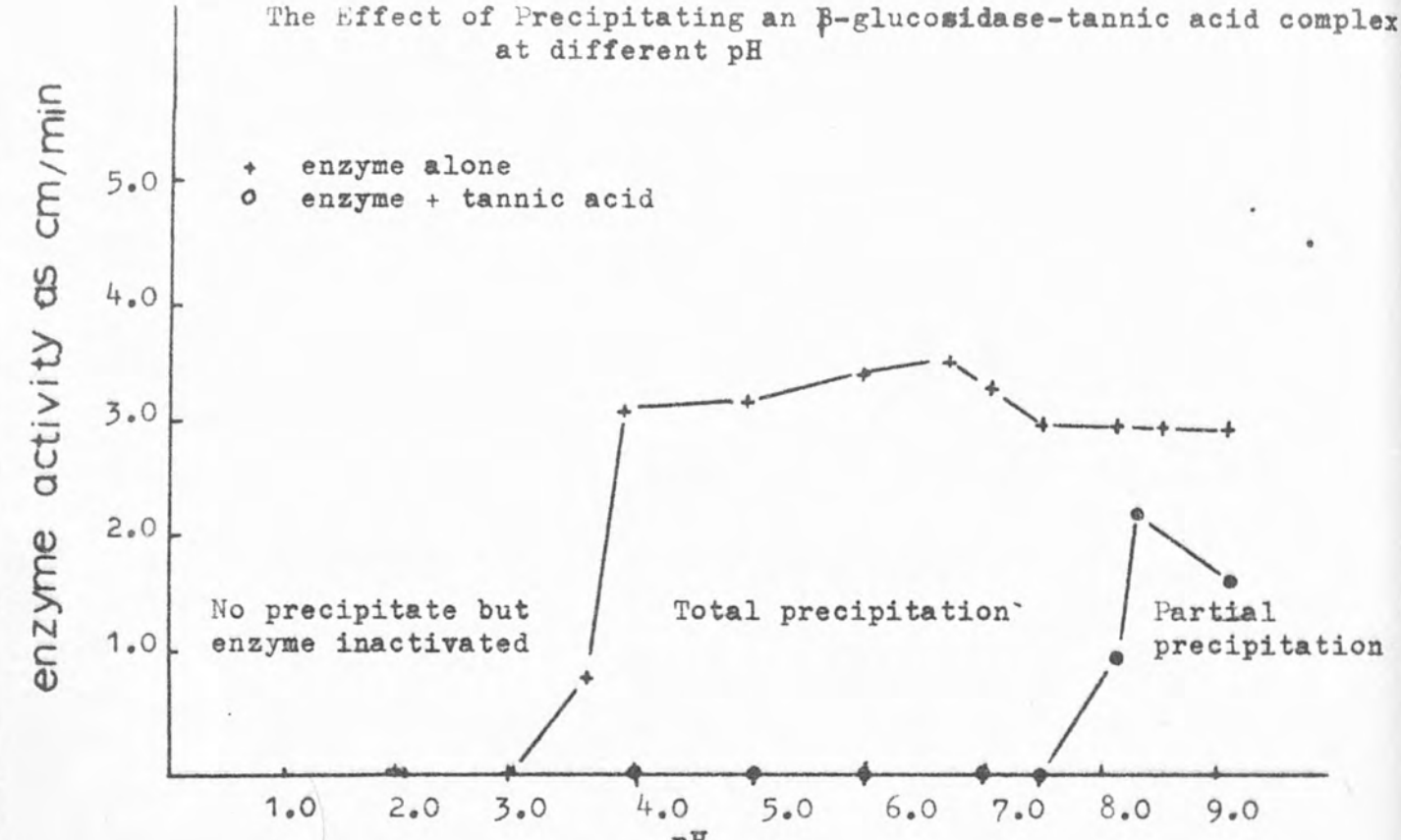
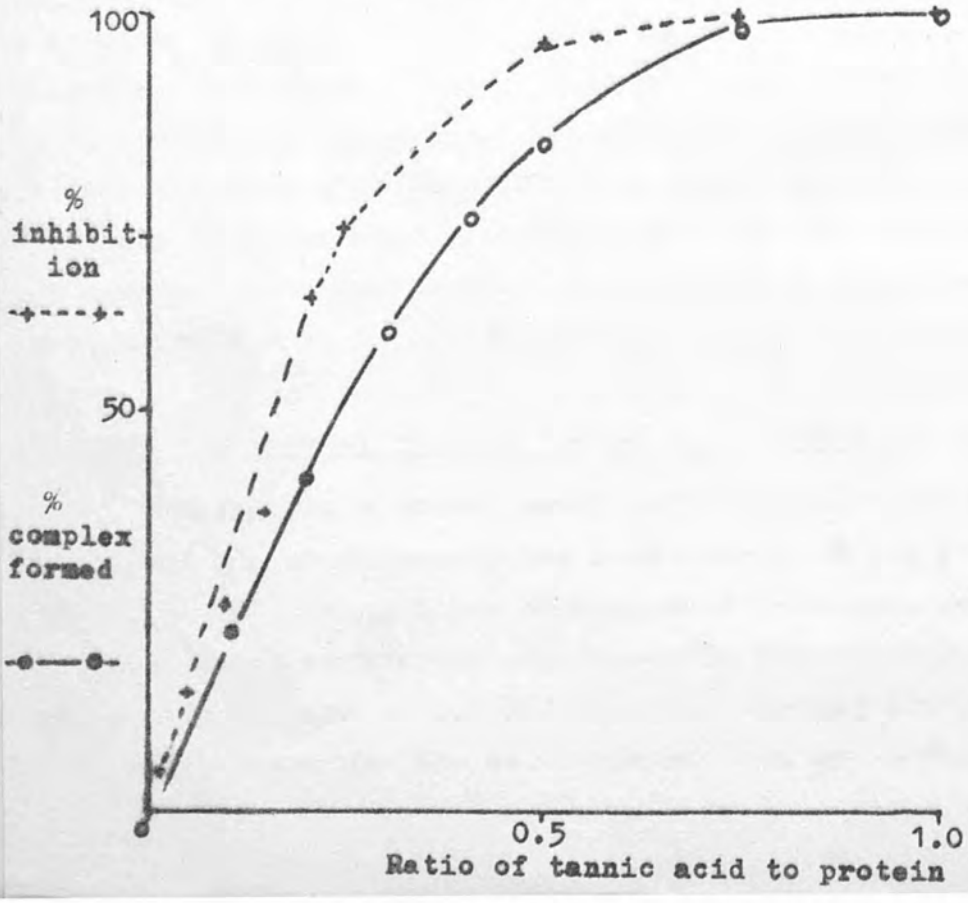


FIGURE 5[2]

The inhibition of β-glucosidase by tannic acid



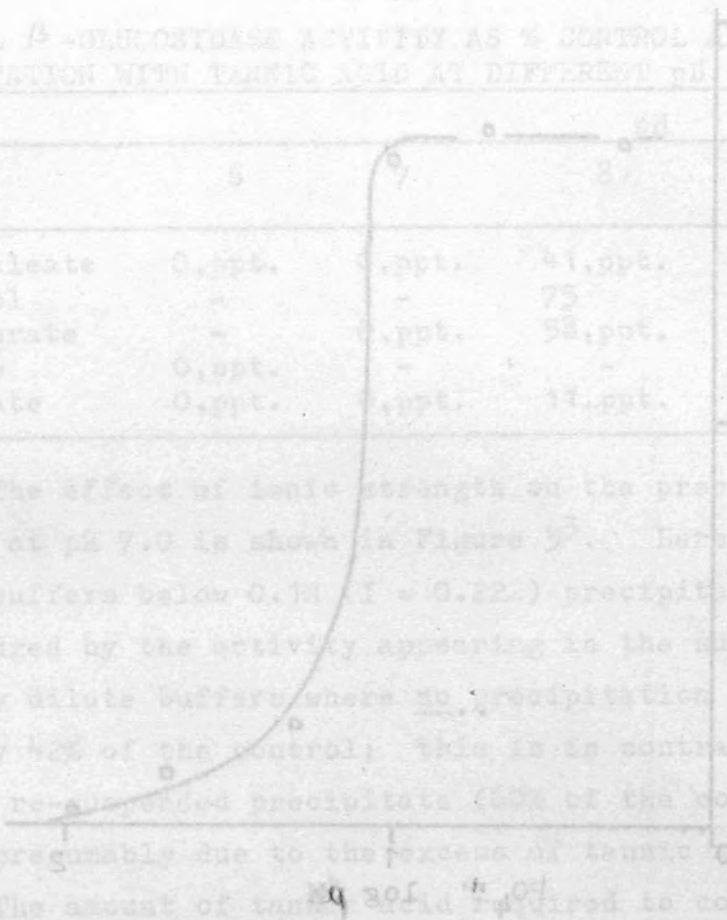
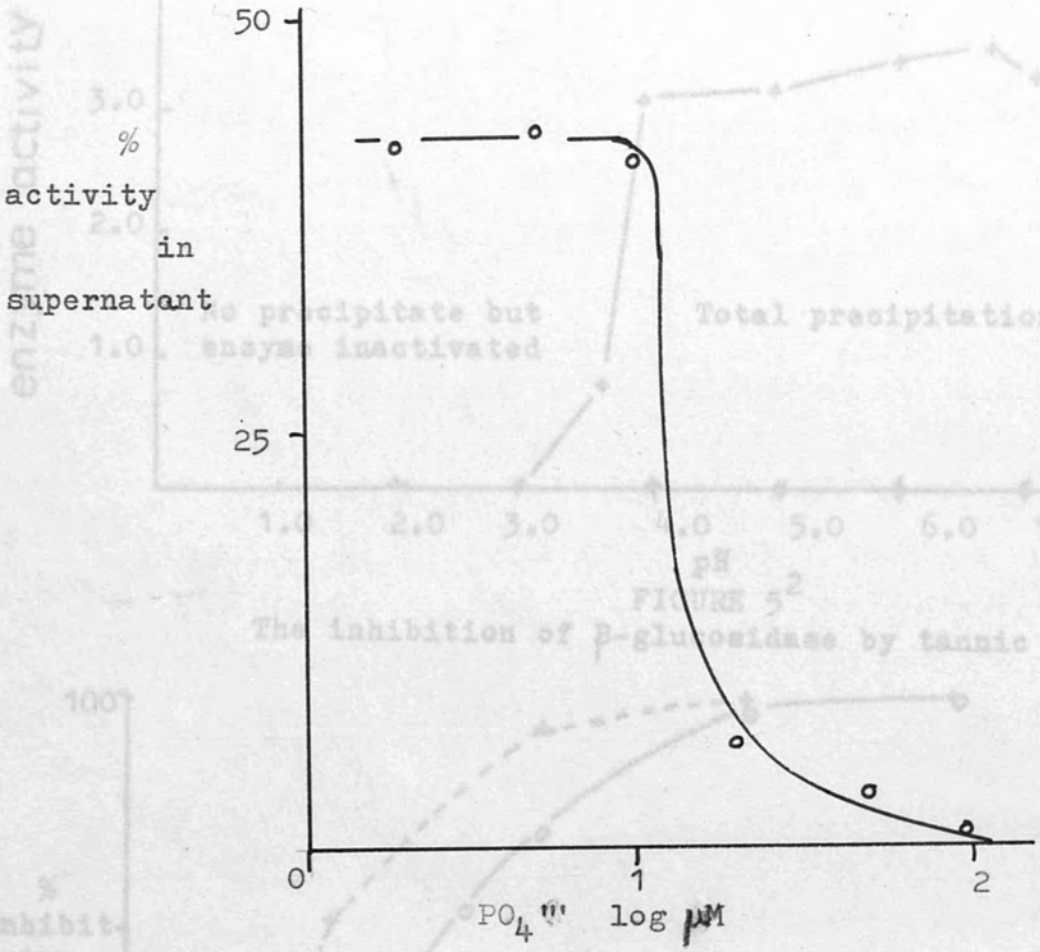


FIGURE 5[3]

Effect of phosphate concentration on the precipitation of β-glucosidase by tannic acid.



fraction obtained by centrifuging at 1500G for five minutes, was tested for activity at pH 4.8 as described above. The results are shown in figure 5[1]. This shows that precipitation is virtually complete between pH 2.0 and 7.5, but that the enzyme is denatured if allowed to stand below pH 4.0. The effect of various alkaline buffers on precipitation is shown in Table 5[6].

Table 5[6]

RESIDUAL β-GLUCOSIDASE ACTIVITY AS % CONTROL AFTER PRECIPITATION WITH TANNIC ACID AT DIFFERENT pH.

	pH			
Buffer	6	7	8	9
Tris-maleate	0,ppt.	0,ppt.	41,ppt.	-
Ammediol	-	-	75	59
Barbiturate	-	0,ppt.	58,ppt.	60
Citrate	0,ppt.	-	-	-
Phosphate	0,ppt.	0,ppt.	11,ppt.	-

The effect of ionic strength on the precipitation using phosphate at pH 7.0 is shown in Figure 5[3]. Here it can be seen that with buffers below 0.1M (I = 0.222) precipitation is incomplete, as judged by the activity appearing in the supernatant, but even in very dilute buffers where no precipitation occurs the activity is only 42% of the control; this is in contrast with the activity of the re-suspended precipitate (60% of the control Vide infra) and is presumably due to the excess of tannic acid present.

The amount of tannic acid required to completely precipitate the enzyme was determined by adding incremental amounts of tannic acid to the enzyme in phosphate buffer (0.1M pH 7.0) and determining the activity of the supernatant as before. It was found under these conditions that 1 mg of β-glucosidase in 1.0 ml. was completely precipitated by 0.75 mg tannic acid, Figure 5[2]. This compares well with the results obtained by Swain (1961 unpublished) who determined the amount of tannic acid spectrophotometrically in the supernatant in a similar experiment. He showed that 460µg of tannic acid was bound to each 1mg of β-glucosidase, but that in order to obtain this maximum binding, 700µg tannic acid was required per mg protein.

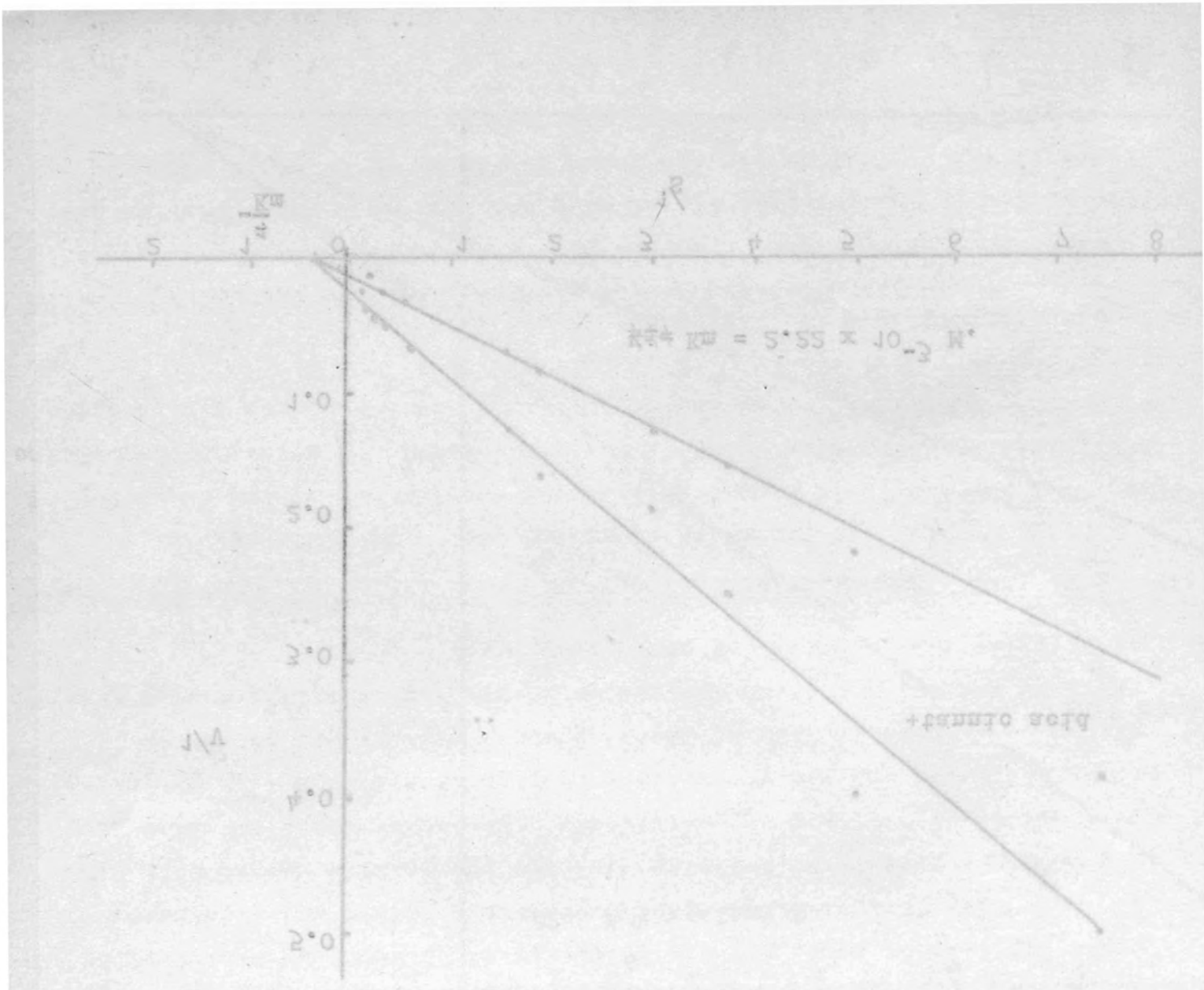


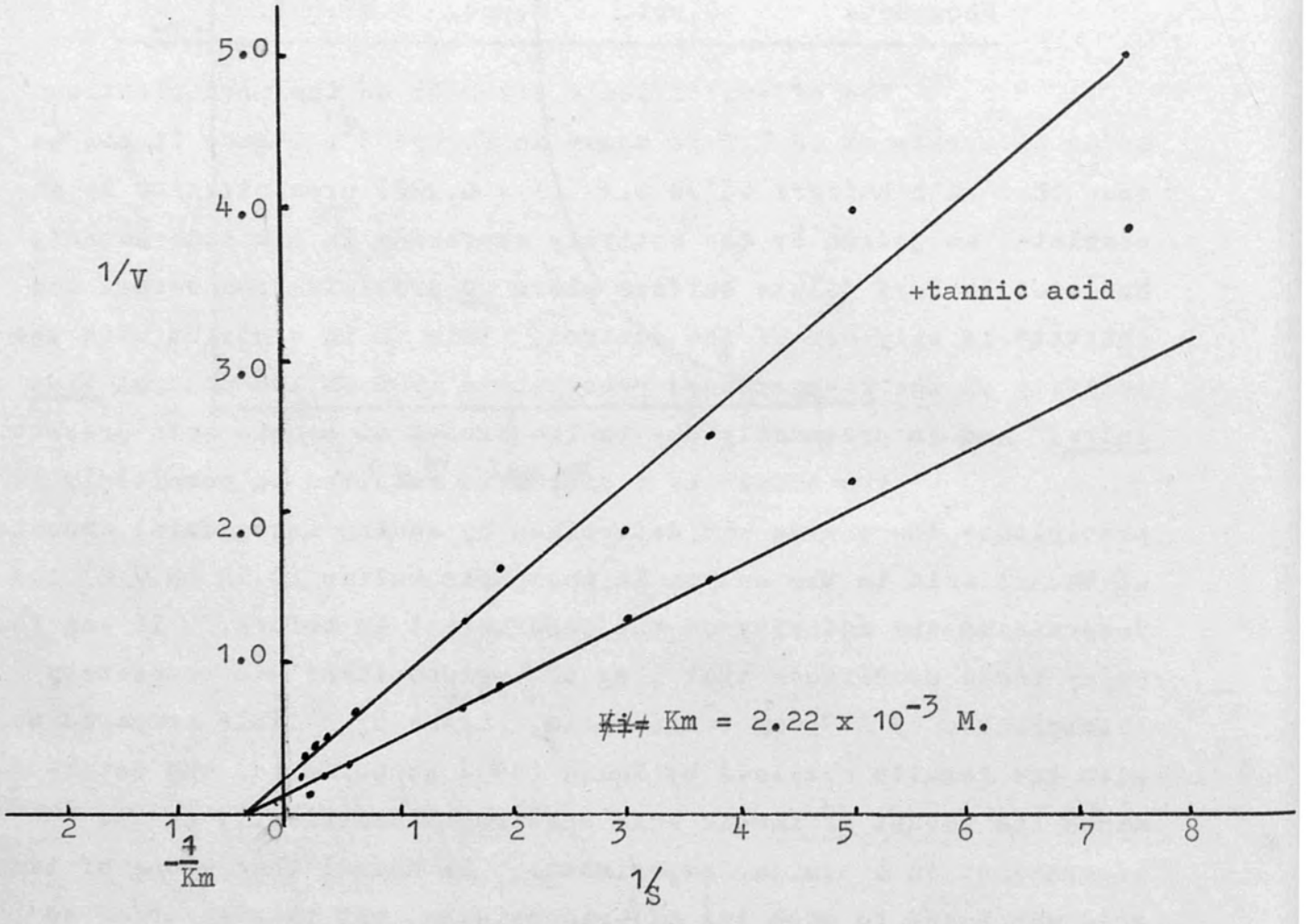
FIGURE 24

Three different plots to show the Michaelis constant K_m and the inhibitor constant K_I of β-Glucosidase and formic acid

FIGURE 5^4

Three different plots to show the Michaelis constant K_m and the inhibitor constant K_i of β-glucosidase and tannic acid

Plot of $1/S$ against $1/V$



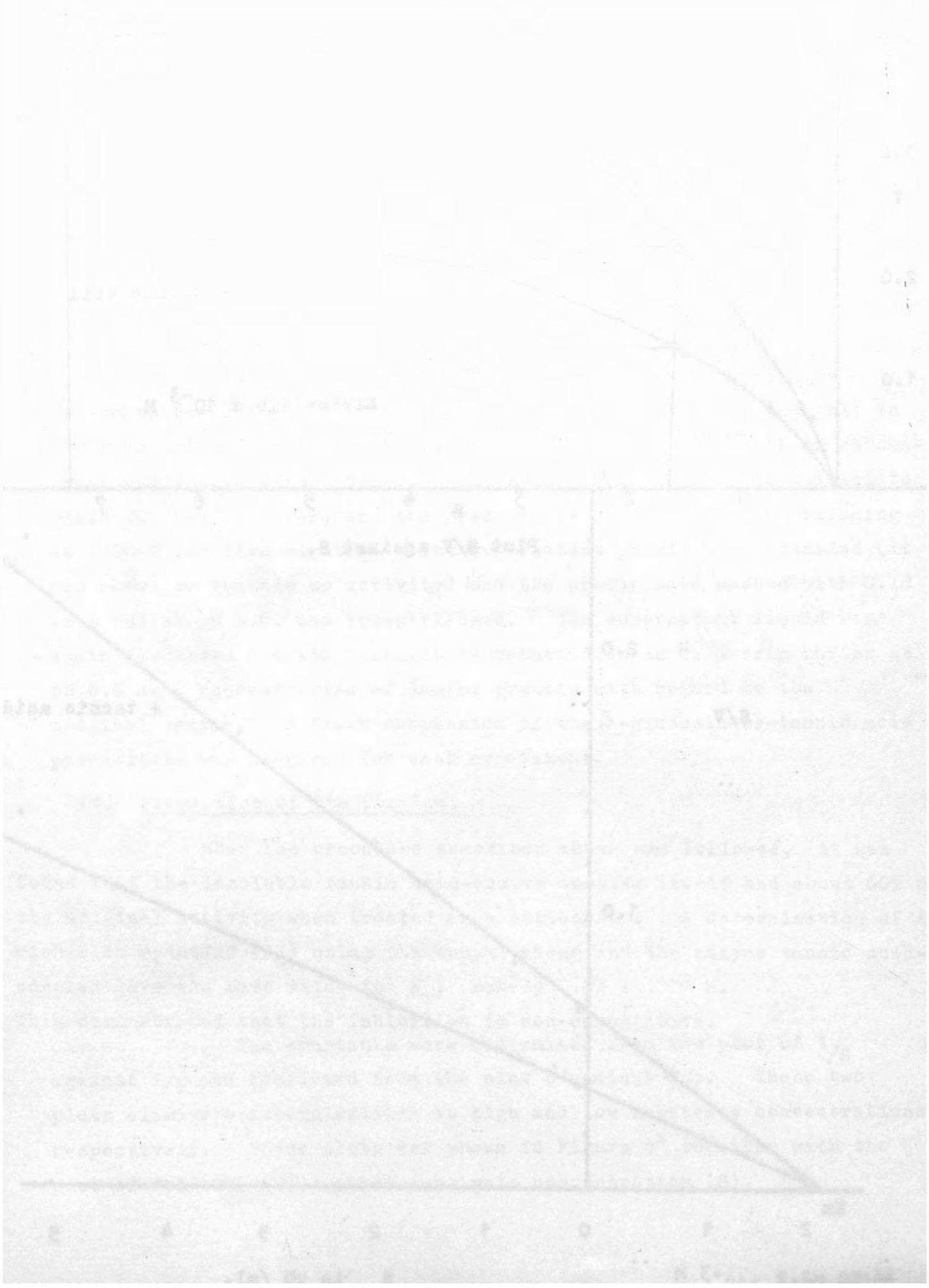
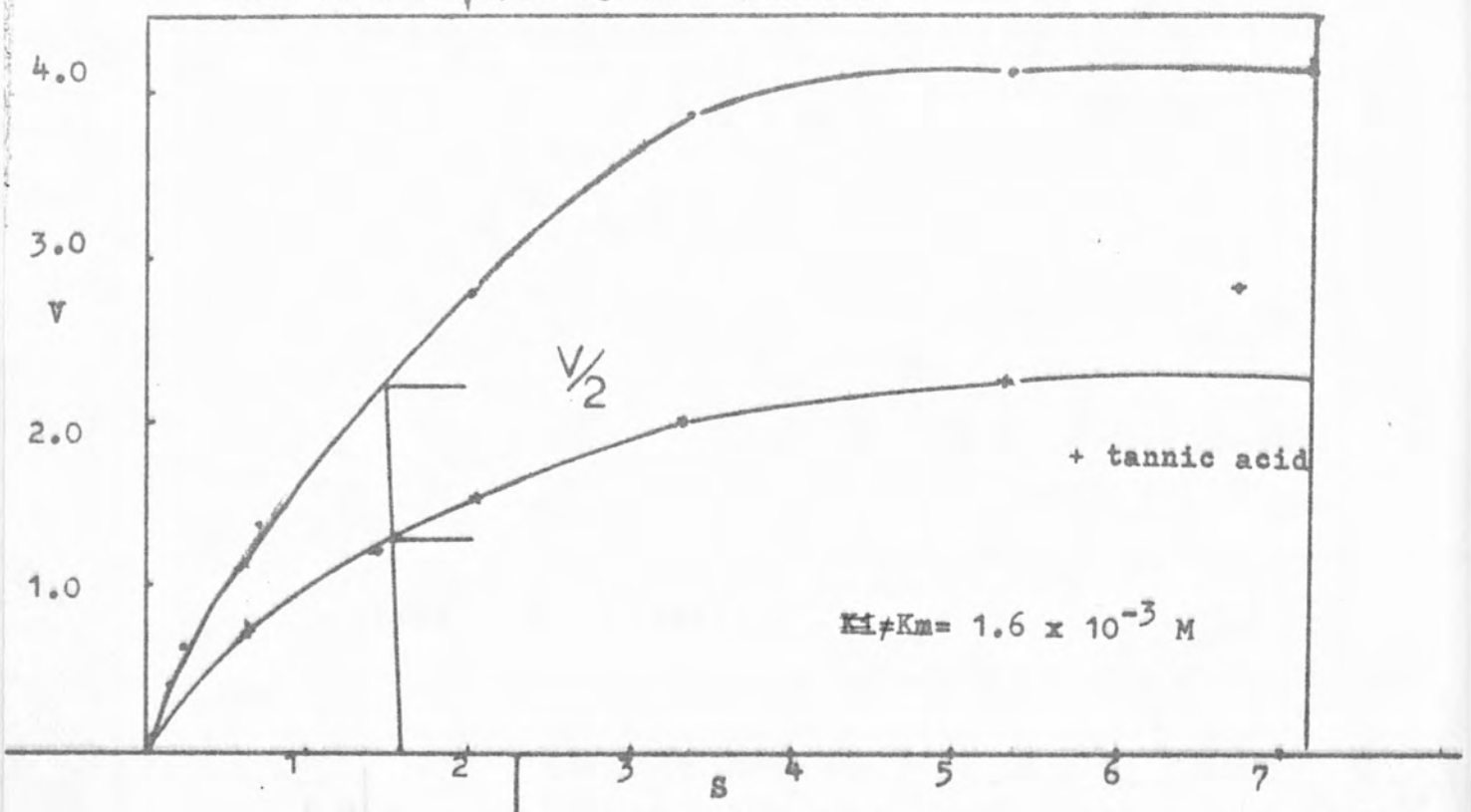
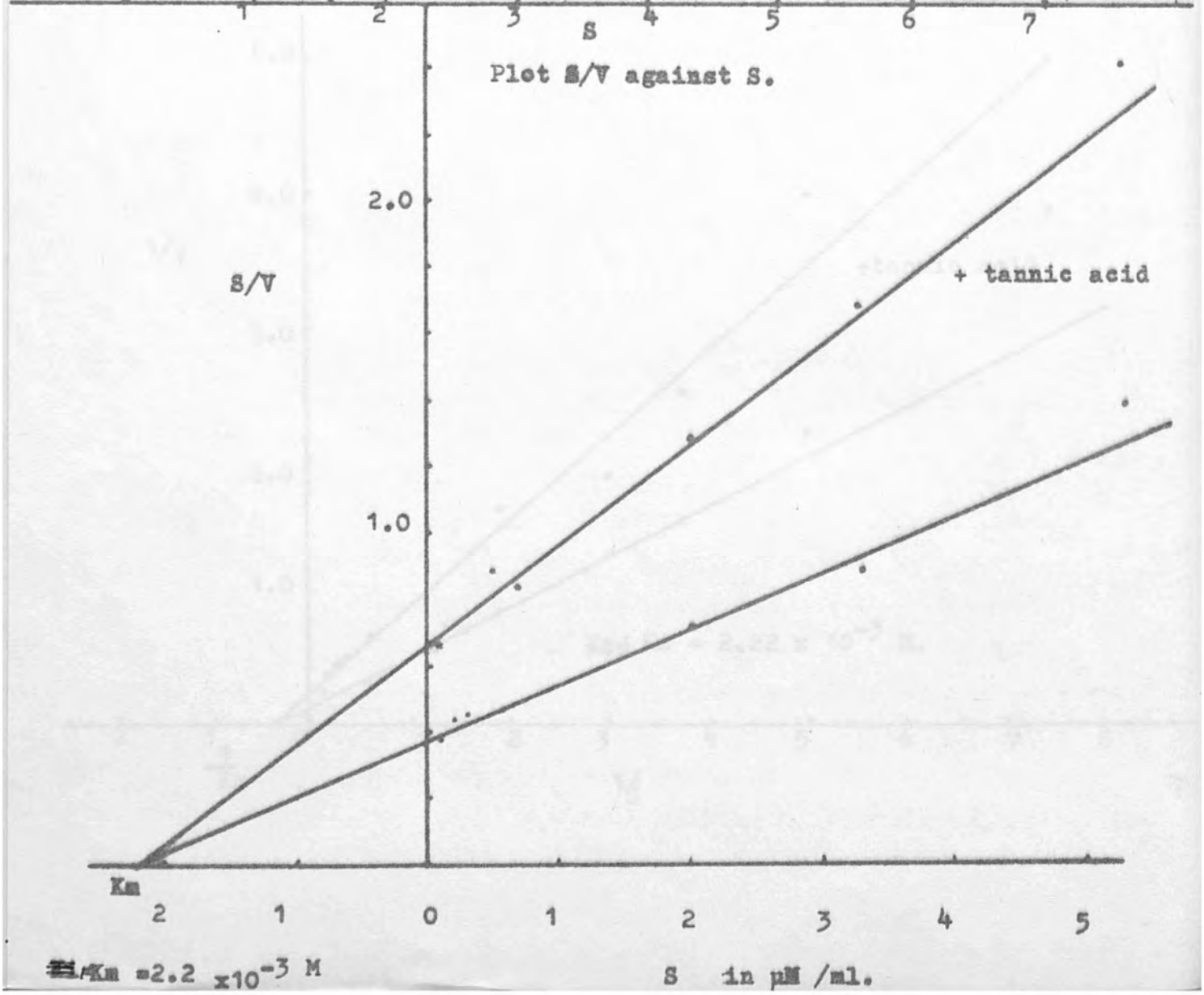


FIGURE 5[4]

Plot of S in μM/ml against V/minute.



Plot S/V against S.



As a result of these experiments it was decided to carry out the precipitation in 0.1M phosphate buffer at pH 7.0. It was thought that these conditions where precipitation was complete but where slight changes in the pH, concentration or type of buffer would lead to dissociation were the most suitable for producing a complex for a comparative study of the effect of reactivating agents. For the study of reactivation, however, pH 6.0 was chosen since under these conditions, any slight change in pH on addition of reactivating reagents would have a minimal effect per se.

(iv) Details of complex preparation.

An equal volume of β-glucosidase (2mg/ml) in 0.1m phosphate buffer pH 7.0 was added to tannic acid solution (2mg/ml) in the same buffer. (I am indebted to Dr. T. White for a gift of chromagraphically pure tannic acid). The resulting solution was allowed to stand for half an hour, and the precipitate collected by centrifuging at 1500×G for five minutes. The supernatant liquid was discarded (it was shown to contain no activity) and the precipitate washed with 0.1M tris buffer pH 6.0, and recentrifuged. The supernatant liquid was again discarded and the precipitate resuspended in 0.1M tris buffer at pH 6.0 at a concentration of 1mg/ml protein with regard to the original enzyme. A fresh suspension of the β-glucosidase-tannic acid precipitate was prepared for each experiment.

(v) Properties of the Complex.

When the procedure described above was followed, it was found that the insoluble tannic acid-enzyme complex itself had about 60% of the original activity when treated as a suspension. A determination of the Michaelis constant (K_m) using the enzyme alone and the enzyme tannic acid-complex gave the same value for K_m, namely 2.22×10^{-3} M. This demonstrated that the inhibition is non-competitive.

The constants were determined from the plot of $1/S$ against $1/V$ and confirmed from the plot S against S/V. These two plots eliminate irregularities at high and low substrate concentrations respectively. These plots are shown in Figure 5[4] together with the plot of velocity (V) against substrate concentration (S).

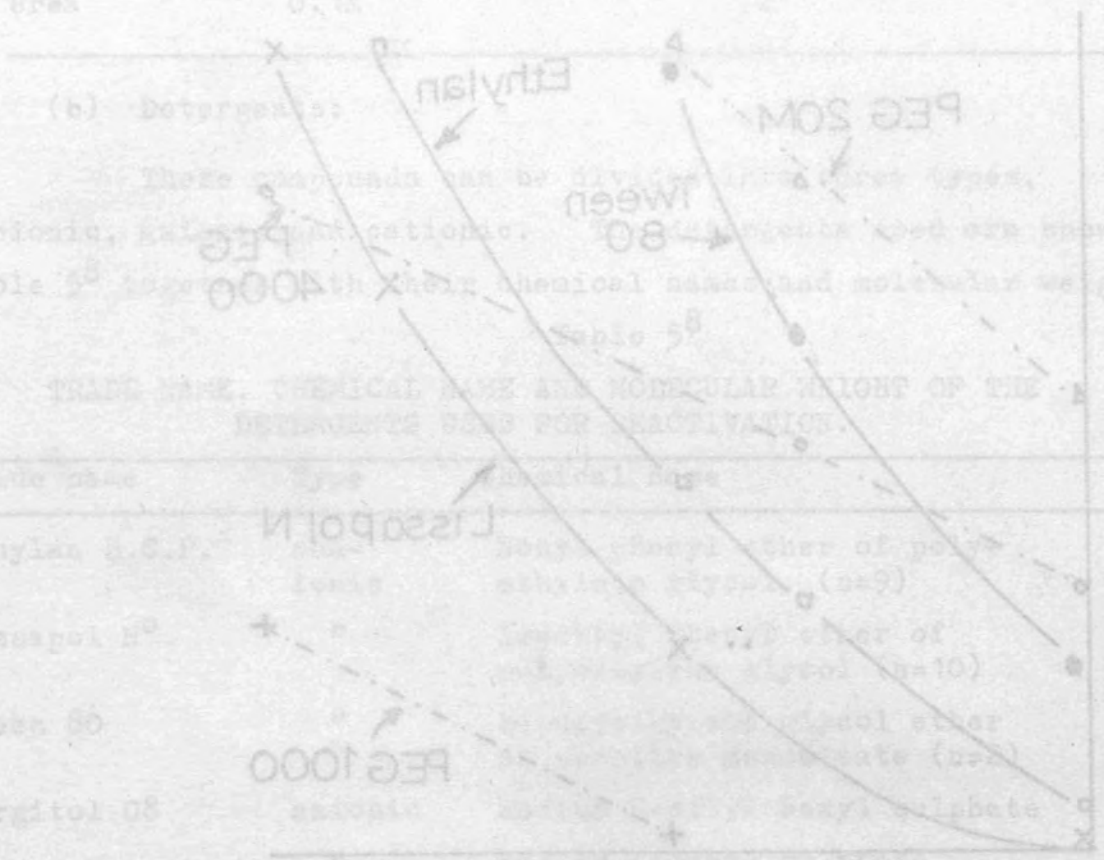

100
50
Activity recovered %
2
3
4
log ppm

FIGURE 5[5]

Reactivation of β-glucosidase from tannic acid precipitates

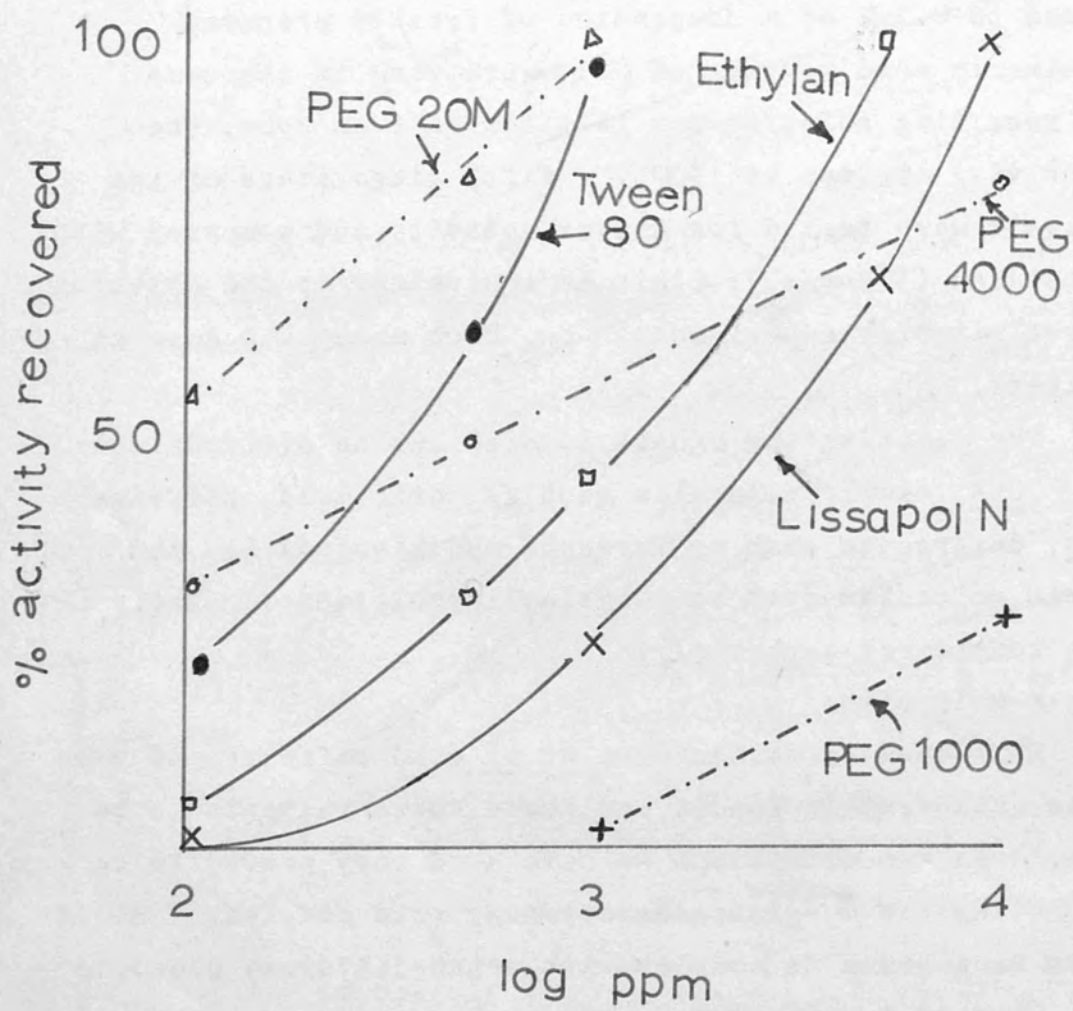


Table 57

THE REACTIVATION OF THE β-GLUCOSIDASE-TANNIC ACID COMPLEX WITH SIMPLE MOLECULES.

Compound		Activity recovered as % of the control
Boric acid	0.1M	53
Caffeine	0.01M	6
Urea	0.1M	2

(b) Detergents:

These compounds can be divided into three types, nonionic, anionic and cationic. The detergents used are shown in Table 58 together with their chemical names and molecular weights.

Table 58

TRADE NAME, CHEMICAL NAME AND MOLECULAR WEIGHT OF THE DETERGENTS USED FOR REACTIVATION.

Trade name	Type	Chemical name	M.W.
Ethylan B.C.P.x	non-ionic	Nonyl phenyl ether of poly-ethylene glycol, (n=9)	616
Lissapol N°	"	Isooctyl phenyl ether of polyethylene glycol (n=10)	646
Tween 80	"	Dipolyethylene glycol ether of sorbitan monooleate (n=2)	604
Tergitol 08	anionic	Sodium 2-ethyl hexyl sulphate	210+
	"	Sodium dodecyl sulphate	266+
Manoxol OT	"	Sodium salt of di (2 ethyl hexyl) ~~succiniate~~ succinate sulphonic acid	422+
Cetavlon°	cationic	Cetyl trimethyl ammonium bromide	364

n = number of units of monomer in the chain,
x kindly supplied by Lankro Chemicals,
o kindly supplied by I.C.I.
+ as free acid.

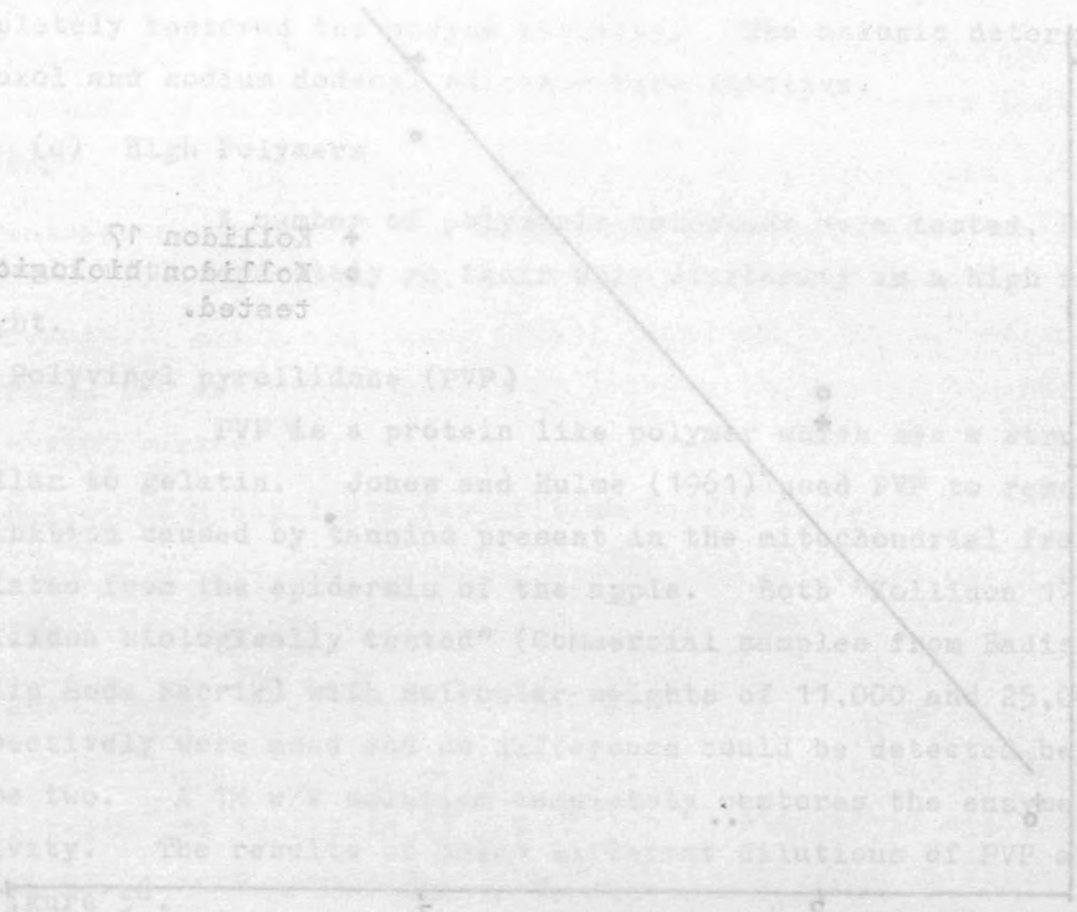
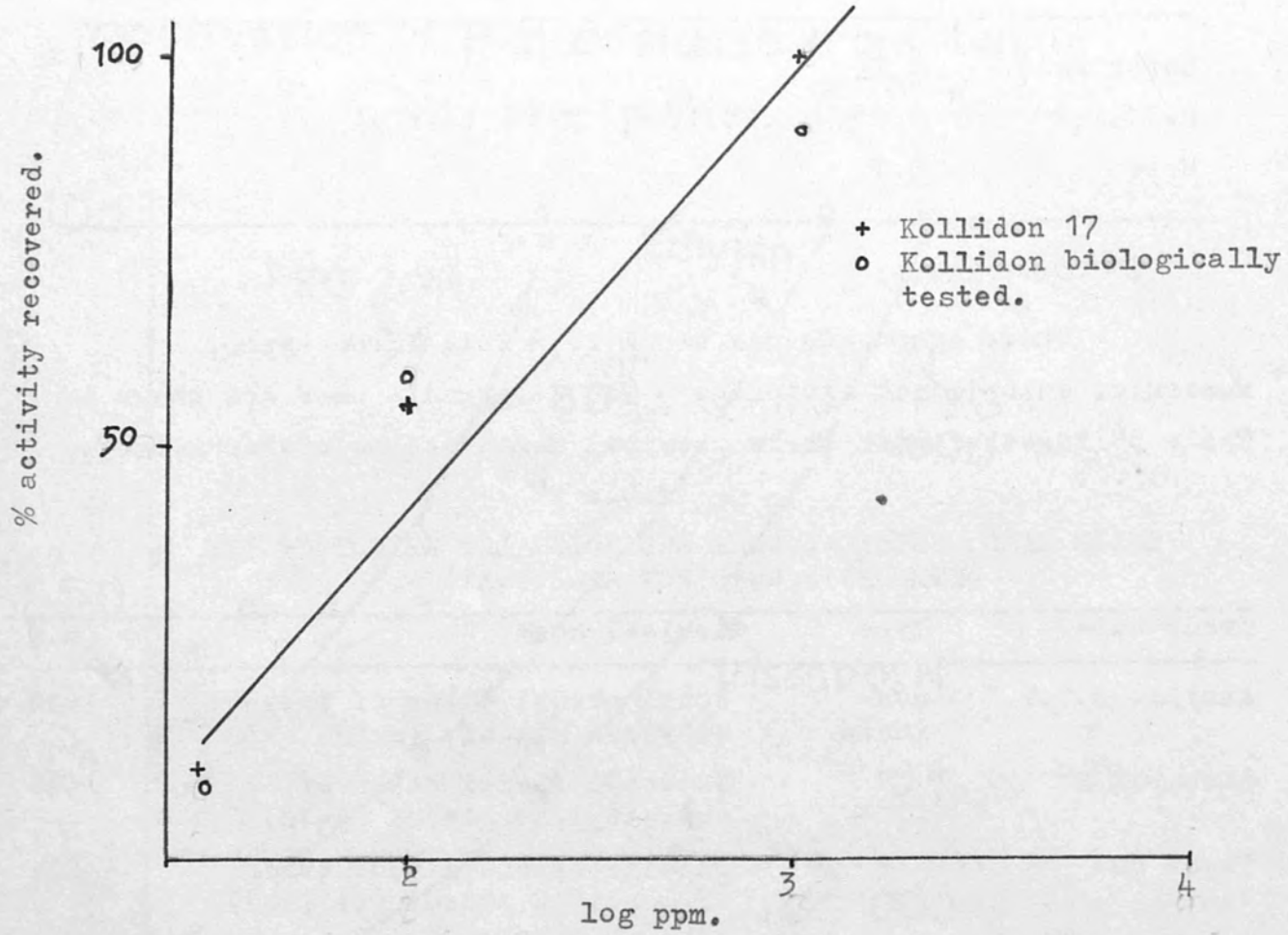

100
50
log ppm.

FIGURE 5[6]

Reactivation of β-glucosidase from tannic acid precipitates with PVP.



It was found that only non-ionic and cationic detergents were effective. The effect of Ethylan B.C.P., Lissapol N and Tween 80 at different dilutions are shown in Figure 5[5] and compared with various polyethylene glycols (PEG). Cetavlon at 1% w/v completely restored the enzyme activity. The anionic detergents, Manoxol and sodium dodecyl sulphate were inactive.

(c) High Polymers

A number of polymeric compounds were tested, these will be dealt with separately as their only similarity is a high molecular weight.

A. Polyvinyl pyrollidone (PVP.)

PVP is a protein like polymer which has a structure similar to gelatin. Jones and Hulme (1961) used PVP to remove the inhibition caused by tannins present in the mitochondrial fraction isolated from the epidermis of the apple. Both "Kollidon 17" and "Kollidon biologically tested" (Commercial samples from Badische Anilin Soda Fabrik) with molecular weights of 11,000 and 25,000 respectively were used and no difference could be detected between these two. A 1% w/v solution completely restores the enzyme activity. The results of using different dilutions of PVP are shown in Figure 5[6].

B. Polyethylene glycol (PEG) (ex Union Carbide).

A range of PEGs with increasing molecular weights were used. The molecular weights were as follows, 200, 400, 1000, 4000 and 20,000 (20 M). The small molecules up to MW 1000 were inactive in restoring the enzyme activity. The results are shown in Figure 55. The failure of the low molecular weight PEG polymers to reactivate the complex is in contrast with the non-ionic detergents tested in Table 58, all of which contain a short polyethylene glycol. The concentration curves for the active PEG types are shown in Figure 55 and it appears as though their action is different from that of the detergents. Biale and Young (1961) found that PEG was effective in reversing the inhibition caused by leuco-anthocyanins on aldolase in the unripe banana.

C. Polyvinyl alcohol PVA (ex British Oxygen Company).

Only 17% of the activity was recovered using this reagent at 1% w/v.

D. Substituted celluloses (ex. I.C.I.).

A range of methyl celluloses, (Methofas M), methyl ethyl cellulose (Cellofas A) and Sodium carboxymethyl cellulose (Cellofas B) were examined. The results are shown in Table 59. As with the detergents (Table 58) the anionic reagent was without effect.

Table 59

THE EFFECT OF VARIOUS SUBSTITUTED CELLULOSES ON REACTIVATING A β-GLUCOSIDASE TANNIC ACID COMPLEX. REAGENTS AT 0.5% w/v.

Reagent	Activity recovered in the supernatant
Methofas 450	80
Methofas 4500	84
Methofas 20M	82
Cellofas A	55
Cellofas B 3500	0

4. DISCUSSION OF THE RESULTS

It has been demonstrated that pH, ionic strength, and the concentration of both the protein and the tannic acid are important in the formation of a β-glucosidase tannic acid complex and consequent inhibition of the enzyme. This inhibition has been shown to be non-competitive. The fact that the precipitate itself is active is interesting and indicates that total inhibition of enzymes by tannins may not take place directly, but is due to further (oxidative ?) changes in the complex during "aging". The effect of the different reactivation agents strongly suggests that the protein and tannin are joined only by hydrogen bonds. These could be broken by pH, by changing the fine structure of the tannin (as with borate), or by competition with other polymers with a higher affinity than β-glucosidase for the tannin (e.g. PVP). The reason for the differences in the effect of the various detergents is not clear since they all can act as H-bond breakers in other systems (e.g. activation of latent phenolases of beans, Kenten 1958). The inactivity of the anionic detergents may of course be due to localised changes in pH which render the precipitate more stable.

5. OTHER ENZYMES

As mentioned earlier the inhibition of a number of enzymes was examined in a preliminary manner using both tannic acid and a fraction of condensed tannins from Wattle. (I am indebted to Dr. T. Swain for the preparation of this tannin).

The enzymes and the methods used for assaying their activity are listed in Table 5[10] together with the concentration of tannin used to obtain complete precipitation.

Table 5[10]

TESTING OF ENZYME TANNIN COMPLEXES

Enzyme	Method of assay	Ratio of protein to tannin in mg/ml Wattle	Tannic acid
°Anthocyanase	β-glucosidase Chapter 5	No precipitate	No precipitate
Alcohol dehydrogenase (ADH)	Racker (1950)	0.2/0.4	0.2/0.4
Lactic dehydrogenase (LDH)	Racker (1952)	50µl/1.0	50µl/1.0
Catalase	Beers and Sizer (1952)	0.4/1.0	0.4/1.0
Peroxidase	Worthington Biochem. Manual No. 11 (1961)	0.1/2.0	0.1/1.0

°Crude enzyme preparation (β-glucosidase) from Aspergillus spp. (ex. Rohm and Haas Co.).

(i) Method for obtaining the protein tannin precipitate

The complex was precipitated in polythene vials in 0.5ml of solution in 0.1M phosphate buffer pH 7.0. The quantities of the reagents are summarised in Table 5[10]. The tannin and enzyme were left for half an hour. The precipitate was obtained by centrifuging for 2 minutes at 20,000 and discarding the supernatant. 0.5 ml of the reactivating agents in 0.1 M tris pH 6.0 was added, and left for an hour and a half. The supernatant was tested for activity after centrifuging at 20,000 for 2 minutes.

The results are summarised in Table 5[11].

Table 5[11]

REACTIVATION OF ENZYME TANNIC ACID COMPLEXES

Results as percentage control

Enzyme	Suspension	0.1M Borate	0.1M Caffeine	25%v/v PEG (400)	8%v/v PVP°	1%w/v PVA	1%w/v Me. Cellulose	25% v/v Lisapol N 100
ADH	71	31	65	0*	94	54	0	-
LDH	0	0	2	3	65	6	66	-
Peroxidase	59	14	27	29	23	35	42	31
Catalase	58	0	24	19	79	26	0	-

REACTIVATION OF ENZYME WATTLE TANNIN COMPLEXES

ADH	24	0	30	0*	35	24	0	24
LDH	0	0	34	25	10	-	-	-
Peroxidase	50	6	5	33	30	36	3	44
Catalase	20	2	2	24	51	-	-	-

* inhibits the enzyme. ° kindly supplied by British Oxygen.

It can be seen from these results that as with the sweet almond β-glucosidase the enzyme tannin precipitates (with the exception of lactic dehydrogenase) are active. In this case borate was found to be less effective as a reactivating reagent, and there were differences in the behaviour of the various polymers. The most important finding, however, is that the condensed-tannins appear to give more "stable" precipitates than the hydrolysable tannins, and in no case could more than half the activity be regained on reactivation. The differences in inhibition and reactivation of the various enzymes are also noteworthy and presumably result from differences in the fine structure of the protein. Although more work requires to be

done, these results give promise that the methods could be useful in determining changes in astringency in ripening of fruits. The reactivating agents used to split the tannin protein complex may well be useful in extracting tannins from plant tissue, as a means of fractionation, or as a means of protecting enzymes from inhibition by tannins.

Summary to Chapter 5

1. The inhibition of β-glucosidase by tannic acid has been studied, and the formation of an insoluble complex has been shown to be dependent on pH, ionic strength and concentration of the reagents.

2. This inhibition has been shown to be non-competitive, and surprisingly the insoluble complex contains over 60% of the original activity.

3. A number of reagents have been used to reactivate the enzyme; these can be divided into three groups, simple molecules, detergents and polymers. Compounds in class 1 are not very effective, and the activity of compounds in class 3 increases with increasing molecular weight. All the detergents tested were found to work except the anionic group.

4. A number of other enzymes have been examined with both tannic acid and a condensed tannin and it is suggested that the measurement of the degree of inhibition and reactivation could be used as a measure of astringency in fruit extracts.

Chapter 6.

Factors affecting the production of tannins by plant tissue cultures.

Chapter 6

Factors affecting the Production of Leuco-Anthocyanins in Sycamore (Acer Pseudoplantanus L.) Cambial Cell Cultures

1. INTRODUCTION

Lamport (1961), in a doctoral thesis submitted to the University of Cambridge, stated that he found compounds present in the ethanolic extract from sycamore cambial cell cultures, which absorbed strongly in the ultra-violet at 280 mμ. Swain (1961) identified these compounds as leuco-anthocyanins and showed that by treatment with hot mineral acid they yielded cyanidin (see Chapter 1). This is not the first time that leuco-anthocyanins have been demonstrated in tissue culture extracts since the French workers Cronenberger, Vallet, Netier and Mentzer (1955) showed the presence of leuco-cyanidin and a trace of leuco-delphinidin in virginia creeper tissue.

Swain and Tjhio (unpublished work, 1961) found that when sycamore tissue was grown as a cell suspension, it produced both 100% methanol and 50% methanol soluble leuco-anthocyanins, demonstrating the presence of monomeric and polymeric substances. They therefore decided to use this tissue for biosynthetic studies on the factors affecting both synthesis of the monomers and polymerisation of these compounds. Unfortunately, they found that the concentration of leuco-anthocyanins in the tissue varied in an unpredictable manner. Thus in one experiment a number of cultures were established and harvested at 10, 20, 30, 40, 50 days after inoculation and analysed for tannins as shown in Table 6[1].

Table 6[1]

The Change in Tannins on Aging in Sycamore Suspension Tissue from Swain and Tjhio

Age of Tissue in Days	Extraction % Methanol	Folin-Denis	Vanillin	Leuco-Anthocyanin	Ratio V/LA
10	100 50	30 3	25 2	14 0	1.8 ∞
20	100 50	28 3	13 3	24 8	0.55 0.37
30	100 50	19 8	9 4	19 3	0.47 1.3
40	100 50	16 4	8 3	18 4	0.45 0.75
50	100 50	14 3	7 2	11 2	0.63 1.0

Folin-Denis as mg catechin per g. dry weight

Vanillin as mg catechin per g. dry weight

Leuco-anthocyanins as mg cacao leuco-anthocyanin per g. dry weight

It can be seen that whereas the Folin-Denis and vanillin reacting phenols fall progressively, the leuco-anthocyanins first increase and then fall, the variations in the ratio of vanillin/leuco-anthocyanin showing anomalous polymerisation behaviour. Repetition of this experiment gave completely different results (e.g. at 40 days, the values in the 100% methanol extract were 6, 14 and 8 respectively for Folin-Denis, vanillin and leuco-anthocyanins).

It was therefore decided to investigate this phenomena to see if some reasons for this variation could be found. It was hoped that one could thereby take advantage of studying the biosynthesis of tannins in tissue culture in which the material is uniform and over which one could exert some degree of environmental control. Also, as the cells, when rapidly growing, have a mean generation time of three days, this gives a more satisfactory system than a seasonal crop like fruit.

2. METHODS USED FOR GROWING THE TISSUE CULTURE

(a) Medium

The medium was based on White (1955) with the addition of coconut milk and 2,4-dichlorophenoxy acetic acid. Four stock solutions containing inorganic and organic salt solutions were made up as follows:

Solution A

Inorganic nutrients	Final concentration in mg/l
$CaNO_3$	200
$MgSO_4.7H_2O$	360
KCl	65
Na_2SO_4	80
$MnSO_4.4H_2O$	200
$ZnSO_4.7H_2O$	4.5
H_3BO_3	1.5
KI	0.75

Solution B final concentration in $NaH_2PO_4.2H_2O$ 10.5 mg/l

Solution C $Fe_2(SO_4)_3$ 3.5 mg/l

Solution D

Organic nutrients	mg/l
Glycine	3
Ca D-pantothenate	0.1
Thiamine	0.1
Cystine	1.0

Stock solutions of A (10 x final dilution), B, C and D (100 x final dilution) were diluted, using de-ionised water and made up to contain 2% w/v sucrose 2.5 mg/l 2,4-dichlorophenoxy acetic acid (Steward and Caplin, 1951), and 20% coconut milk which had been sterilized and deproteinised by heat treatment. The medium was made up freshly as required, and 1% agar was added for growth of the callus

tissue on slopes. All apparatus and media were sterilised in the normal microbiological manner.

(b) Method for growing the cell suspension tissue

The cell suspension tissue was grown at 25°C in 1 l. Fernbach flasks containing 200 ml media and plugged with cotton wool. The flasks were placed on a rocking type shaker. Originally, the cell suspensions were produced by adding approximately 1.5 g. of callus tissue (I am indebted to Dr. D.H. Northcote for the original isolate), and once it was established (30 days), 10 ml. aliquots were removed with a pipette and used to reinoculate fresh media. The amount of tissue transferred was determined by centrifuging another aliquot in a graduated tube for five minutes at 1500 g and measuring the packed cell volume (PCV).

(c) Method for growing the callus tissue

The callus tissue was grown on slopes (7 mls) in 1 oz. screw top McCartney bottles in the dark at 25°C. Approximately 20 mg. was used as the inoculum.

(d) Method of Extraction

Since the sycamore tissue contains about 92-95% water, the results were expressed on fresh weight or volume (PCV) basis. The cells from the suspension cultures after determination of the PCV were well washed with distilled water and extracted with methanol. The callus tissue was blotted on filter paper and weighed directly into methanol. The tissue was usually extracted three times with 80% methanol, in a similar way to that described in Chapter 4. In some experiments the callus tissue was treated directly with the butanol-hydrochloric acid reagent (cf. Hillis and Swain, 1959).

PHOTO MICROGRAPHS OF SYCAMORE CELL TISSUE CULTURES.

Taken with Leitz Panophot equipment, with arc -lamp illumination, using a green filter, bright field illumination , 4mm achromatic objective and x 8 eyepiece. Ilford Pan F 35mm film; magnification on the negative of x 680.

FIGURE 6[1]

Sycamore cell suspension tissue. cells unstained.

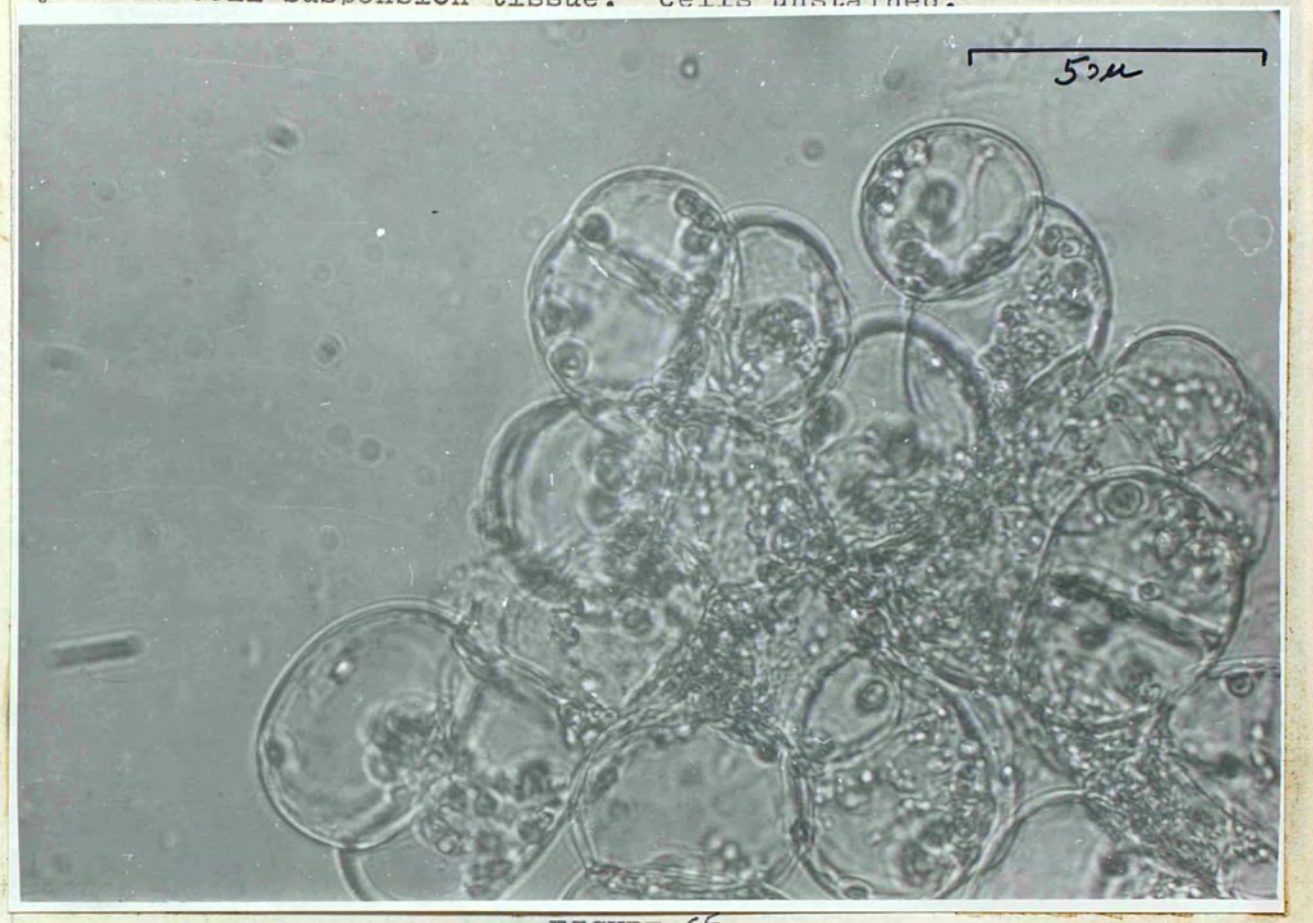


FIGURE 6[2]

Sycamore cell suspension tissue, cells stained with iodine.

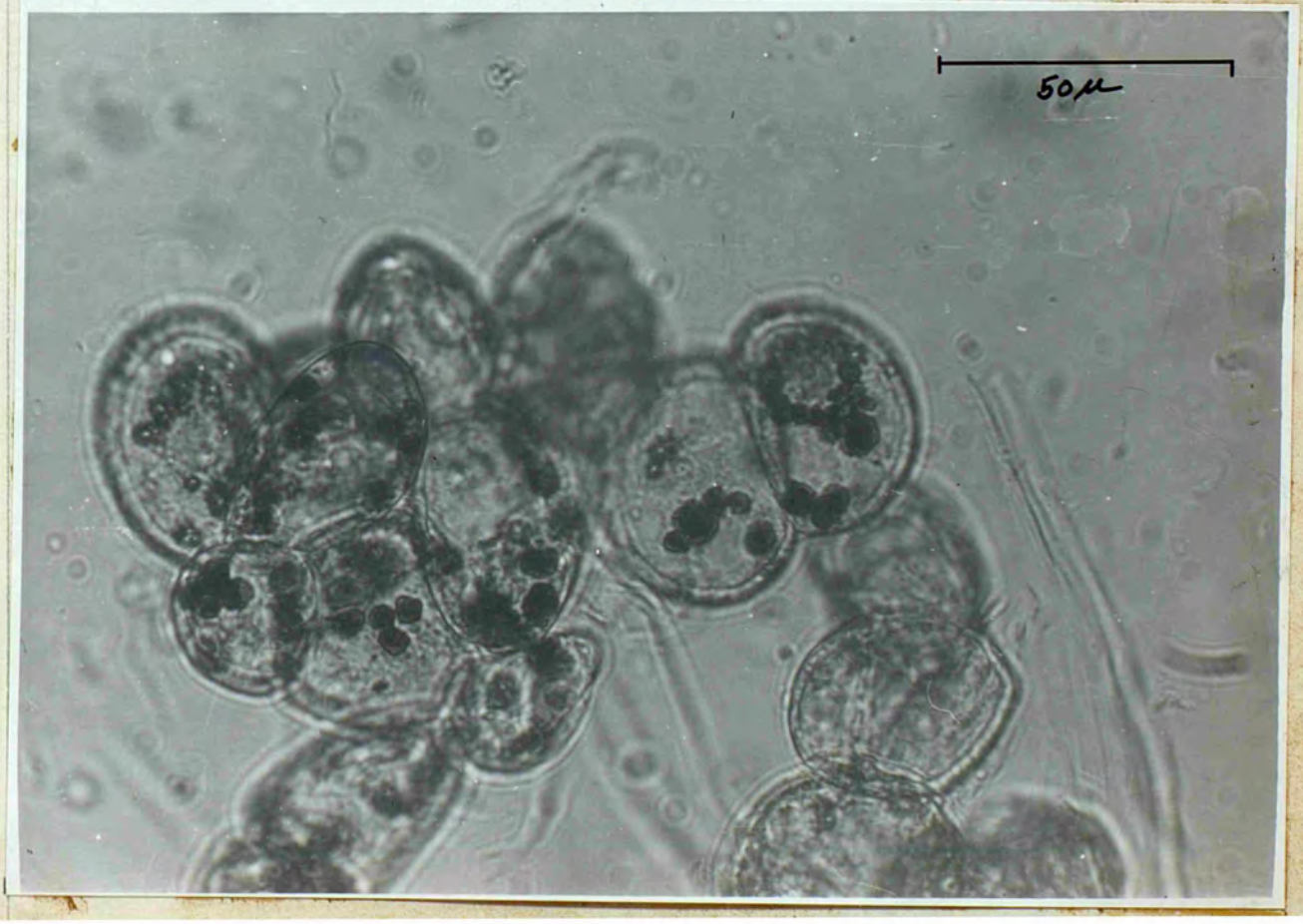


FIGURE 6[3]

Sycamore cell suspension tissue, cells unstained.

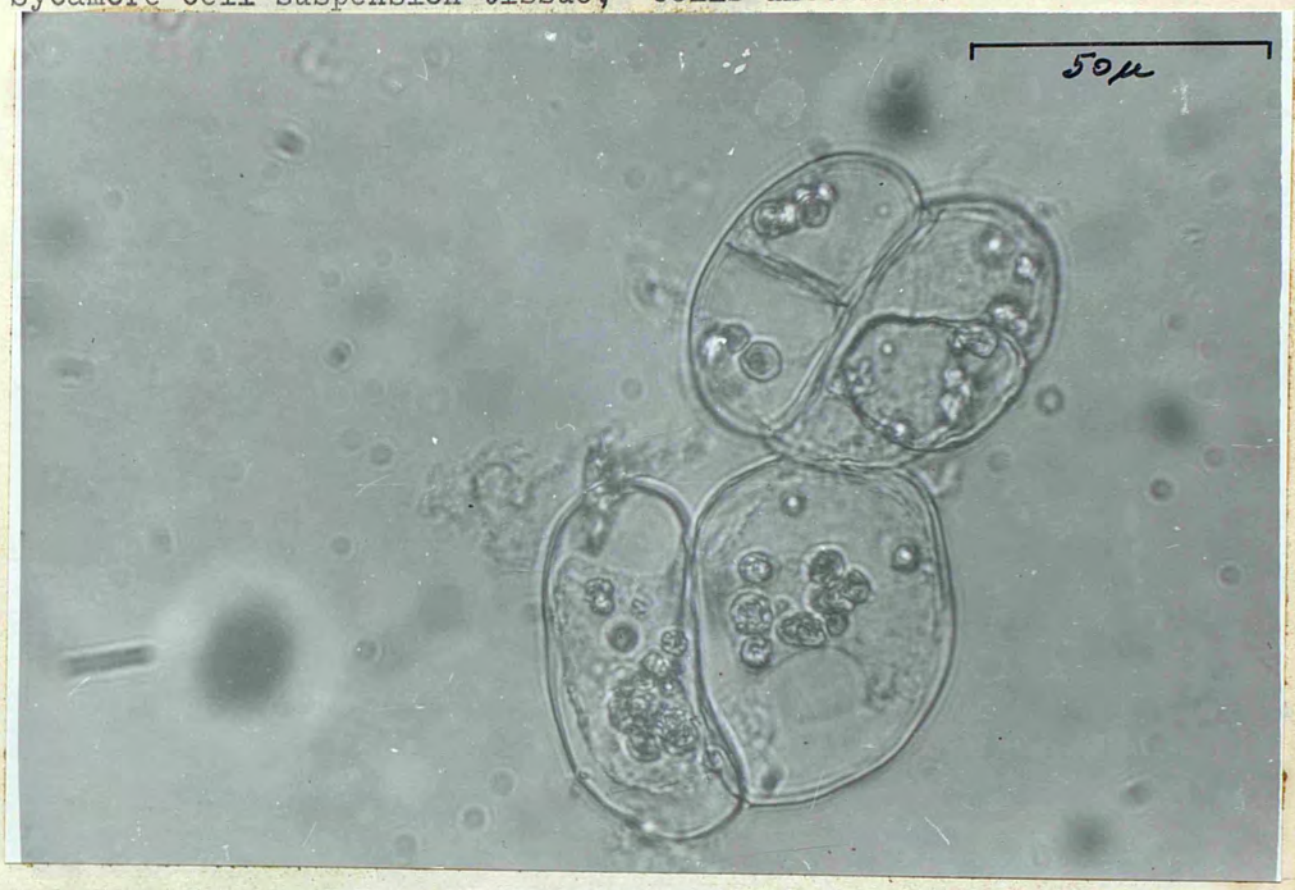
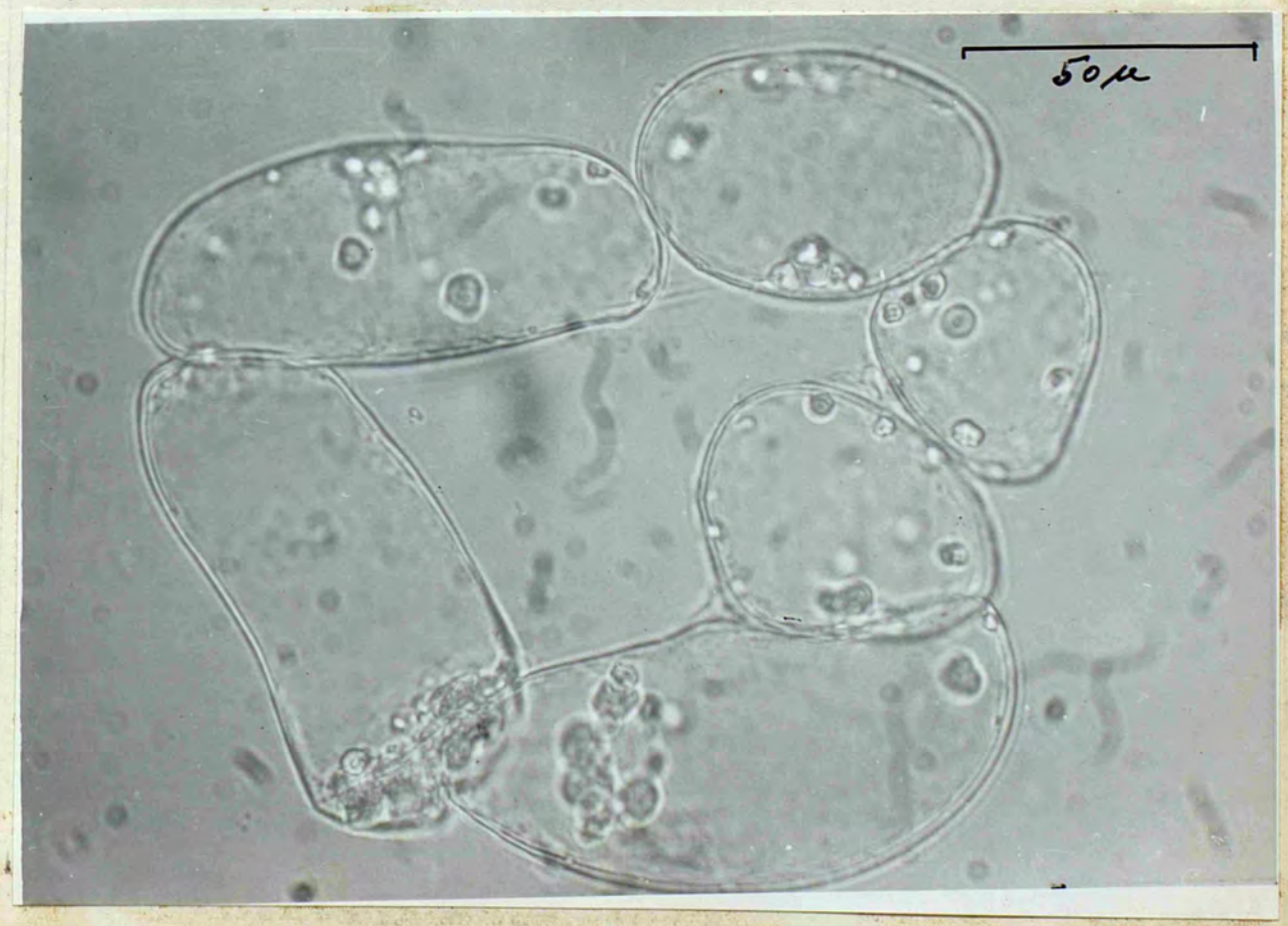


FIGURE 6[4]

Sycamore callus tissue, cells unstained.



(e) General observations on growth

The growth of the cell suspension tissue follows a general curve of an exponential type (Lamport, 1961). Actively growing sycamore cells are white in appearance but, on aging, turn brown and finally die. The actual anatomy of the cells varies with their physiological age. "Young" cells are 50-150 μ long, both spherical and long-oval cells being present. The cells are highly vacuolated, the nucleus being present near the edge of the cells or sometimes in the centre connected to the cytoplasm by numerous cytoplasmic strands. The cell wall is thin and there are numerous starch grains present. In the older cultures there are fewer spherical cells and more of the long-oval cells. There are starch grains and the cell wall appears to be thicker. It was almost impossible to stain the nucleus using either aceto-carmine or Feulgen stains. Figures 6^1, 6^2, 6^3 and 6^4 show photomicrographs of a selection of actively dividing cells taken both from callus tissue and from cell suspension tissue.

(f) Description of Experiments

(i) Experiments to test the effect of "strain" on leuco-anthocyanin production.

From two original calluses, a number of sub-cultures were made. One line was established as a cell suspension culture, the other as a callus tissue culture. In the case of the cell-suspension, both a high and a low leuco-anthocyanin containing "strains" were selected, and the change in concentration of leuco-anthocyanins determined through five sub-cultures (20-25 generations of cells, Figure 6^5). The cells were always harvested while they were still in the exponential phase of growth, about 12-15 days after inoculation, cells grown in six different flasks being extracted from both high and low "strains" each time, two samples being taken from each flask. After the fifth re-inoculation, that is about 20-25 generations, the high and low line were still distinct, Figure 6^1. In the callus tissue, an original high and low "strain" were again selected and these were grown through three sub-cultures. Twelve

calluses were selected for the analysis of each strain and the analyses were done in duplicate. In this case, the high and low leuco-anthocyanin containing "strains" came to equality after only 3 sub-cultures. It seems, therefore, that the callus tissue is not as "biochemically" stable as cell suspension tissue, and perhaps differentiates more easily. This is shown by the variation in the level of leuco-anthocyanin for separate pieces of the same callus (Table 6^2).

Table 6^2

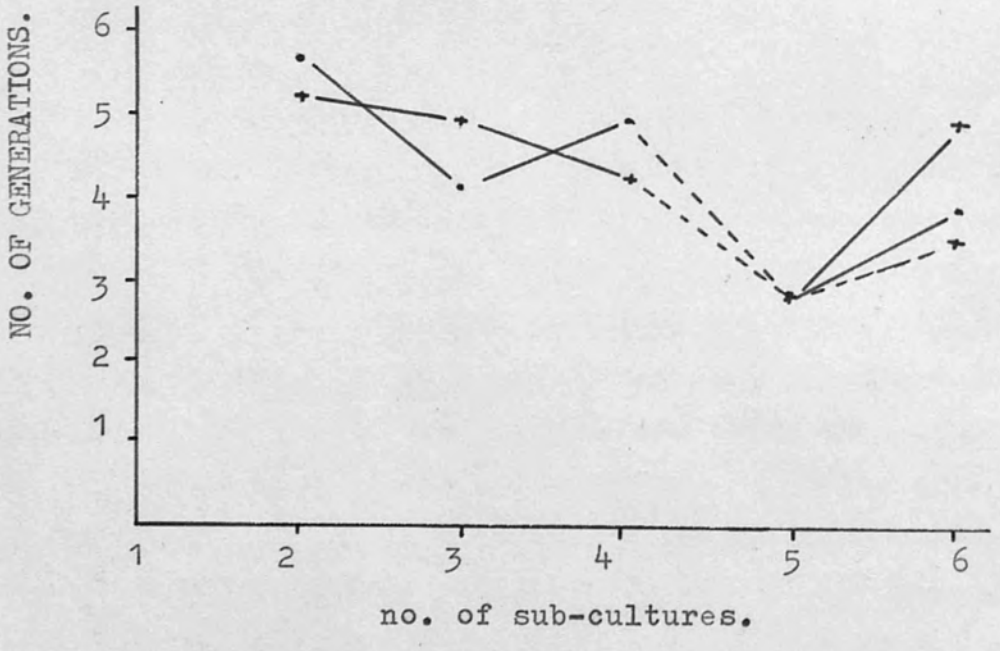
Leuco-Anthocyanins as mg cacao leuco-anthocyanin per 10 g. fresh weight from two separate calluses

(1)	(2)
90	36
42	50
34	21
28	30
74	56
55	58

FIGURE 6[5]

The number of cell generations in high and low leuco-anthocyanin containing "strains" in sycamore suspension tissue on each sub-culture.

—normal aeration, _ _ _restricted aeration, + high "strain", • low "strain".



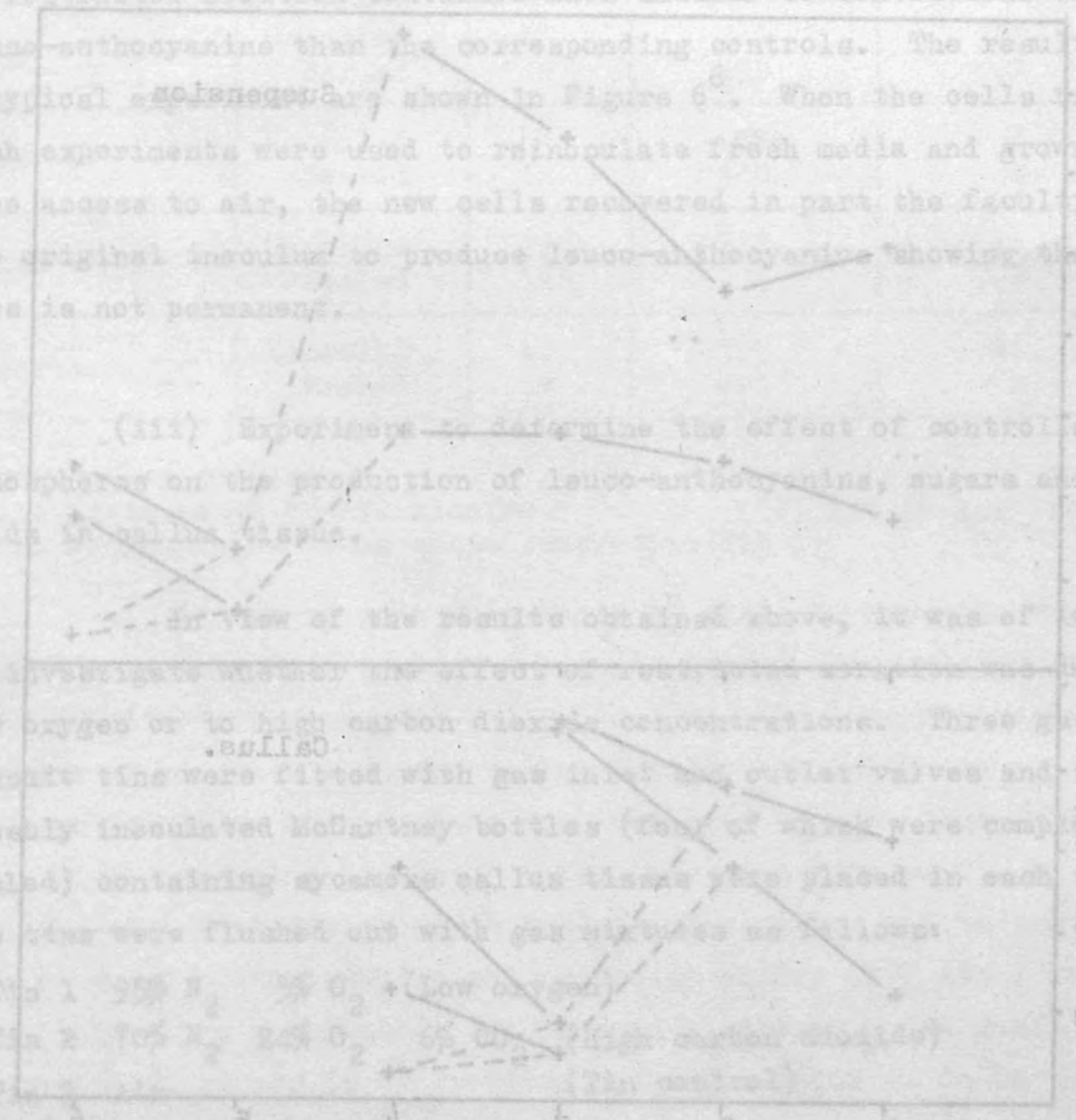
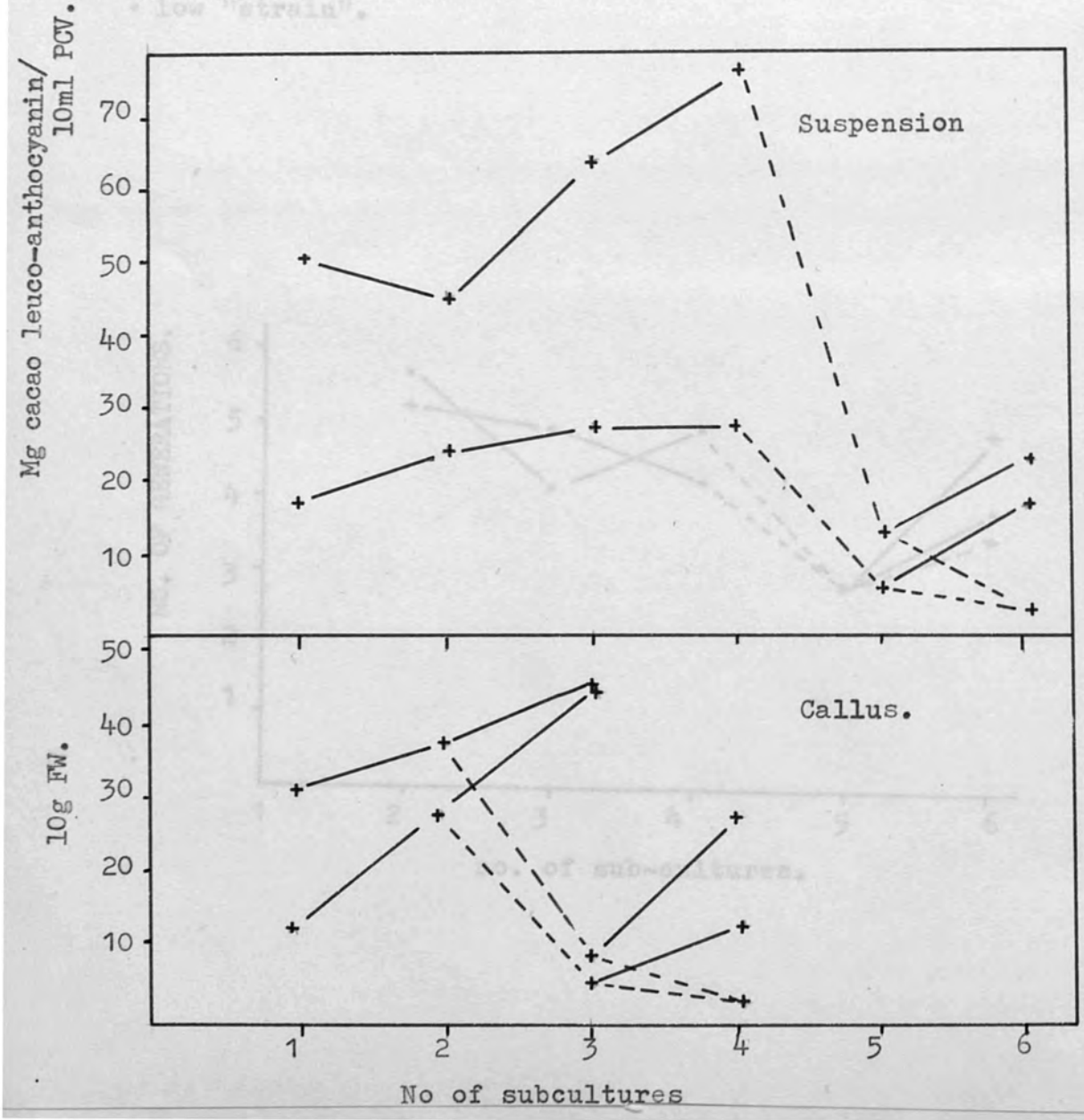


FIGURE 6[6]

The effect of "strain" and restricted aeration on the production of leuco-anthocyanins in sycamore tissue culture.

(cell suspension tissue the results are the mean of 6 flasks per "strain" and 24 analysis each. Callus tissue, the results are the mean of 12 calluses per strain and 24 analysis.)

— normal aeration.
--- restricted aeration.



(ii) Experiment to test the effect of restricted aeration on leuco-anthocyanin production.

It was noticed that cells which had been grown in a rotating 5 l. flask (by K.H. Tjhio) in which ingress of air may have been limiting contained surprisingly small amounts of leuco-anthocyanins. A repetition of this experiment with a high leuco-anthocyanin "strain" gave similar results. It was decided, therefore, to test the effect of restricted aeration on leuco-anthocyanin synthesis. Consequently, the tops of the McCartney bottles were fitted with a rubber seal, screwed down tightly and covered with paraffin wax tissue (para-film) and the cotton wool plugs of the Fernbach flasks were likewise covered with parafilm. In both cases cells grown under these conditions of restricted aeration contained much smaller concentrations of leuco-anthocyanins than the corresponding controls. The results of a typical experiment are shown in Figure 6[6]. When the cells from such experiments were used to reinoculate fresh media and grown with free access to air, the new cells recovered in part the faculty of the original inoculum to produce leuco-anthocyanins showing that this loss is not permanent.

(iii) Experiment to determine the effect of controlled atmospheres on the production of leuco-anthocyanins, sugars and amino acids in callus tissue.

In view of the results obtained above, it was of interest to investigate whether the effect of restricted aeration was due to low oxygen or to high carbon dioxide concentrations. Three gas-tight biscuit tins were fitted with gas inlet and outlet valves and twelve freshly inoculated McCartney bottles (four of which were completely sealed) containing sycamore callus tissue were placed in each tin. The tins were flushed out with gas mixtures as follows:

Tin 1 95% N_2 5% O_2 (Low oxygen)
Tin 2 70% N_2 24% O_2 6% CO_2 (High carbon dioxide)
Tin 3 Air (Tin control)
Control outside (in the dark)

The tissue was grown for three weeks at 25°C, the gas being flushed twice weekly. The tissue from each treatment was bulked and extracted with 80% methanol. Leuco-anthocyanins were estimated as previously, amino-acids were estimated by the method of Moore and Stein (1948), and the sugars (aldohexoses) were estimated by the method of Leopold (1962). The results are shown in Table 6^3.

Table 6^3

Variation in sugars, amino acids and leuco-anthocyanins in cells grown under controlled atmospheres

Gas mixture	Tissue	Sugars	Amino-acids	Leuco-Anthocyanins
5% O_2	Unsealed	17	112	31
	Sealed	9	499	0
6% CO_2	Unsealed	8	69	7
	Sealed	12	500	0
Air	Unsealed	12	138	14
	Sealed	11	535	0
Control	Unsealed	12	100	21
	Sealed	15	400	0

Sugars as mg glucose)
Amino acids as μM phenyl alanine) per 10 g. tissue
Leuco-anthocyanins as mg cacao leuco-cyanidin)

It can be seen from Table 6^3 that, whereas the hexose sugars only vary ∓ 40%, the amino acids vary ∓ 80%, and the leuco-anthocyanins vary even more. In each case, the leuco-anthocyanin content of the tissue in the sealed bottles has fallen to zero, whereas the amino acid content has increased four to five-fold from that of the unsealed bottles. This effect has been confirmed for the suspension tissue grown in restricted aeration and is probably due to there being insufficient metabolic energy (ATP etc.) available to utilize the amino-acids for protein synthesis. The small variation in sugars is interesting and was shown also to be the case

for the high and low leuco-anthocyanin producing "strains" grown in suspension, both in normal and restricted aeration.

From the viewpoint of leuco-anthocyanin production, it would appear that CO_2 simulates restricted aeration more closely than does low oxygen. However, the amino-acid content of the tissue in the high carbon dioxide treatment is somewhat lower than that of the control, whereas restricted aeration gave, as mentioned above, a fourfold increase (cf. Table 6^3). It is obvious, therefore, that the effect of restricted aeration is not due to increase in carbon dioxide concentration alone, although this may be a contributory factor to the lowering of the leuco-anthocyanin content.

(iv) Experiment to determine the change in lignin content of cells with age.

Lignin is an aromatic polymer closely related biosynthetically to the condensed tannins. In fact, it is based on the condensation of C_9 units similar to those of the $C_6(B)C_3$ units of the flavonoids. Since lignin is present in all woody plants and is taxonomically associated with the leuco-anthocyanins (Chapter 2, Bate-Smith and Lerner, 1954), it was of interest to examine whether concentration varied with leuco-anthocyanin concentration of sycamore tissue. An opportunity kindly afforded by Dr. F.A. Isherwood enabled the analysis to be carried out and the results are shown in Table 6^4.

Table 6^4

Percent of Lignin Breakdown Products in Dried Methanol Extracted Sycamore Cells

	% p-hydroxy benzaldehyde	% Vanillin
High LA cells 3 weeks old	0.12	0.09
Low LA cells 3 weeks old	0.14	0.18
High LA cells 2 months old	0.18	0.57

From these results, it would appear that these cells do make lignin. As expected, the old cells contain more lignin than the young cells, but surprisingly the low leuco-anthocyanin containing "strain" contains more than the high leuco-anthocyanin "strains". The amount found in the old cells is similar to the concentration in oat coleoptiles (Dr. F.A. Isherwood personal communication).

3. DISCUSSION

It has often been assumed that the precursors of tannins and lignin are formed in different regions of the plant from that in which they ultimately are found. This work demonstrates that in a relatively undifferentiated tissue all the enzymes are present for the biosynthesis of these substances from primary metabolites and there is no need to suggest that translocation has to occur.

It has been shown that strain difference (i.e. a genetic difference) is important in determining the level of leuco-anthocyanin produced by a given population. It has also been demonstrated that in restricted aeration leuco-anthocyanins are produced in much lower quantities or not at all. The cause of this block in leuco-anthocyanin synthesis is not yet known. Restricted aeration also causes an accumulation of amino acids in the tissue, and it may be that the high concentration of these compounds inhibits leuco-anthocyanin production, or that the enzymes necessary for some of the biosynthetic steps are not synthesised, due to lack of ATP.

The other strange feature of the effect of restricted aeration is that once the cells have lost the faculty to synthesise leuco-anthocyanins, it is only regained slowly, and a number of cell generations have to pass before leuco-anthocyanins are produced at the maximal rate.

The experiments described here were of a preliminary nature to determine means of obtaining uniform cell colonies in order to examine the biosynthesis of leuco-anthocyanins. It is believed, however, that the results are of general interest, and

point to the need for much further work on factors influencing the synthesis of secondary plant products.

The results suggest that free access of air may be one of the prime rate-controlling processes in the formation of leuco-anthocyanins in woody tissues. However, the differences in the faculty to synthesize these compounds in "strains" grown in shaken culture indicates that other factors of importance exist. The results also stress that the biosynthesis of plant secondary substances is more susceptible than that of primary metabolites to changes in environment. This is especially important in connection with any investigation carried out on tissue culture or whole plants, which contain leuco-anthocyanins. These compounds have been shown to inhibit enzymes (Chapter 5) and are adsorbed onto cell walls and particulate fractions (Jones and Hulne, 1961). Thus observation of differences in enzymic activity and the like in such cases might only be a reflection of changes in the leuco-anthocyanin content.

- THE END -

Summary to Chapter 6

1. The synthesis of leuco-anthocyanins in sycamore cambial cell tissue culture has been investigated and "strain" differences have been shown to be important in cell suspension tissue.

2. Restricted aeration has been shown to cause a reduction in concentration of leuco-anthocyanins in both callus and cell suspension tissue. The reasons for this effect are not yet known. It may be due to there being insufficient metabolic energy available to carry out protein synthesis, hence the enzymes responsible for the production of leuco-anthocyanins have not been synthesised.

3. The effect of restricted aeration is not due to low oxygen, but high carbon dioxide may affect the synthesis of leuco-anthocyanins although it shows a different effect from restricted aeration in the accumulation of free amino acid.

4. Lignin is synthesised by sycamore cambial tissue cultures and is produced in larger amounts in the older tissue.

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