# THE CHEMISTRY OF THE WATTLE TANNINS

(Acacia mollissima Willd., A. decurrens Willd., A. pycnantha Benth. and A. dealbata Link)

by

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## DEFINITION OF TERMS

Some confusion is caused by the ambiguous use of various terms in the leather-chemical literature.

In this thesis the term "extract" refers to all the water-soluble extractives obtained from the various portions of plants; the term "tannins" specifically embraces only the polyphenolic constituents in the extract as well as their esters with carbohydrates; and the term "non-tannins" refers to the remainder of the extract, which normally consists of mixtures of carbohydrates together with other constituents which yield only carbohydrates on hydrolysis.

## SUMMARY

Four species of acacia of Australian origin are associated with the wattle industry in South Africa. Black wattle, Acacia mollissima Willd., is the most important of these, and is today almost exclusively cultivated. The tree grows successfully only in a limited area of rich soil and high rainfall, and is easily affected by adverse conditions caused by insect pests, frost damage and drought. Expansion of the area under cultivation is, therefore, not feasible, although the world-demand for vegetable extracts far exceeds the available supply.

The remaining species such as green (Acacia decurrens Willd.) and silver wattles (Acacia dealbata Link.) possess many desirable characteristics which resist such adverse factors. Their barks, however, give reddish extracts, which are considered unsuitable for tannery usage because of the red colour they impart to the leather. Hybridisation studies, involving the crossing of green and silver wattles with the black, and aimed at producing progenies containing many of the desirable characteristics of the parent plants, are thus a natural result, and have been in progress at Cedara and the Wattle Research Institute for a considerable period.

Due to the close relationship between the various species of wattle, such genetic work requires the assistance of chemical studies which show up characteristic differences in the complex extracts obtained from the barks. From the present investigation

it appears that chemical genetics might possibly be applied successfully in this field.

A fundamental knowledge of the nature and chemical behaviour of black wattle extract is also essential in view of its already large and increasing application in industry.

In order to place the present work in its correct perspective, and to serve as a basis for reference and discussion, a comprehensive review of the chemistry of all the important tannins is included.

Commercial black wattle "Mimosa" extract contains tannins, non-tannins and water in the ratio of 3 : 1 : 1, and may be fractionated into gums, sugars and polyphenolic tannins.

The gums (7% of the commercial extract) are separated from the remainder either by addition of alcohol to aqueous solutions of the extract, or by extracting the solid material with 95% ethanol. The gums are large-molecular units of remarkable swelling power, which give on hydrolysis, galactose, arabinose, rhamnose and a uronic acid. They thus appear identical with the natural exudate of the bark already investigated by Stephen (377).

Four methods were examined for the separation of the sugars from the polyphenolic tannins, which together form the remainder of the extract. These are :

(a) a lead-salt method

(b) a salting-out method

(c) a hide-powder method

and (d) mixed solvent methods.

The three first-mentioned methods give tannins of 97 - 98% "purity". The lead-salt method, by which almost quantitative recovery of the polyphenols is possible, was preferred. The salting-out and hide-powder methods give only partial recoveries of the tannin fraction, and are, therefore, less suitable. The salting-out and mixed solvent methods are useful for fractionating the tannins.

The sugars, separated by the lead-salt procedure were identified as sucrose, glucose, and fructose. Sucrose was found to be the only carbohydrate constituent of the fresh-bark extract, and it thus appears that the invert sugars are produced during the drying of the bark, and/or the commercial extraction process.

The polyphenolic tannins are a complex mixture of similar amorphous high molecular-weight bodies, and were investigated both by paper chromatography and as a group.

Paper chromatography of the condensed tannins presents special problems and the use of conventional solvent mixtures is often less effective. A detailed study was therefore made of a wide variety of solvent mixtures and the best of these utilised for a one- and two-dimensional chromatographic study. n-Butanol-acetic acid-water mixture was suitable for resolving three phenolic bodies, which are associated with the tannins in very low proportion, and which fluoresce brightly when the developed chromatogram is viewed under ultra-violet light. One of these, RF = 0.72, was isolated and identified as fisetin for the first time, and another, RF = 0.90, appeared to be aphloroglucinol derivative. These

could be separated from the tannin by repeated ether extraction, or by the method which Perkin and Gunnell (120) used for isolating fisetin from quebracho extract. Gallic acid and resorcinol also resulted from the action of concentrated sulphuric acid on the extract during the latter process. The abnormal behaviour of yellow fluorescent bodies on paper chromatograms was observed.

One-dimensional chromatography was not sufficiently effective for the resolution of the complex polyphenolic mixture in the tannin. Two-dimensional chromatography, using water-saturated sec-butanol for the first direction and water-saturated phenol for the second, gave excellent separations. This combination appears to be superior to those recently (1952) used by White and co-workers (168), and the developed chromatogram shows the presence of at least nine constituents in the mature fresh-bark extract. Five of these predominate and occur in a pattern characteristic of black wattle tannin.

Chromatograms of the tannins obtained from the commercial extract differ from the above in that the spots, although evident as areas of high concentration, are joined by a more pronounced and dark trail to the point of application, and also to each other. The appearance of this chromatogram could be simulated by subjecting the fresh-bark extract to atmospheric oxidation before chromatography.

As oxidation of the tannin mainly during the drying of the bark, appears to have a marked influence, a study was made of the effect of atmospheric oxidation on pure catechins at elevated

temperatures. From pure gambier-catechin a variety of additional compounds of lower RF values were formed, and could be separated on a two-dimensional chromatogram. A spot at the origin as well as a pronounced trail was prominent. The oxidation-products of highest concentration were two discrete spots in close proximity to that of catechin. Casuarin from the bark of Casuarina equisetifolia, identified by Osima (200) as a gallocatechin, but now found to contain an admixture of catechin, behaves similarly, and condenses to form a single spot at the point of application of the mixture.

It is evident that the unheated and unoxidised fresh-bark extract provides the best starting-material for further investigation, which calls for the separation of all the individual compounds constituting the tannin. The indications are that these may be closely related, and they could conceivably represent oxidation or condensation-states of at least two nuclear species, resulting from enzymatic action. Fractionation of the tannins by the afore-mentioned methods has been attempted by White and co-workers (loc. cit.), and also in the present investigation.

The above indications are supported by a study of the tannins as a whole, which preceded the above work. The tannin fraction, freed of sugars and gums, has in the past at the hands of numerous investigators, given abnormally low yields of degradation products and some significance has been attached to this phenomenon. An improved oxidation technique has now been found

to increase the yield of veratric and O-trimethylgallic acid to above 20%. A study of carefully controlled alkaline fusions of the tannins has been made, and under optimum conditions a 43% yield of ether-soluble phenolic degradation products was obtained. These included β-resorcylic acid (4.2%), resorcinol (10.5%), gallic acid (about 10%), pyrogallol (about 1%), protocatechuic acid (low proportion), phloroglucinol (trace), and some oxidised material. Opposed to this, Stephen previously obtained resorcinol (1 - 2%) and gallic acid (trace) only. β-resorcylic acid, pyrogallol, protocatechuic acid and phloroglucinol were obtained directly from the tannins for the first time, and the high yield of phenolic bodies isolated has cleared away much of the uncertainty which resulted from the previously-obtained low yields. The phloroglucinol is considered to originate from gallic acid, and the phenols are believed to be formed from the decarboxylation of the corresponding phenolic carboxylic acids. Only three nuclear types, potential β-resorcylic, gallic and protocatechuic acids, appear to be present, of which the first-mentioned pair form the major proportion. The pyrogallol and catechol nuclei survive permanganate oxidation whereas the resorcinol moiety is destroyed. In this respect the tannin resembles catechins in behaviour. A spraying reagent for identifying phenols was developed during the course of this degradative investigation.

The tannin fraction as a whole has an average composition $C_{15}H_{13}O_6$, and the fully-substituted acetyl and methoxyl

derivatives both correspond to $C_{15}H_9(OR)_{4.1}O_{1.9}$. About two oxygen atoms do not acetylate and methylate even under drastic conditions and about four hydroxy groups are present per hypothetical $C_{15}$ unit.

Diazomethane has been found, contrary to Stephen's (149) findings, to react easily and rapidly with the tannin to produce an almost fully substituted methylated derivative. From a parallel methylation study of other phenols, it appears that all the reactive hydroxy groups are phenolic in character. From this evidence the predominant presence of catechin bodies or their condensates appears doubtful.

Terminal methyl groups, ethylenic links, and carbonyl groups were shown to be absent. From a colorimetric study with buffered ferrous tartrate reagent, a high proportion of "free" pyrogallol nuclei, (one per hypothetical $C_{15}$ unit) appears present in the tannins. The tannins condense in the presence of mineral acids to form red insoluble phlobaphenes which differ only slightly analytically from the starting-material.

Previous molecular-weight determinations were shown to give an erroneous impression of average values for the tannin fraction, and also for other tannins. The hitherto accepted value of 1700 for the tannin probably represents an average value for the highest molecular-weight fraction, which could also include some gums, due to the method of "purification". The methylated and acetylated derivatives represent far more satisfactory material for such determinations, as these are benzene-

soluble. Values of 1133 - 1173, 1318 ± 85 and 1084 for the molecular weight of the methylated tannin, have been obtained by one accurate ebulliometric and two different and approximate cryoscopic methods. With the gentle methods of methylation and acetylation now available, accurate and reproducible results should be obtainable.

Two photometric methods of tannin analysis have been evolved and found to give accurate results. One depends on an absorption peak in the ultra-violet region (280 mμ) caused by the phenolic chromophoric groups in the extract. The other depends on a blue-violet complex formed by buffered ferrous tartrate solutions with ortho-hydroxy groups in the extract ( $\lambda$ max. = 545 mμ). The non-tans (gums and sugars) do not interfere. Both methods are rapid and should supercede the laborious hide powder method where large numbers of estimations are necessary.

As previously mentioned, the green wattle tree has numerous advantages over black wattle, but afforestation has been discontinued due to the red colour of the extracts. Fresh green wattle bark shows higher peroxidase activity than that of black wattle, and green wattle tannin also darkens more rapidly over a wide pH range. Identical degradation products are produced from the tannins and their methylated derivatives, and the two tannins also agree analytically. Green wattle tannin, however, differs from black wattle in (a) that it contains no associated yellow fluorescent bodies such as fisetin, (b) the appearance of its two-dimensional chromatograms and (c) its

slightly greater astringency. From two-dimensional chromatograms it appears that many identical tannin constituents are common to both extracts.

Silver wattle bark has a lower tannin content than black and green wattle barks. The tree is reputed to be frost-resistant and capable of growth under relatively arid conditions. Two-dimensional chromatograms of silver and green wattle tannins closely resemble each other. Silver wattle tannins also contain no yellow fluorescent constituents, but identical degradation-products were isolated from both black and silver wattle tannins. Many similar tannin and non-tannin constituents are present in these extracts.

Golden wattle bark has a higher tannin content than black wattle, but the golden wattle tree produces a lower yield of bark per acre, and the extract has the further disadvantage of redness also common to green and silver wattle extracts. The two-dimensional chromatogram of the extract appears to be distinctive.

Chromatographic studies have shown many differences, which it is hoped will assist genetic investigations, when comparing the green, silver and golden wattle tannins with black wattle. Many tannin and non-tannin constituents, however, appear to be common to all the extracts and this re-emphasises the close relationship which obviously exists between the various species.

The effects of various non-tan constituents on the practical tanning process may be studied as a direct result of

this work. The high proportion of gums seems likely to exert a retarding effect on the penetration-rate of the polyphenolic tannins because of their large molecular weight and swelling properties. The tannins may be separated in a "pure" state by the lead-salt method, and the effects of various admixtures of non-tannins assessed by trial tannages. The separation of the tannin into mellow and astringent fractions is also feasible and is receiving attention at this Institute.

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# CHAPTER I.

## THE DEVELOPMENT AND IMPORTANCE OF THE WATTLE INDUSTRY IN SOUTH AFRICA, WITH SPECIAL REFERENCE TO THE DIFFERENT SPECIES OF ACACIA.

From the sustained cultivation of exotic acacias in the Union of South Africa there has developed an organised forest industry covering over half a million acres and providing annual exports exceeding £6,000,000 in value. The wattle industry, concerned primarily with the production of wattle bark and the manufacture of solid extract, has for many years maintained its position as producer and exporter of one of the Union's most valuable agricultural crops. Exports are at present confined mainly to the sterling area (1) within which it constitutes the largest source of natural tanning materials, while those to hard currency areas, provide a substantial amount of valuable exchange.

Due to the increased demand for all vegetable extracts, and to the diminishing potential supplies of such extracts as quebracho, wattle is assuming increasing economic importance and possibly represents the only tanning material afforested on a continuous and sustained-yield basis. World demand for wattle extract far exceeds the available supply, thus providing an excellent opportunity for the continued development and expansion of the industry. Overall augmentation of wattle extract production will depend largely on an extension of the areas under cultivation, but could also be based on an improvement in the

tannin content and yield of bark per acre. These problems, as well as others occasioning tannin losses constitute the research programme of the recently-established Wattle Research Institute (2). Other investigations concerning the practical application of the extract during tannage are carried out at the Leather Industries Research Institute as well as elsewhere.

Such work would be facilitated by fundamental investigations into the nature of the extract. A thorough knowledge of the chemistry of the extracts produced by the different species of acacia is of importance not only to the tanner and extract manufacturer, but also to those investigators concerned with finding new and improved applications of the raw material, as well as others engaged in hybridisation studies. These aspects will be discussed fully at a later stage.

In the past, four species of Acacia have been associated with the wattle industry. These are :-

Acacia mollissima Willd., the black wattle;

Acacia decurrens Willd., the green wattle;

Acacia dealbata Link., the silver wattle;

and Acacia pycnantha Benth., the golden wattle.

In order to convey the relative importance of the different species a brief historical survey follows.

(a) Origin and Development of the Wattle Industry.

Although a wide variety of indigenous tannin-containing plants are to be found in South Africa (See Williams (3)) and

have been utilised from time to time by farmers and small-scale tanners, their economic exploitation is not warranted due either to the dark colour of the extracts, to their relatively slow growth or to their comparatively low tannin content. The most promising of these are Elephantorrhiza burchelli (the "elandsboontjie"), a leguminous plant which contains high proportions of soluble tanning and non-tannins in both rhizomes and leaves and grows plentifully over the wide and arid north-western regions, and also the Rhus tunbergii, or so-called "kliphout".

Exotic acacias of Australian origin belonging to this important genus of leguminous plants included in the family Mimosacae, thus form the basis of the Union's wattle industry. The term "wattles" as applied to these trees, originates from the Anglo-Saxon word "watel" meaning a wicker hurdle, and is today used for fencing, roofing or building walls. As the acacias were employed largely for the construction of such structures in Australia the term "wattle" became by common usage a local name for the trees themselves. In British and Continental leather trade the term "mimosa" is also freely used for tanning materials originating from the various acacias, although its use should be restricted to the family of that name.

Of the above-mentioned, Acacia dealbata was probably the first to be introduced into South Africa during the middle of the last century (4), closely followed by A. mollissima. The seeds of the latter were reputed to have been brought from Australia by the brothers Vanderplank and first planted at Camperdown, Natal in

1864. A pycnantha and A. decurrens were introduced shortly afterwards and the seeds from the original plantings were distributed throughout Natal in later years. These rapidly-growing trees were then primarily used for ornamental purposes, as shelter-belts for stock and the wood for fuel and fencing.

The utilisation of wattle bark as a tanning agent has been traced as far back as 1824 from articles published in Australian and Tasmanian journals. Long before its value as a tanning material was known in South Africa, wattle bark was already a recognised article of commerce in Australia, and had been exported as such to Europe. Its use for this purpose in Natal was, therefore, not long delayed after its first introduction. Sir George Sutton, generally regarded as the pioneer of the wattle industry in South Africa, was the first to encourage the large-scale planting of wattle trees and supplied a mixture of black and silver wattle barks to Lyle's tannery in Pietermaritzburg in 1884. As yet the superior qualities of black wattle bark were unknown, but these soon became evident from trial tannages. Today the sale of silver wattle bark under the name "black wattle" is prohibited by government proclamation in order to obtain a uniform product for foreign trade. Plantations of silver wattle previously established in northern Natal were partly eradicated due to the very inferior qualities of the bark and extract, and afforestation discontinued. Subsequently black wattle was almost exclusively cultivated.

At that time the local price for black wattle bark was far below the London quotations for Australian bark, and a trial

shipment was consequently sent to England in 1886. This realised only £11, but in the following year excellent prices were obtained and large-scale commercial afforestation was started almost immediately. The phenomenal growth of the industry can be gauged from recent production figures (Table I) and available values of the export trade (Fig. I and Table II).

TABLE I.

QUANTITY AND VALUE OF WATTLE BARK, CHOPPED OR GROUND IN THE UNION

| Year | Quantity Short Tons | Value £ |
|---|---|---|
| 1943/44 | 52,426 | 436,600 |
| 1944/45 | 54,552 | 492,543 |
| 1945/46 | 55,517 | 499,804 |
| 1946/47 | 60,000 ※ | 672,000 ※ |
| 1947/48 | 62,000 ※ | 888,000 ※ |
| 1948/49 | 67,000 ※ | 1,056,000 ※ |

※ Preliminary.

Source of above information : Industrial census bulletins compiled by Bureau of Census and Statistics.

In 1915 extract factories were established for the first time, with the result that the solid extract exports today far exceed that of the bark in value (Fig. I). The value of local consumption

TABLE II.

EXPORTS OF WATTLE BARK AND BARK EXTRACT

| YEAR | WATTLE BARK | | WATTLE BARK EXTRACT | |
|---|---|---|---|---|
| | Quantity lb. | Value £ | Quantity lb. | Value £ |
| 1940 | 162,450,324 | 547,281 | 131,403,185 | 960,281 |
| 1941 | 122,723,309 | 421,802 | 115,779,876 | 866,760 |
| 1942 | 114,348,330 | 400,642 | 162,717,106 | 1,249,037 |
| 1943 | 110,022,076 | 424,960 | 141,442,141 | 1,160,077 |
| 1944 | 97,464,732 | 410,142 | 132,466,293 | 1,160,075 |
| 1945 | 95,876,592 | 423,325 | 147,196,053 | 1,411,071 |
| 1946 | 118,227,814 | 547,824 | 143,556,572 | 1,418,075 |
| 1947 | 112,234,458 | 634,848 | 174,169,713 | 2,192,443 |
| 1948 | 135,432,669 | 1,040,854 | 201,185,593 | 3,359,134 |
| 1949 | 139,845,199 | 1,180,882 | 209,513,542 | 3,779,793 |
| 1950 | 112,073,925 | 982,809 | 183,564,203 | 3,386,017 |
| 1951* | 90,379,621 | 1,111,500 | 186,259,030 | 4,995,198 |

* Provisional figures.

Source : Trade and Shipping.

of wattle extracts in South Africa outstripped that of imported vegetable tannins since 1944 (Fig. II). An index of the increasing use of wattle extract in the United Kingdom is reflected in Table III (5) :

The Value of Exports of Wattle Extracts and Barks from South Africa 1886-1951

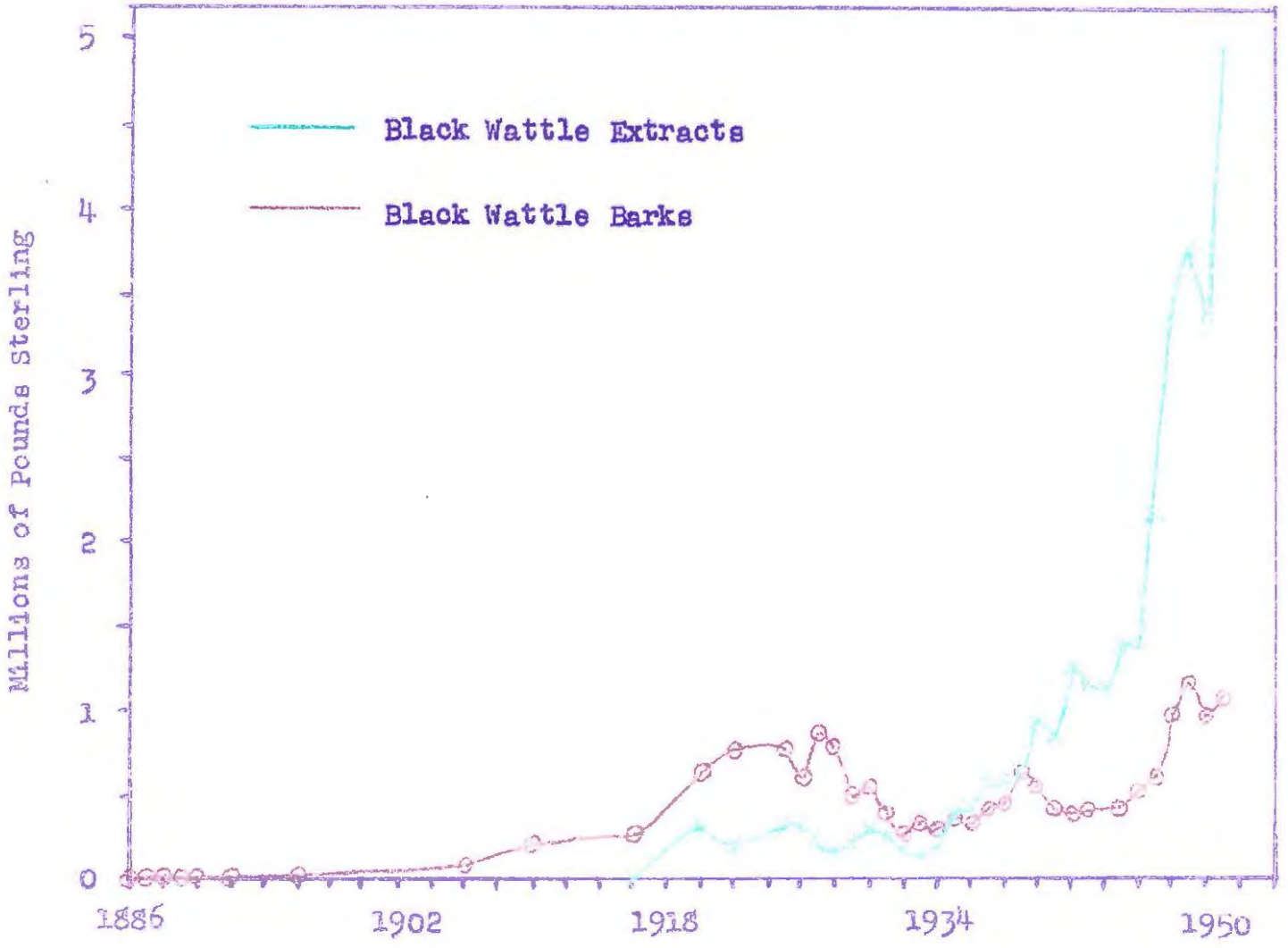



Fig I.

Fig. II.

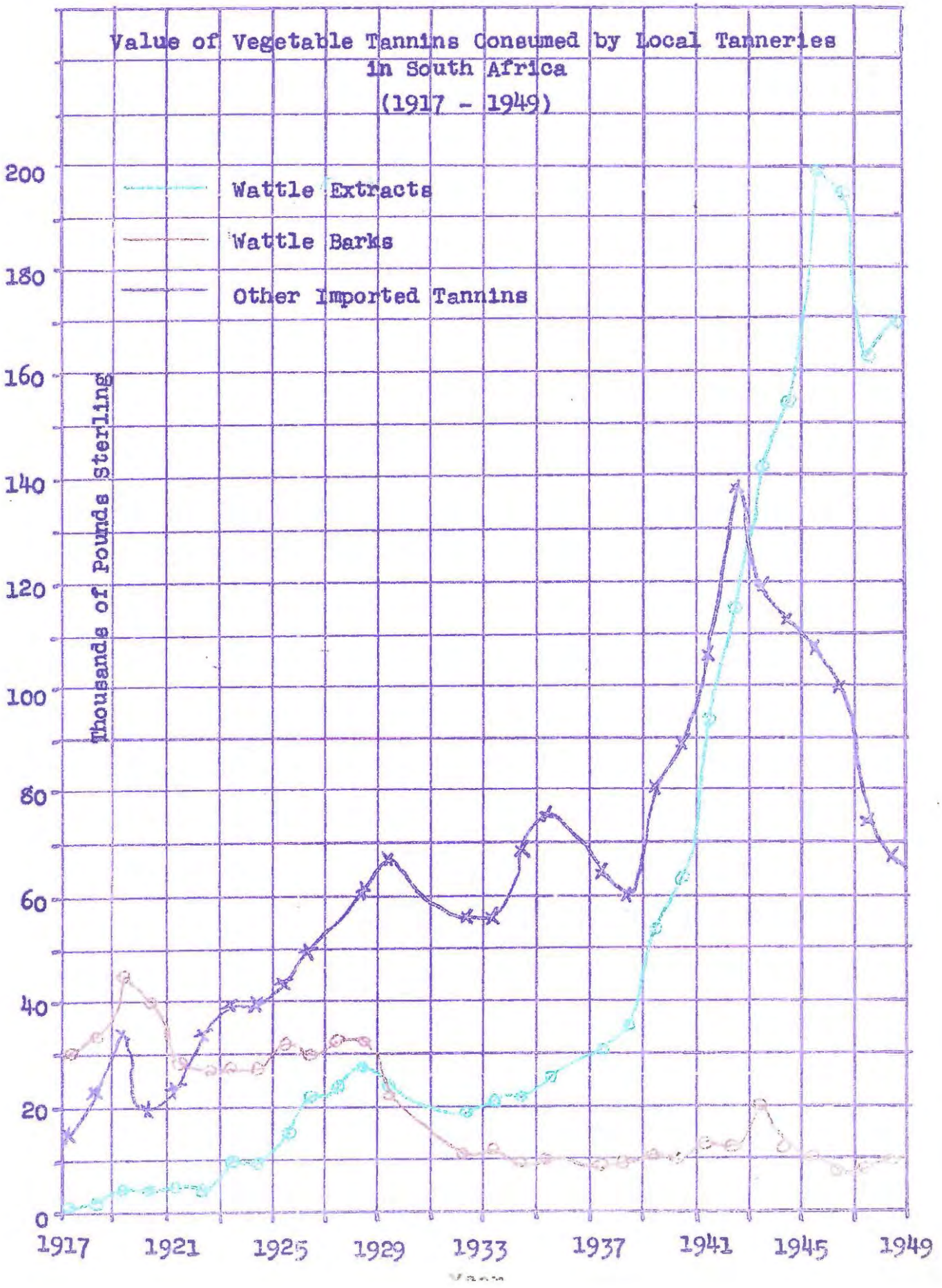

Value of Vegetable Tannins Consumed by Local Tanneries in South Africa (1917 - 1949)
Wattle Extracts
Wattle Barks
Other Imported Tannins
Thousands of Pounds Sterling
200
180
160
140
120
100
80
60
40
20
0
1917
1921
1925
1929
1933
1937
1941
1945
1949

TABLE III.

U. K. IMPORTS OF VEGETABLE EXTRACTS IN 1000 TONS

| Year | Mimosa | Quebracho | Myrabolam | Others | Total |
|---|---|---|---|---|---|
| 1945 | 21.5 | 15.3 | 6.3 | 4.1 | 47.2 |
| 1946 | 18.0 | 23 | 4.3 | 4.2 | 49.5 |
| 1947 | 22.5 | 21.5 | 6.4 | 6.5 | 56.9 |
| 1948 | 30.4 | 7.3 | 5.9 | 4.6 | 48.2 |
| 1949 | 28.3 | 3.9 | 7.2 | 2.6 | 42.0 |
| 1950 | 29.5 | 5.9 | 7.8 | 5.2 | 48.4 |

"Others" include oak, chestnut and valonia.

Since 1947 a noticeable increase in the importation of wattle and a corresponding decrease in the use of quebracho has occurred.

South Africa, although the largest producer and exporter to world markets at present, is not entirely free from potential competition. At an early date wattles were also introduced into other countries with suitable climatic conditions, and relatively large plantations exist in Brazil, India, Ceylon and Madagascar. On the African continent the main competition is centred in Kenya where a well-established wattle industry produces both bark and extracts. More recently extensive plantations have been established in Southern Rhodesia and others projected in Southern Tanganyika and Northern Nyasaland. Australia naturally led the field as exporters of wattle bark in the early days with Britain

as the main market. Later her exports dwindled considerably due partly to the depletion of natural forests on which the Australians mainly depended, and partly also to increased labour costs. Such factors have not been surmounted and Australia, although the home of the tannin-producing acacias, is today one of the large importers of the Union's wattle barks and extracts, being unable to produce sufficient for her own requirements.

Due to the lack of mechanical methods for stripping the bark, the labour requirements in wattle production are considerable. The African continent is particularly fortunate in possessing large and cheap labour reserves at present. This factor, coupled with the ideal climate and excellent conditions of growth should enable the South African wattle industry to consolidate the strong position already established.

(b) Climatic Requirements and Distribution

Black wattle trees grow most successfully mainly in the deep fertile soil of the so-called "mist-belt" areas of Natal located within the altitudinal range of 2,000 to 4,500 feet above sea-level, and having a minimum annual rainfall of 35 inches. At higher altitudes frost and snow damage limits growth, while in the milder coastal regions recurrent cycles of insect pests are difficult to control. A fair proportion (about 25%) is also cultivated in the south-eastern corner of the Transvaal, and in the Cape small plantations are confined mainly to the eastern area where conditions are drier than in Natal. The distribution of

areas under cultivation is reflected in Tables IV (from Sherry (4) and Craib (6)) and V.

TABLE IV.

ACREAGE AFFORESTED WITH WATTLES IN SOUTH AFRICA

| Province | Year | | | | | |
|---|---|---|---|---|---|---|
| | 1908 | 1921 | 1926 | 1930 | 1937 | 1946 |
| Natal | 147,778 | 222,211 | 230,618 | 379,351 | 362,811 | 408,928 |
| Transvaal | | 49,163 | 49,051 | 111,866 | 136,489 | 139,268 |
| Cape | | 15,071 | 27,776 | 36,043 | 35,418 | 41,452 |
| O. F. S. | | 1,245 | 1,938 | 2,389 | 1,279 | 3,586 |
| Totals | 147,778 | 287,690 | 309,383 | 529,649 | 535,997 | 593,234 |

Source of Information : Agriculture census bulletins.

From the above it is evident that after 1930 the previously rapid rate of expansion was not maintained. This is due to a variety of factors which not only seriously limit significant expansion but also actually tend to reduce the area under cultivation (2). The afore-mentioned susceptibility of black wattle to frost damage and insect attack, as well as poor soils and lower or irregular rainfall are mainly responsible.

The silver wattle, however, is reputedly resistant to frost damage, and is capable of vigorous growth in areas of lower rainfall. Yet the reddish colouration of the extract as well as the lower yield of bark per acre militates against its large-scale

## TABLE V

## DISTRIBUTION OF WATTLE PLANTATIONS IN THE UNION

(31st August, 1946)

| | Black and Green Morgen | Other Morgen | Total Morgen |
|---|---|---|---|
| A. Private European Farms | | | |
| Cape | 7,350 | 1,461 | 8,811 |
| Natal | 172,001 | 10,840 | 182,841 |
| Transvaal | 58,666 | 6,065 | 64,731 |
| O.F.S. | 973 | 691 | 1,664 |
| UNION | 238,990 | 19,057 | 258,047 |
| B. Native Reserves | | | |
| Cape | 4,279 | 169 | 4,448 |
| Natal | 6,627 | 677 | 7,304 |
| Transvaal | 94 | 18 | 112 |
| O.F.S. | - | 29 | 29 |
| UNION | 11,000 | 893 | 11,893 |
| C. Government Plantations | | | |
| Cape | 5,204 | - | 5,204 |
| Natal | 169 | - | 169 |
| Transvaal | 267 | - | 267 |
| O.F.S. | 1 | - | 1 |
| UNION | 5,641 | - | 5,641 |
| D. Municipal Plantations and Forests | | | |
| Cape | 1,074 | 98 | 1,172 |
| Natal | 3,359 | 30 | 3,389 |
| Transvaal | 869 | - | 869 |
| O.F.S. | 1 | 4 | 5 |
| UNION | 5,303 | 132 | 5,435 |

Source : 1945/6 Agriculture census bulletins.

afforestation. Hybridisation studies have thus been instituted, aimed at producing frost-resistant strains from the progenies of black and silver wattle crosses.

The green wattle tree, due to its vigorous growth, is known to be more resistant than black wattle to bagworm attack. This tree is, therefore, also receiving attention in genetic studies at the Wattle Research Institute.

A fundamental knowledge of the extract of each of the acacia species would assist in, and might even prove an essential prerequisite for, such work.

(c) Distribution of Tannin in the Black Wattle Tree

In all acacias the tannins and their associated non-tannins are concentrated mainly in the bark. Williams determined the tannin content of the leaves, twigs, wood (7), roots (8) and pods (9) from mature trees (Table VI).

TABLE VI

TANNIN CONTENT OF VARIOUS PORTIONS OF THE MATURE BLACK WATTLE TREE

| Portion of Tree | % Tannin | % Non-tannins | % Insolubles | % Moisture |
|---|---|---|---|---|
| Leaves only | 4.9 | 8.4 | 44.8 | 41.9 |
| Leafless twigs | 3.6 | 5.9 | 44.6 | 45.9 |
| Upper Stem | 0.4 | 6.7 | 92.9 | - |
| Stem Base | 1.6 | 3.3 | 95.1 | - |
| Roots | 12.7 | 4.2 | 73.1 | 10.0 |
| Pods | 21.6 | 13.5 | 51.8 | 13.1 |

Due to the low tannin content none of these portions of the tree are utilised for extract manufacture. The proportion of tannins to non-tannins in the leaves and twigs is remarkably low.

Mature black wattle bark (8 - 10 years' growth) contains on an average 35% tannin. The tannin content of the bark varies within a single tree, being highest in the thick basal bark and gradually decreasing with increasing height. The bark of lowest tannin content is, therefore, to be found in the youngest portions of the trunk and branches. Tannin content usually increases with increasing age (7)(10) and thickness of the bark, but variations in soil conditions, rainfall and other environmental factors also exercise a marked influence. Sherry (4) recently found a significant although not very high correlation between age and tannin content. A higher correlation exists between the diameter of the tree at breast height, and tannin content.

(d) The Stripping of the Bark and the Extraction Processes.

Black wattle trees after reaching maturity are felled and stripped of bark during the period October to June when the soil moisture-content is high. The trees strip easily and almost continuously in areas of high rainfall, but with difficulty in dry areas, or after a sudden drop of temperature.

The stripped bark may either be sent directly to the extract factories, where bark is preferred in the green condition, or else prepared for storage or export by drying. Preliminary drying is usually carried out in the plantations for two

or three days. The colour of dried bark is of great importance in grading (10). Open-air drying is thus satisfactory under fine conditions, but as the stripping of the trees usually takes place in the wet season, the drying bark may be subjected to rain if not covered. Continued moist conditions cause serious discoloration of the bark, especially on the inner surfaces, and may result in non-tan losses (Williams (7)). Direct sunlight also accelerates discoloration, and shade-drying is recommended. Once thoroughly dried the tannin in the bark appears stabilised to oxidative darkening and any subsequent colour increase is very gradual.

Black wattle bark extractives are soluble in hot water and their removal is easily effected. In tanneries the bark is broken up and extracted by a hot countercurrent leach. Alternatively, the bark is first cold-leached and finally hot-leached to remove all solubles. The hot and cold extractives are said to have different tanning properties.

The commercial manufacture of the extract may be divided into three phases :

1) The mechanical conversion of the dry stick bark into small chips and coarse powder.
2) The extraction of the solubles with superheated wattle extract and water, and
3) the concentration of the liquid extract.

The air-dried or relatively moist fresh bark is rapidly broken into small pieces suitable for extraction by mechanical

metal flails. The extraction of the chips occurs with water as solvent in a countercurrent process using a battery of large copper-lined autoclaves. In this process the fresh chips are brought into contact with hot solutions already concentrated by the repeated leaching of partly extracted barks, while the soluble extractives from the almost completely leached bark are removed by fresh water, constituting the first stage of extraction. The resultant solution is concentrated in copper evaporators under vacuum. This "thin liquor" contains about 15% solids and after passing continuously through multiple-effect evaporators, it is concentrated to a "thick liquor" containing 50% solids (11). Finally single-effect evaporators reduce the moisture content to about 17%. Calcium oxalate normally deposits from the liquor during the first stages of evaporation, but in practice its deposition is largely prevented by the addition of sodium hexa meta-phosphate (Calgon) to the system (11).

The final concentrate is discharged in the hot state from the last evaporator in the form of a thick pliable solid, into jute bags holding on an average 112 lbs. After cooling over 36 hours the extract sets into a hard mass, and is suitable for shipment or transport in this condition.

Strict analytical control is exercised both as regards tannin content and the colour of the product. It is guaranteed to contain not less than 60% tannins (62% in the case of exports to the United States (1)) by the manufacturer. The commercial extract breaks up into an amorphous mass, and is easily soluble in hot

water. The solution does not sludge appreciably and contains a very low proportion of insolubles. A typical analysis of present-day production is :-

| | | |
|---|---|---|
| Tannins | = | 61.7% |
| Non-tannins | = | 19.1% |
| Insolubles | = | 1.2% |
| Moisture | = | 18.0% |

A portion of the moisture is lost on further exposure to the atmosphere resulting in an extract which crumbles easily to the touch.

Extract manufacturers use a slightly modified version of the standard hide-powder technique of tannin estimation, which is slow and laborious.

(e) By-products of the Black Wattle Tree

The exploitation of the black wattle has been justified solely by the valuable bark it produces. Wattle timber, however, is a valuable by-product which has been used on a large scale for many years as pit props in the mine shafts of the Reef gold-fields, and also as fuel. More recently wattle timber has found a new use in the manufacture of hardboard, but this utilises only a fraction of the total production. The size of the tree when felled (about 4 - 5 inches diameter) unfortunately limits the uses to which it may be put. Parquet flooring manufacture for which it is ideally suited, and also paper making, are two projected industries. Plastic products have been formed by incorporating

a proportion of ground bark into a tannin-formaldehyde mixture (12) and the latter also forms an excellent water-resistant adhesive for wood. (13).

(f) Comparison of Wattle Tannins with Other Vegetable Extracts

It is common knowledge that the quality of leather produced during any tanning process depends on the chemical constitution of the extract as well as the manner in which it is used.

Significant chemical and physical properties of extracts may be listed as follows :-

1) The constitutional nature of the tannins.
2) The stability of the tannins to enzymatic and atmospheric oxidation, which also determines their colour-stability.
3) The stability of the tannins to condensation at different pH values and temperatures.
4) The relation between average molecular weight, surface tension and
5) The affinity of the tannins for collagen.
6) The chemical nature of the non-tannins, including their mineral content; their acid content and acid types present; and their stability to fermentation.
7) The insolubles content of the extract, and ease of formation of insolubles in relation to time, temperature and concentration.
8) The viscosity of the extract solutions.

Some of these have already been related to the physical properties of the resultant leather, but due to the complexity of the extracts the leather chemist still awaits the analysis and structural elucidation of almost all the commercially important vegetable extracts now in use.

Wattle extracts have a relatively high pH value (4.6 at 50 Bk); low salt and acid content (35 and 60 mgm. equivs/litre of 100 Bk. solution); relatively low viscosity (8.2 poises at 16°C. and 3.8 at 37°C. for 100 Bk. solutions) and high solubility. The last-mentioned property makes wattle extract particularly useful for tanning blends, where by the appropriate additions, the composition of the wattle liquors may be adjusted for any purpose. Unlike other condensed tannins, wattle extracts do not readily precipitate over the pH range 1 - 4, and compare favourably with hydrolysable tannins in this respect. On account of their high solubility, wattle extracts are not easily precipitated from aqueous solution on the addition of salts, as compared with other condensed tannins.

Leather chemists have from time to time published tables comparing the speed of tannage and the amounts of tannins fixed using various vegetable extracts. Shuttleworth (208) has reviewed this work and has demonstrated that much of it has failed to take into account the effects of pH and the various non-tannins (acids, salts, sugars and gums). He has shown that where reasonable cognisance is taken of these differences, wattle extracts compare favourably with others when correctly used in the tannery. It is evident, however, that a thorough study of the composition of non-tannins is desirable.

Wattle-tanned leather tends to redden when exposed to sunlight or ultra-violet light. This disadvantage is common to all condensed tannins, and some practical aspects of colour control are discussed in this thesis.

# CHAPTER II

## ORGANIC CHEMISTRY OF THE VEGETABLE TANNINS

The vegetable tannins are amorphous polyphenolic substances of high molecular weight, and often of semi-colloidal nature, capable of combining with the proteins present in hides and skins to form non-putrescible leather. Numerous theories are extant regarding the nature of such combination, but the hydrogen-bond theory of Shuttleworth and Cunningham (14) is most generally accepted today, and has found the support of other workers (15). This theory, and subsequent comparative work by Shuttleworth (209) on synthetic tannins, groups the naturally occurring tannins together as materials which

a) are soluble in water because of hydrogen bond formation with water molecules,

b) are capable of forming strong hydrogen bonds with collagen protein,

c) have molecules which are spatially orientated in such a manner that sufficient of the polar groups become hydrogen bonded to collagen protein to remove the molecules from aqueous solution.

Many compounds capable of hydrogen bonding to water (e.g. sugars and small phenolic bodies) are not tannins, and it appears desirable to consider the structural evidence available on all the natural tannins as a general guide to the structural elucl-

dation of wattle tannin. The successful techniques used and the difficulties encountered are also considered useful as a guide to subsequent work on wattle tannin.

Tannins are weakly acidic, and have an astringent taste. They form coloured complexes with certain salts; are precipitated by albumin, gelatin and various alkaloids; and are used extensively in the manufacture of leather. They are widely distributed in nature and occur in varying proportion in almost all plants. Those of commercial importance are often highly concentrated in certain portions of the plant e.g. in the bark of the acacias; in the heartwood of Schinopsis lorentzii Engl. and S. balansae Engl. (quebracho tannin); in the leaves of Rhus coriana (sumac) and Thea sinensis L (green tea); in the fruits of Terminalia chebula Retz. (myrabolam); in the pods of certain leguminosae (divi-divi and algarobilla) and in galls produced by the action of insects on plants (tannic acid).

The function of tannins in plants is not known. They are most likely by-products of photosynthetic processes and merely incidentally furnish a protective action against insects and animals by virtue of their astringent taste. Tannins are invariably associated with minor proportions of sugars and other carbohydrates and are considered (16) to arise from such sugars by photosynthesis. Shaded shoots are known to have a higher tannin content than those exposed to the sun (17), and tannins disappear from spirogyra when kept in the absence of sunlight (18).

The natural tannins are constituted of one or more of

the following phenolic nuclei : phenol, resorcinol, phloroglucinol, catechol and pyrogallol, and yield these on drastic degradations such as uncontrolled alkali fusions. Sugars, chiefly glucose, also constitute part of the unit in some tannins. The presence of a phloroglucinol nucleus may be detected by the colour produced with a pine shaving in the presence of hydrochloric acid; and catechol and pyrogallol nuclei by the green and blue complexes produced in aqueous solution with ferric chloride or ferric alumn. Where both ortho-hydroxy nuclei are present, the more intense blue colour of the latter masks the weaker green of the former and hence the colour reaction gives no clear-cut distinction.

Tannins are precipitated with gelatin-salt reagent (19). Their classification is based on chemical behaviour. Some are hydrolised by mineral acids or enzymes to produce hydroxycarboxylic acids (e.g. gallic and p-hydroxy-benzoic acids) and neutral carbohydrates (such as glucose); while others are not hydrolised by mineral acids but converted to red insoluble precipitates termed "phlobaphenes" or "tanners' reds".

Freudenberg (20) accordingly classified tannins into two main groups :-

(1) Hydrolysable Tannins

and (2) Condensed Tannins.

Freudenberg's hydrolysable tannins include the following principal types :

a) the depsides or mutual esters of phenolic carboxylic acids.

b) esters of phenolic carboxylic acids and sugars,

c) glucosides of depsides

and d) the ellagitannins.

The condensed tannins are more complex and much less information regarding their nature has been gleaned from chemical investigation. They include two types :

a) hydroxybenzophenones

and b) compounds hitherto considered to be constituted of polymers or condensates of catechins, with which they are often associated in nature e.g. in gambier extract and others.

There are a few exceptions to the above subdivision. Most notable of these are the "tannins" in green tea which contain a galloyl ester of catechins hydrolysable by tannase (21), and also by acid hydrolysis, to form a phlobaphene and gallic acid. The tannin from Quercus cirris (22) yields ellagic acid and a phlobaphene on acid hydrolysis.

Tannin chemistry represents a difficult field due to the amorphous nature of almost all tannins. The consequent lack of criteria of purity and purification difficulties, together with the large molecular size and the ease of condensation of polyphenolic compounds, have all caused much confusion, and clear-cut reactions, analyses and conclusions are rarely attainable. As a result much work has been done on material of doubtful uniformity. This has led to conflicts regarding analytical results, degradation products and the resultant structural

implications.

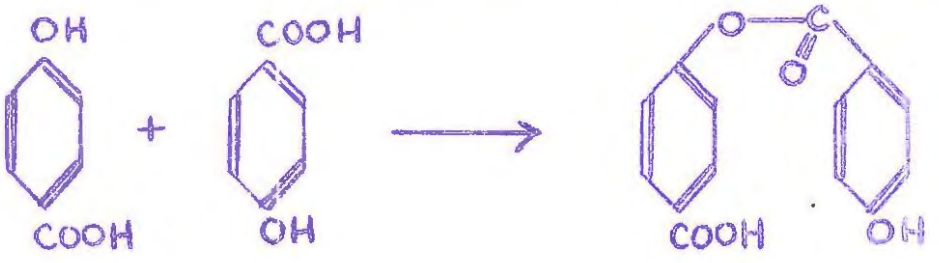
The hydrolysable tannins, a few of which are known to exist in the crystalline state, have thus far received most attention. This is so probably because their easy fission gave readily identifiable products and the method of fission could be related to simple ester linkages.

Condensed tannins by comparison have so far presented an even more complex field of investigation. Low yields of phenolic bodies are obtained only through drastic degradations and their mode of linkage is still subject to speculation. A large number of theories have resulted, some of which have been disproved while others appear improbable.

## HYDROLYSABLE TANNINS

### (a) THE DEPSIDE GROUP

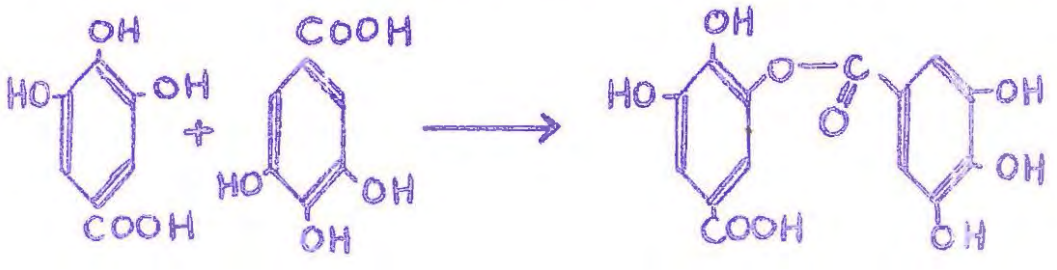
The depsides are structurally the simplest of the hydrolysable tannins. The term "depside" was first introduced by Fischer (22) and represents those compounds formed by the mutual esterification of hydroxy-benzenecarboxylic acids. Thus I where two molecules of p-hydroxybenzoic acid are joined by an ester bridge is called a didepside, and where three or four such




I

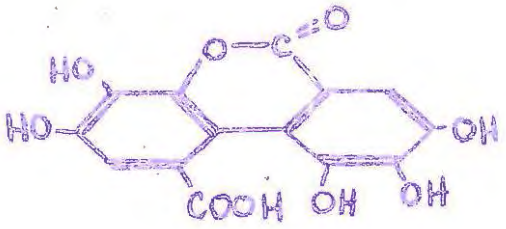
units are joined the names tri- and terta-depside are used. The terminology for each type is similar to that used for polypeptides. A dedepside formed from p-hydroxybenzoic acid is known as p-hydroxybenzoyl-p-hydroxybenzoic acid, and a tridepside as di-(p-hydroxybenzoyl)-p-hydroxybenzoic acid.

m-digallic acid II (23)(24), formed by the mutual esterification of two gallic acid nuclei, is a crystalline tannin

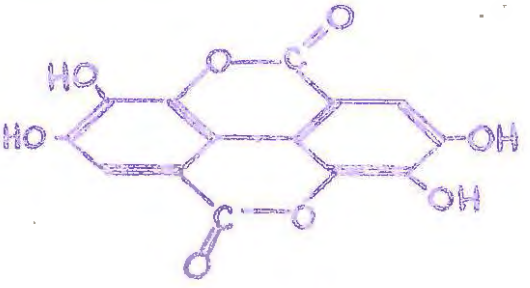



II

which plays an important part in the constitution of hydrolysable tannins where it is combined with glucose to form amorphous tannins. Lutecic acid III (25) and ellagic acid IV (26) are also considered to be didepsides of gallic acid, but will be discussed later under the groups gallotannins and ellagitannins.

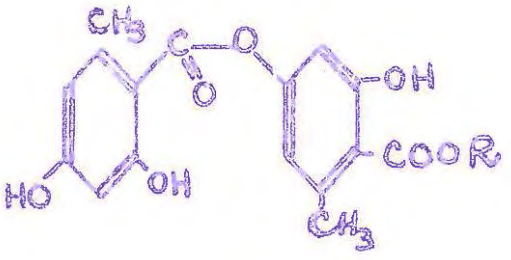


III



IV

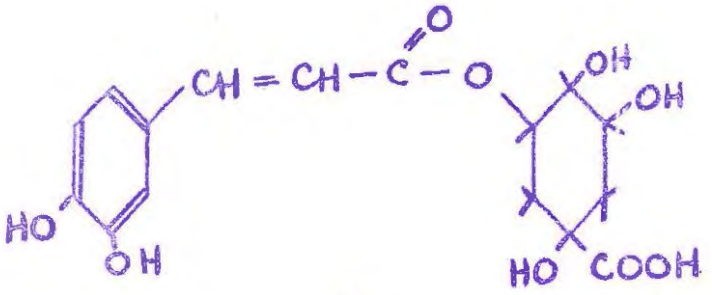
Many of the organic acids obtained from mosses and lichens belong to the depside group. Examples are lecanoric acid V and erythrin VI (27) which possess tanning properties.



V R = H

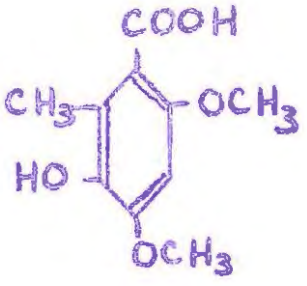
VI R = $-CH_2-\underset{OH}{CH}-\underset{OH}{CH_2}$

Chlorogenic acid VII (28)(29) is a crystalline tannin which occurs in combination with sugars to form amorphous tannins.

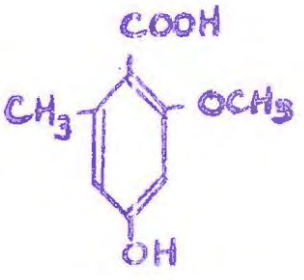


VII

Hiascinic acid (30) an example of a tridepside, is found in the acetone-soluble portion of the extract of Cetraria hoascins, a lichen. It forms a penta-acetyl, but only a tetra-methoxy derivative as one hydroxy group is resistant to methylation. The tetra-methyl ether forms 5-hydroxyorsellinic acid VIII, isoevirninic acid IX and its methyl ester on hydrolysis with ice-cold sulphuric acid.

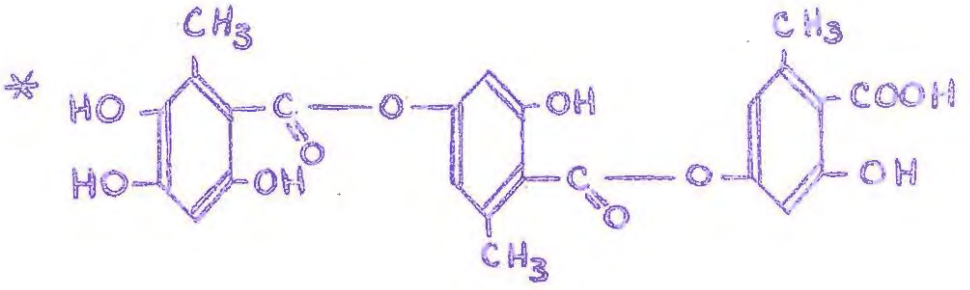


VIII



IX

From this and other evidence, the structure of the tannin is considered to be X of which the hydroxy group[X] is

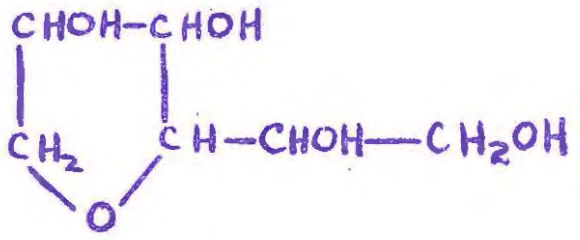


X

resistant to methylation with diazomethane and methyl iodide/ potassium carbonate.

(b) ACERTANNIN

This is a crystalline tannin obtained from the leaves of Acer ginnala Maxim, the Korean maple, and investigated by Perkin and Uyeda (31)(32). It crystallises from water in colourless needles $C_{20}H_{20}O_{13}.2H_2O$; is optically active $[\alpha]_D^{15^\circ}$ in acetone = + 20.55; precipitates gelatin and gives a blue coloration with ferric chloride solution. It forms an octa-acetyl derivative $C_{20}H_{12}O_5.(C_2H_3O_2)_8$. On hydrolysis with 5% sulphuric acid it yields two molecules of gallic acid and one molecule of a new crystalline sugar $C_6H_{12}O_5$ called aceritol (33). The latter is dextrarotatory $[\alpha]_D^{19^\circ}$ = + 39° in water, and gives a tetra-acetyl derivative $C_6H_8O_2(C_2H_3O_2)_4$, but is neither an aldose or a ketose. Aceritol was provisionally represented as an anhydrohexitol XI, probably derived from the hexahydric alcohol sorbitol.



XI

Methylation of the tannin with diazomethane followed by hydrolysis yields only O-trimethylgallic acid. The two gallic acid nuclei therefore take part in ester formation with the aceritol independently and not as a digallic acid unit. Acertannin is, therefore, a digalloyl aceritol in which the position of the galloyl groups and the exact constitution of the sugar molecule has yet to be established.

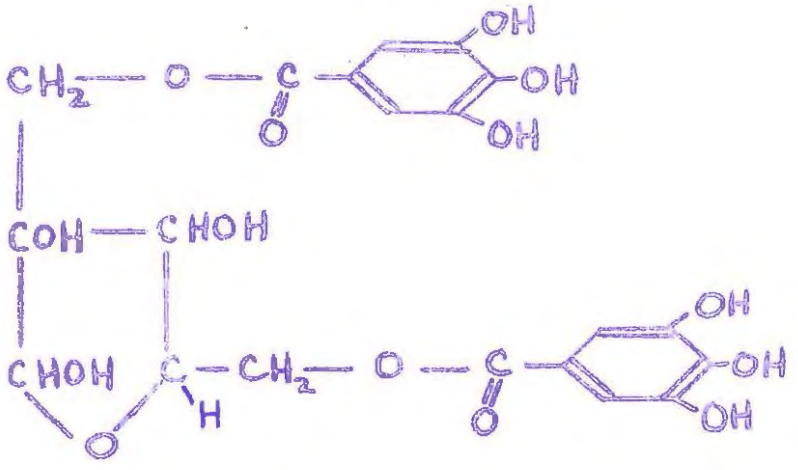
Acertannin is accompanied by digallic acid, traces of a glucoside of quercetin and an amorphous tannin of unknown constitution. It is used in Japan in conjunction with an iron mordant for dyeing cotton and silk.

A related tree, Acerspicatum Lam., contains a crystalline tannin also consisting of independent galloyl nuclei joined by ester links to aceritol, and is thus identical with or similar to acertannin.

(c) HAMAMELI TANNIN

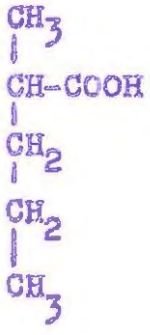
A crystalline tannin occurs in Hamamelis viginica Linn., which resembles acertannin in many respects. As a result of the

work of Vollbrecht (34), Freudenberg and Blümmel (35), Kurmeier (36) and Schmidt (37) a tentative formula XII has been proposed.

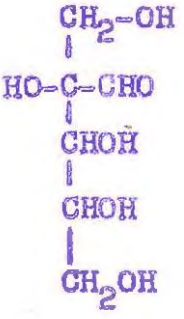


XII

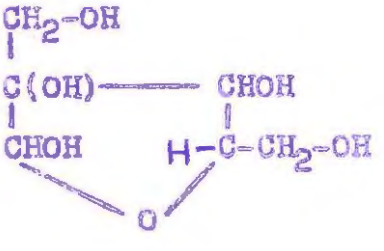
It is considered to be a di-galloyl derivative of a new sugar, an aldohexose, to which the name hamamelose has been given. Hamamelose forms no osazone, but on oxidation gives a hexuronic acid which is reduced to methyl-n-propyl acetic acid XIII on boiling with HI and phosphorus. The isolation of a methyl hexoside which behaves as a $\gamma$-glucoside, from the tannin, coupled with the above properties suggest the structure XIV for the hexose.



XIII



XIV



XV

Hydrolysis of the tannin with the enzyme tannase yields two molecules of gallic acid and one of hamamelose. Hydrolysis of the tannin after methylation with diazomethane yields only O-trimethylgallic acid and the sugar as in the case of acertannin. From the above Freudenberg et Al. suggested the above constitution XV in which the exact constitution of the sugar and the positions of the galloyl radicals still require proof.

(d) CHEBULINIC ACID

Chebulinic acid is a crystalline tannin which occurs together with other amorphous tannins in myrobalans from the fruit of Terminalia chebula. Fischer and Bergmann (38) obtained glucose and gallic acid from the acid hydrolysis of the tannin. They methylated the tannin with diazomethane but the product was capable of further reaction with p-bromobenzoyl chloride showing that some hydroxyl groups (probably of the aliphatic type) remain unmethylated.

Freudenberg and Frank (39) studied the tannin more carefully and suggested a tentative formula based on the following data :

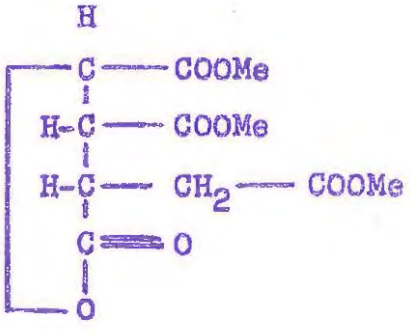
Chebulinic acid may be represented by the formula $C_{41}H_{34}O_{27} \cdot 9H_2O$, and on hydrolysis it yields glucose, gallic acid and a second acid to which the name "spaltzsaure" was given. The hydrolysis may be represented by the reaction :

$$C_{41}H_{34}O_{27} + 5H_2O = 3C_7H_6O_5 + C_6H_{12}O_6 + C_{14}H_{14}O_{11}$$

gallic acid — glucose — "split acid"

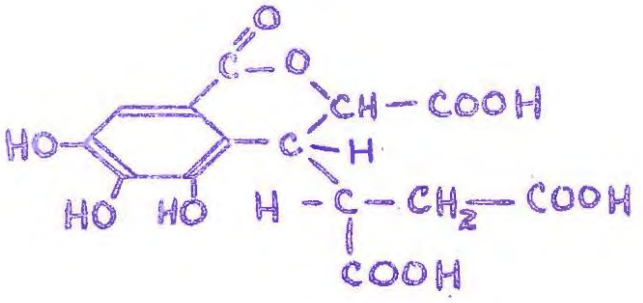
This "spaltzsaure" is also obtainable from chebulagic acid, and is converted by diazomethane into a hexamethyl derivative (40). The latter when hydrolised forms a trimethyl derivative. Schmidt and Mayer (41) very recently established its constitution as follows :

The hexamethyl "split acid" gave very low values with the Zerewitinoff determination and therefore contains no free hydroxy groups. When the trimethyl derivative is treated with alkali, three equivalents of alkali are used in neutralisation. When treated with excess NaOH at 100°C. the presence of a fourth acidic group was indicated by back-titration and an amorphous tetra-salt $C_{17}H_{16}O_{12}Na_4$ may be recovered. This behaviour indicated the presence of an aromatic lactone in the "spaltzsaure". The trimethyl derivative of the acid when oxidised with permanganate under controlled conditions, and the product methylated with diazomethane yielded XVI




XVI

The structure of the corresponding acid was proved by various methods including alkaline degradation products. The "spaltzsaure" also yields pyrogallol on decomposition and the structure XVII was proposed on the following grounds :



XVII

a) The acid corresponding to XVI resists decarboxylation; it is thus not a malonic acid derivative and the three carboxyl and one lactonic group are associated with different carbon atoms.

b) The lactonic group is derived from an $\alpha$-hydroxy acid, as sodium cyanate is obtained by the action of sodium hypochlorite on the triamide of the trimethyl "split acid".

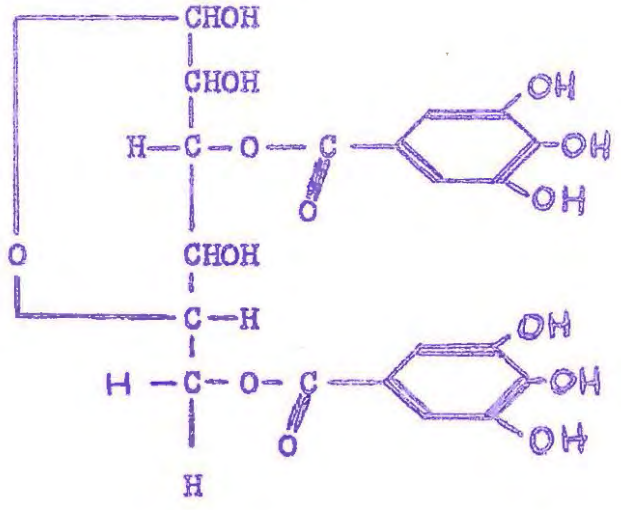
c) The acid form of XVI gave a mixture of acetic, oxalic, and succinic acids on fusion with potassium hydroxide.

d) The proposed formula for the "split acid" XVII was attractive because it could be derived from ellagic acid on the oxidative fission of one of the phenolic rings.

Conclusive support of this formula was furnished very recently by Haworth and de Silva (42) who obtained 3 : 4 : 5-trimethoxy phthalic acid in yields exceeding 40% by the oxidation

of the trimethyl derivative of the "split acid" with alkaline potassium ferricyanide.

Partial hydrolysis of chebulinic acid yields equimolecular proportions of a digalloyl glucose, gallic acid and "spaltzsaure" $C_{14}H_{14}O_{11}$ (43). The digalloyl glucose behaves as 3,6-digalloyl glucose XVIII (44) and its constitution was proved by synthesis from diisopropylidene glucose and tribenzyl galloyl chloride (45). The gallic acid is attached by an ester linkage



XVIII

to one of phenolic hydroxyl positions on the "spaltzsaure" nucleus. The points of attachment of the "spaltzsaure" to the glucose residue are most likely the 1 and 2 positions.

(e) CHEBULAGIC ACID $C_{41}H_{30}O_{27} \cdot 10H_2O$.

Chebulagic acid which might also be classified as an ellagitannin, is a crystalline tannin recently isolated by Schmidt

and Nieswandt (46) from myrobalans and by Schmidt and Lademan (40) from divi-divi. It occurs with chebulinic acid which it resembles in many respects. On complete hydrolysis it yields one mole each of ellagic acid, glucose, gallic acid and "split acid". The difference between chebulagic and chebulinic acid is thus obvious.

With crystalline tannins a fair amount of progress has, therefore, been made in recent years, and the structures of the major fragments constituting such tannins, have been satisfactorily elucidated. Their points of attachment to each other in most instances are not known with certainty. The transition from crystalline to amorphous tannins introduces uncertainty regarding the purity or homogeneity of the material under investigation, and consequently also regarding the significance of degradation products isolated in low proportion.

Two amorphous hydrolysable tannins which have received much attention are tannic acid or Chinese tannin found in Chinese galls produced by the green fly, Aphis chinensis, on the leaves of Rhus chinensis Mill. of the family Anacardiaciae, and Turkish tannin found in Turkish or Alleppo galls on the leaves and twigs of Quercus infectoria Oliver of the family Fagaceae, caused by the cynipid fly, Cynips tinctoria.

(f) CHINESE TANNIN

Attempts at the extraction of gallotannins from galls started towards the end of the 18th century. Kunsemüller (47) was the first to extract appreciable quantities using ethanol.

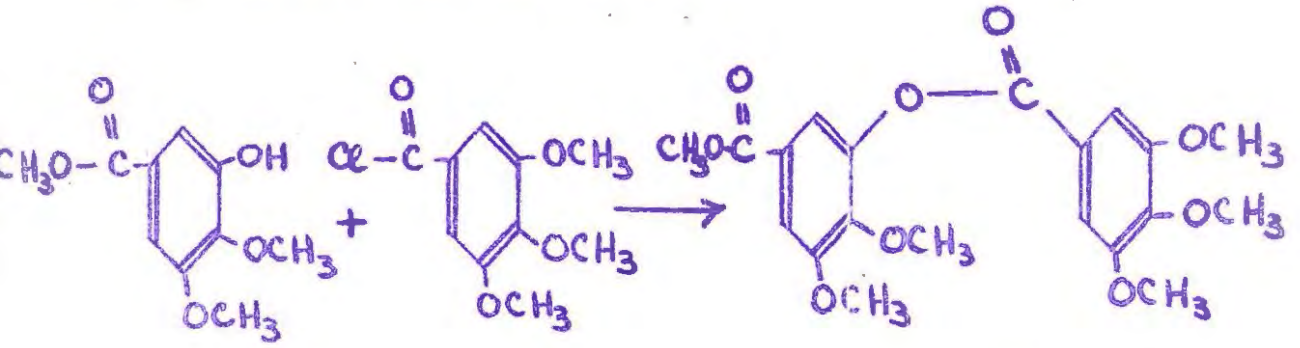
Pelouze in 1834 (48) used a mixture of water, alcohol and ether which is still employed today in industrial processes. The extract obtained in this way was heterogeneous, consisting of gallotannin contaminated with gallic acid, glucose, chlorophyll and mineral salts.

Attempts were made to isolate a homogeneous substance for constitutional investigation from the earliest times. In 1827 Berzelius (49) removed the gallic acid by precipitating the alcoholic extract with sodium carbonate and extracted the product with alcohol. Other methods were also attempted but only the one used by Paniker and Stiasny (50) gave satisfactory results and was subsequently employed by Fischer and Freudenberg (51). This consists of extracting the gallotannin with ethyl acetate, removing the acids from this solution with alkali, and finally removing the solvent under vacuum. Even after such purification, however, the tannin remained amorphous and no definite proof of purity could be established. As the same material was obtained from various commercial samples from different sources, the purified sample was considered more or less homogeneous.

Strecker (52) in 1852, as a result of extensive research, concluded that the gallotannin was constituted of glucose and gallic acid nuclei in the ratio of 1 to 3, and that the presence of glucose explained the optical activity of the tannin.

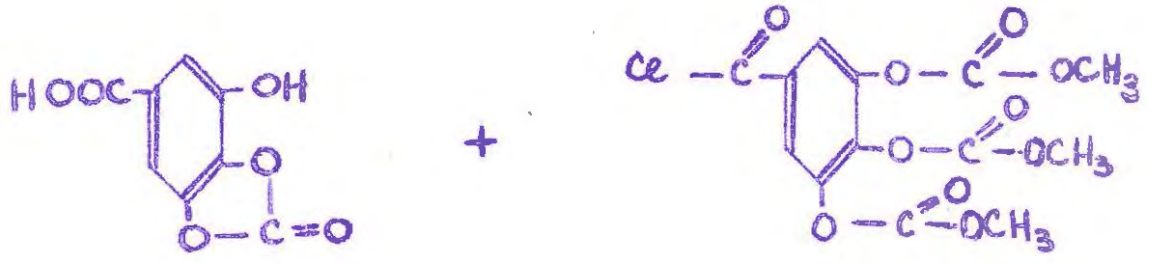
Strecker's findings were contested by Kawalier (53), Robiquet (54), Wehmer and Tollens (55) and Pottevin (56) but supported by Freda (57), Utz (58), Feist (59), Fisher and Freuden-

berg (60), Gaeke and Nierenstein (61) and by Karrer et Al. (62). Hlasiwetz (63) and Schiff (64) went further and claimed that gallotannin was related to gallic acid and consisted of a compounded gallic acid. Schiff formed a digallic acid which behaved very much like gallotannin but was subsequently proved to be m-digallic acid by Maunther's (66) synthesis of the methylated derivative XIX from the methyl ester of 3,4-dimethoxygallic acid and O-trimethyl galloyl chloride :



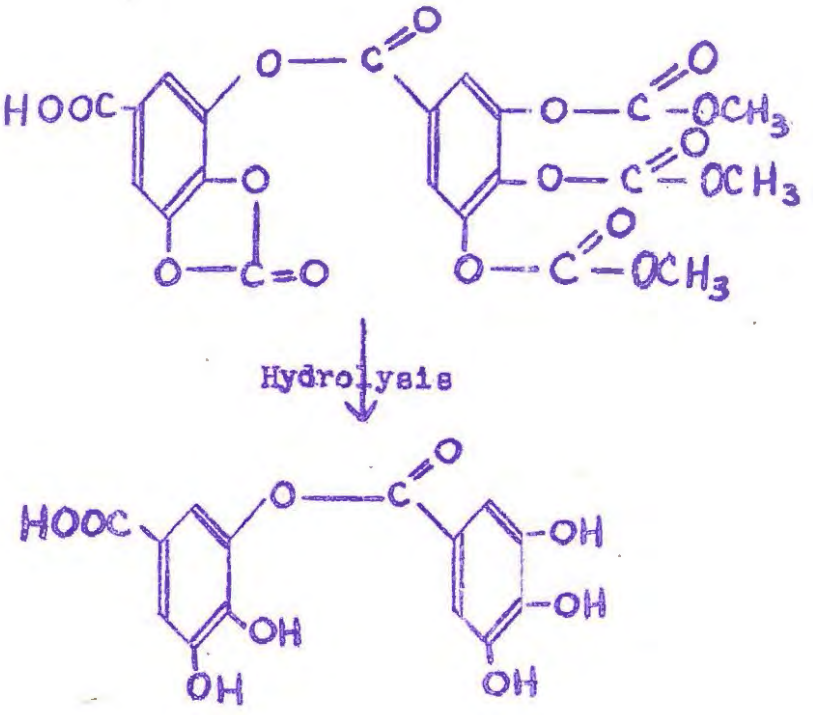
XIX

and by Fischer and Freudenberg's (65) synthesis of the digallic acid itself :



carbonyl gallic acid

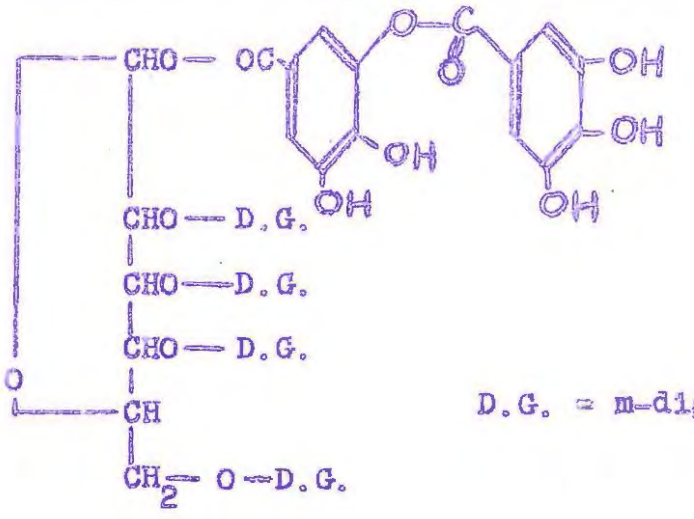
tricarbomethoxygalloyl chloride




Schiff's digallic acid formula, although it failed to explain the optical activity of gallotannin, was only questioned a quarter of a century later by Walden (67) who demonstrated many differences between the two materials.

Fischer and his school commenced work on gallotannin at the beginning of this century and placed the chemistry of gallotannins on a sound basis. They purified (51) the tannin by Paniker and Stiasny's (50) method. Hydrolysis of the purified product gave glucose and gallic acid in the proportion of 1 to 10. From various data they concluded that as glucose contains only five hydroxyl groups, only five galloyl groups could be joined to the sugar by ester bridges. The remaining five gallic nuclei must, therefore, be joined to those galloyl nuclei already attached to the nucleus, forming depside links.

Such a structure would explain the ease of hydrolysis of gallotannin and the absence of free carboxylic groups. Fischer considered that the simplest structure, based on the above reasoning, could be represented as a penta-m-digalloyl glucose. XX.




D.G. = m-digalloyl group

XX.

Such a structure would require proof of the presence of m-digallic acid nuclei in the tannin. Attempts to isolate this from the natural tannin failed at first, but was accomplished in later years by Herzig (68). Herzig (69) also isolated O-trimethylgallic acid, and O-3,4-dimethyl gallic acid from diazomethane methylated gallotannin. This provided additional indirect proof of the presence of m-digallic acid.

Fischer next approached the problem by using synthetic methods. He formed a methylated gallotannin by the reaction of pentamethoxy-m-digalloyl chloride with $\alpha$ - or $\beta$-glucose. The product obtained from $\beta$-glucose resembled diazomethane methylated

Chinese tannin. The formation of synthetic Chinese tannin itself was brought about by the condensation of pentaacetyl-m-digalloyl chloride with β-glucose in a similar way. After the removal of acetyl groups penta-m-digalloyl-β-glucose resulted which proved to be similar to, if not identical with natural Chinese gallotannin by a comparison of elementary analysis, chemical behaviour, optical rotation, solubilities and degradation products of the tannins and their methylated and acetylated derivatives.

Fischer regarded the natural and even the synthetic gallotannin, not as a single substance, but at least a mixture of stereo-isomers. As a result of the work of Fischer with Freudenberg (60)(71) and with Bergmann (72) the following analytical results were obtained for the natural tannin from the leaf-galls of Rhus chinesis (Chinese tannin) :

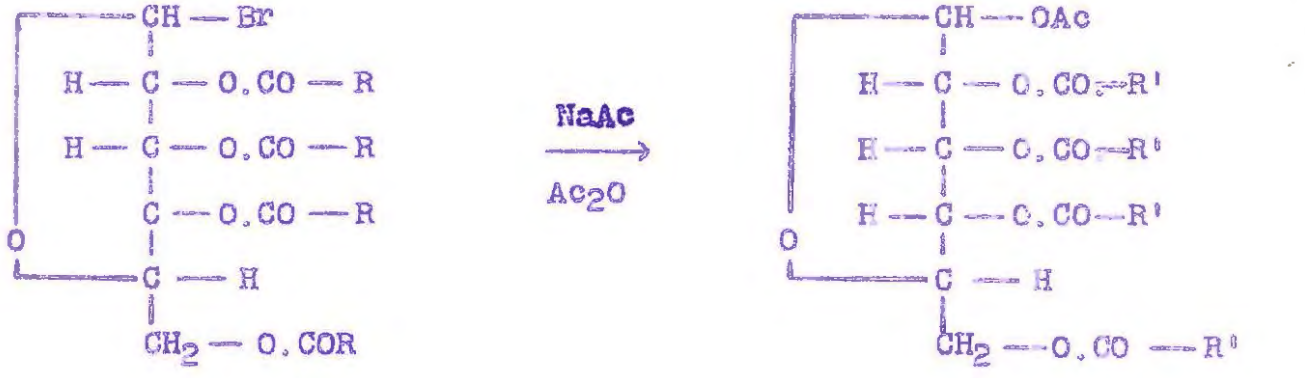
| | Mol. Wt. | % C. | % H. | Gallic Acid | Glucose |
|---|---|---|---|---|---|
| Found : | 1460-1700 | 53.5 | 3.5 | 98.6% | 11.4% |
| Reqd. by : | | | | | |
| Octagalloyl-glucose | 1396 | 53.28 | 3.18 | 97.4% | 13.0% |
| Nonagalloyl-glucose | 1548 | 53.47 | 3.12 | 98.9% | 11.6% |
| Decagalloyl-glucose | 1700 | 53.63 | 3.08 | 100.0% | 10.6% |

The percentages of gallic acid and glucose obtained on hydrolysis of the gallotannin were corrected to allow for losses during hydrolysis. Blanks showed losses of 5% gallic and

55% glucose under identical conditions. The tannin, therefore, corresponds on an average most closely to the nonagalloyl glucose.

Iljin (73) fractionated the tannin with zinc acetate, and Karrer et Al (7C) used aluminium hydroxide. Optical rotation of Karrer's fractions varied from $[\alpha]_D = +30$ to $[\alpha]_D = +157°$.

Karrer subjected the various fractions of high specific rotations to the action of HBr in glacial acetic acid when gallic acid and tetragalloyl-1-bromoglucose XXI were formed. The latter was converted to tetra-(triacetylgalloyl)-1-bromoglucose XXII with acetyl bromide and then with sodium acetate and acetic anhydride to tetra-(triacetylgalloyl)-1-acetylglucose, XXIII.

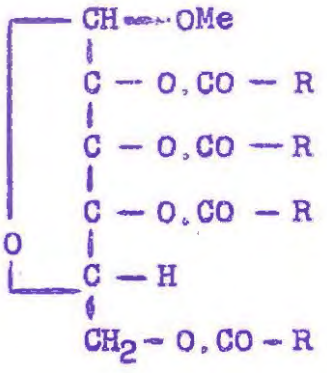



XXI R = pyrogallol

XXII R = triacetylpyrogallol

XXIII R' = triacetylpyrogallol

These products were not crystalline but the analysis of the bromine content indicated purity. Tetra-(triacetylgalloyl)-1-bromoglucose XXII yielded a methyl glucoside XXIV when treated with methanol and silver carbonate.



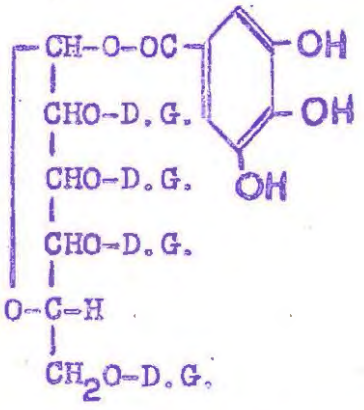
R = Triacetylpyrogallol XXIV.

This product had a similar rotation to that obtained synthetically from the action of triacetylgalloyl chloride on β-methylglucoside and hence the glucose present is likely to have the β-configuration.

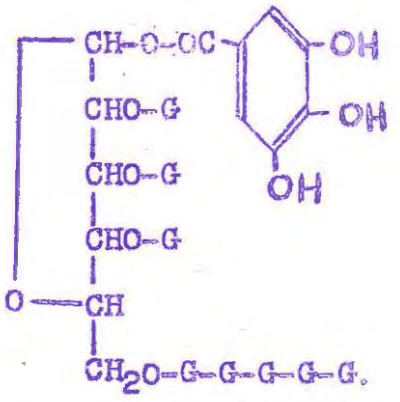
Tetra-(triacetylgalloyl)-l-bromoglucose was also formed from synthetic penta-galloyl glucose by the identical reactions and could similarly be converted to the l-acetyl glucose and l-methyl glucoside derivatives. It was concluded that the tannin fractions of high specific rotation are derived from pentagalloyl glucose, and that the individual fractions differ from each other in the number and arrangement of the depside, gallic acid linkings or in the configuration about the 1-C atom of glucose.

The unfractionated Chinese tannin as well as fractions with low specific rotation give preparations of l-bromoglucose and l-acetyl glucose which differ from that obtained from penta-(triacetylgalloyl)-glucose probably due to naturally occurring impurities. Tetra-(triacetylgalloyl)-l-acetyl glucose was obtained from fractions of high specific rotation in yields

which indicate 8 - 9 moles gallic acid per sugar molecule. It is concluded that Chinese tannin is a mixture of many galloylated glucoses of similar constitution which are responsible for its amorphous character. Many isomeric formulae could be suggested to explain these facts. The two simplest are :




and

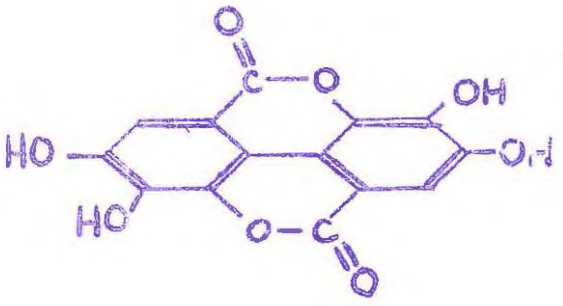



D.G. = m-digalloyl nuclei G = galloyl nuclei

(g) ELLAGITANNINS

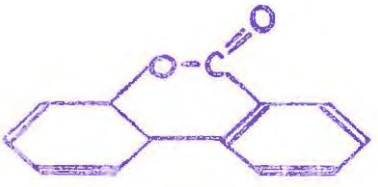
These differ constitutionally from other hydrolysable tannins in that they contain a diphenyl dimethylolid group. Included in this class are tannins capable of depositing the so-called bloom on leather, consisting in most cases of ellagic acid. Ellagic acid XXV generally constitutes a relatively low proportion of the extract and occurs in both free and combined forms.

The principal tannins of this class are valonia (74) which is exceedingly rich in ellagic acid, myrobalans (75), divi-divi (76), algarobilla (77), European oak bark tannin (78) and chestnut (79). Many of these tannins have received little attention, but ellagic acid was recently found to constitute part of chebulagic acid isolated from myrobalans (46) and divi-divi (40).



XXV

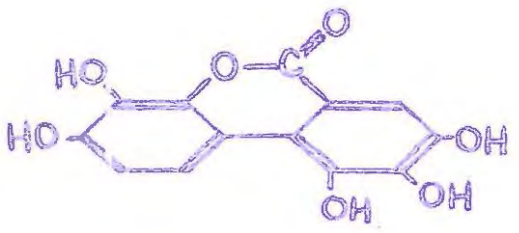
Ellagic acid $C_{14}H_6O_8$ is most readily crystallised from pyridine (80) and converted by alcohol to a pale yellow powder. It may be synthesised by oxidising gallic acid with arsenic acid (81) or with potassium persulphate and sulphuric acid (80). Graebe (82) found that when ellagic acid is distilled with zinc dust fluorene XXVII is obtained. Fluorene was also obtained by the zinc dust distillation of diphenylmethylolid XXVI, and he therefore suggested the structure XXV for ellagic acid.



XXVI

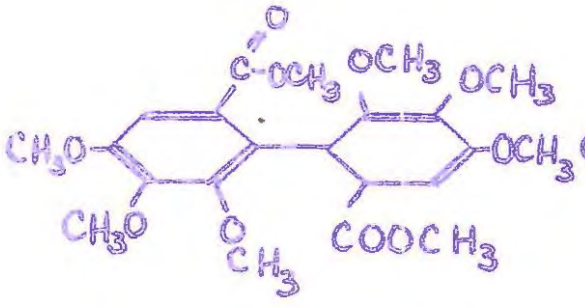
XXVII

Perkin and Nierenstein (80) obtained further evidence by isolating pentahydroxydiphenylmethylolid XXVIII when boiling ellagic acid with KOH solution.

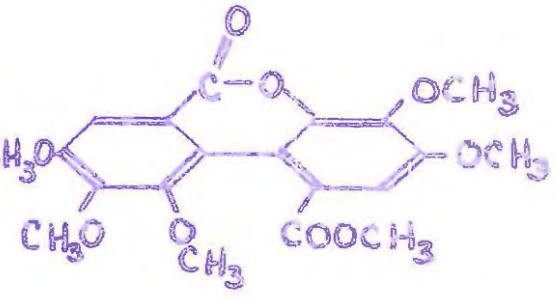


XXVIII

Herzig and Pollak (83) obtained results which further confirmed Graebe's proposed constitution of ellagic acid. When tetramethoxyellagic acid is digested with alcoholic KOH and $CH_3I$ diphenylhexamethoxy-diphenyldicarboxylate XXIX is obtained together with a small quantity of pentamethoxymethylolidcarboxylate XXX.



XXIX



XXX

(h) SYNTHETIC GALLOTANNINS

During their comparisons of synthetic Chinese and Turkish gallotannins with the natural product, Fischer and his school synthesised a number of esters of m-digallic and gallic

acids with alcoholic polyhydroxy compounds, in addition to the penta-m-digalloyl-$\beta$-glucose already mentioned (72). Syntheses were performed in a similar manner by the action of penta-acetyl-digalloyl chloride or tri-acetylgalloyl chloride with various glycols and sugars. Pentagalloyl-$\alpha$-glucose, penta-m-digalloyl-$\alpha$-glucose, pentagalloyl-$\beta$-glucose and trigalloyl glucose were amorphous products although they appeared analytically pure. Digalloyl ethylene glycol, digalloyl trimethylene glycol and tetragalloyl erythritol were, however, isolated in the crystalline state.

Russel and Tebbens (84) also used Fischer and Bergmann's method for preparing a number of synthetic tannins. These were subsequently tested in a number of small-scale tannages and their tanning-ability compared with gallotannin. $\beta$-d-glucose pentagallate, d-mannose pentagallate, aldehydo-d-glucose pentagallate and d-arabinose tetragallate showed good tanning properties and form leather comparable to that tanned by gallotannin. Leathers formed by methyl-d-glucoside tetragallate, d,l-erythritol tetragallate, d-arabitol pentagallate, mannitol hexagallate and sorbitol hexagallate give fair tannages.

A surprising result recorded was that fructose pentagallate is not a tanning material although it precipitates gelatin.

Ethylene glycol digallate and glycerol trigallate gave poor tannages.

## THE CONDENSED TANNINS

The condensed or flavotannins constitute by far the most important commercial vegetable extracts used in the tanning industry today. Gambier, cutch, quebracho, wattle, mangrove, spruce, hemlock, larch, willow, avaram, urunday and tizerah extracts fall under this group, of which quebracho, wattle and gambier are the most important and occur most abundantly in nature.

All condensed tannins are amorphous, and do not break down when hydrolised by acids or treated with enzymes. Strong mineral acids, on the contrary, convert these tannins to characteristic amorphous red insoluble precipitates known as "phlobaphenes" or "tanners reds". Hence they are also known as phlobatannins. With ferric chloride they give either green or blue colorations, and they invariably contain low proportions of crystalline associated 2-phenylchromane or related nuclei. The result is that their structures have invariably been compared with, and based on, that of the catechins, although no conclusive proof has been obtained to confirm or invalidate such an assumption. Very little is known of their constitutions.

Catechins also form red amorphous precipitates when treated with mineral acids. For this reason, and also because they constitute a large proportion of gambier and cutch extracts, the chemistry of the catechins and their interconversions will first be considered.

(a) CATECHIN

Catechin is the crystalline principle in cube gambier obtained from the leaves and twigs of Uncaria gambier Roxb. of the family Rubiaceae. It also occurs in Bengal or acacia catechu obtained from the heartwood of Acacia catechu Willd., and in cutch obtained from the bark of the mangrove Ceriops candolleana. Catechin occurs in these extracts in various stereoisomeric modifications. Gambier contains mainly d-catechin together with traces of dl-catechin and d-epicatechin, whereas acacia catechu contains varying quantities of l-epicatechin and dl-catechin (85).

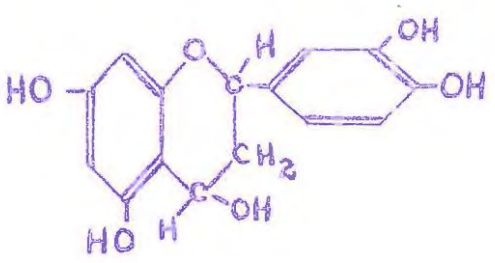
Catechin was first described by van Esenbeck (86) in 1832 and also examined by Berzeluis (87) in 1837. During the course of a century it was examined by numerous eminent chemists but its constitution was only conclusively established about 25 years ago as the result of the work of Freudenberg and co-workers.

Catechin obtained from gambier extract crystallises from water as $C_{15}H_{14}O_6.4H_2O$ (88)(89). It forms a green coloration with ferric chloride and gives the phloroglucinol reaction with pine-wood and hydrochloric acid. Alkaline fusions produce phloroglucinol and protocatechuic acid, and this process has been used for the commercial preparation of the latter.

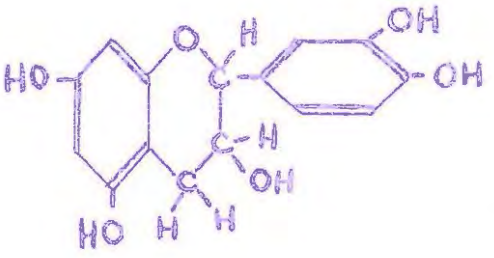
From acacia catechu Perkin and Yoshitake (89) isolated acacatechin, which gave the identical degradation products as catechin. Its melting point and that of its derivatives, however, differed from that of gambier catechin.

In 1905 Perkin (90) isolated veratric acid and possibly

phloroglucinol-dimethylether from the permanganate oxidation of catechin-tetramethylether, and considered catechin to be a reduction-product of quercetin (which occurs in low quantity in acacia catechu (91)(92)), corresponding to either XXXI or XXXII.

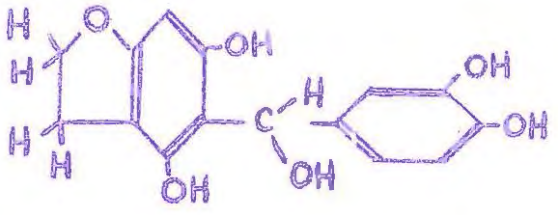


XXXII

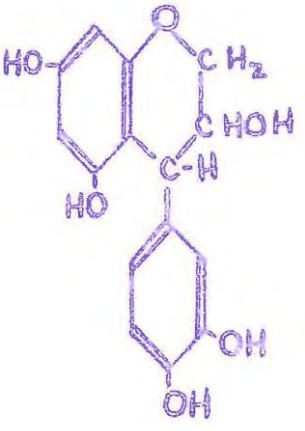


XXXI

Other formulae proposed at the time were those of Von Kostanecki and Lampe (93) XXXIII, and Nierenstein (94) XXXIV.



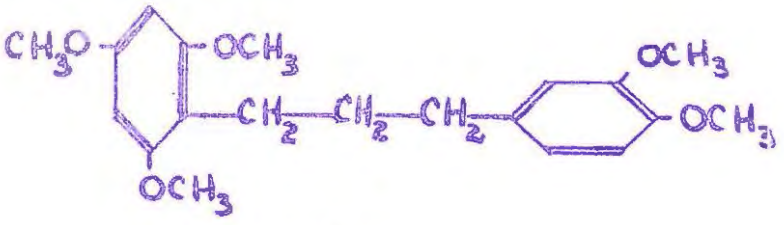
XXXIII



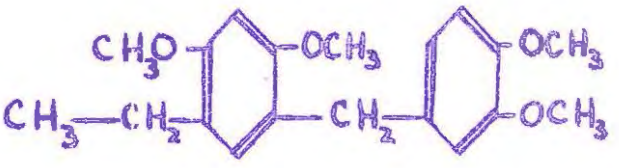
XXXIV

Reduction of tetramethoxycatechin with sodium in ethanol afforded, after remethylation, a pentamethyl ether which Von Kostanecki considered to be 2,4,6-trimethoxy-3-ethylphenyl

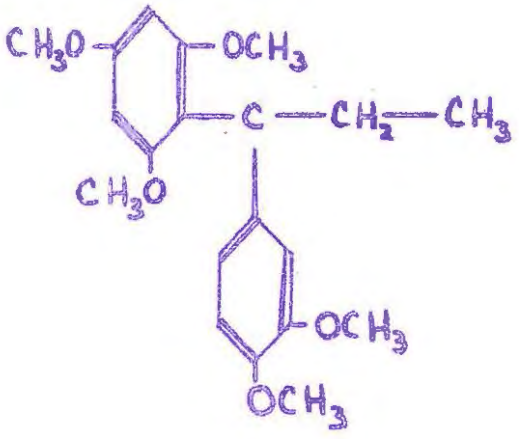
3',4'-dimethoxyphenylmethane XXXV. Based on Perkin's formulae such a reduction-product should be an α,γ-diphenylpropane derivative XXXVI and according to Nierenstein's formula a pentamethoxy-α,α-diphenylpropane derivative XXXVII.



XXXVI



XXXV



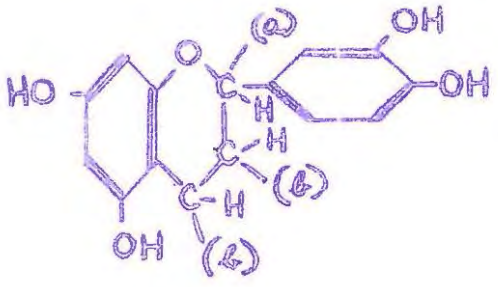
XXXVII

This matter was only clarified in 1920 by Freudenberg's (95) synthesis of 2,4,6,3',4'-pentamethoxy-α,γ-diphenylpropane XXXVI by the condensation of veratraldehyde with C-trimethylphloracetophenone, and the subsequent reduction of the 2,4,6-trimethoxyphenyl-3',4'-dimethoxystyrylketone.

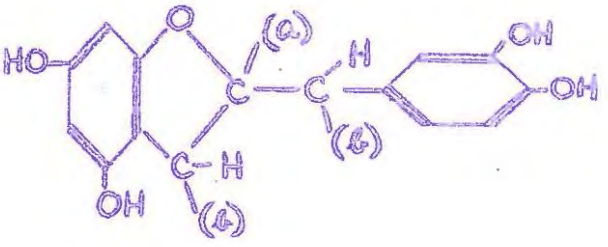
2,4,6,3',4'-pentamethoxy-α,γ-diphenylpropane proved

to be identical with Kostanecki and Lampe's methylated reduction-product of catechin tetramethylether and acacatechin tetramethyl-ether (96)(97) and formulae XXXV and XXXVII were, therefore, incorrect.

Two alternative structures XXXVIII and XXXIX were still possible and the position of the hydroxy group on the pyrane ring or on the aliphatic chain joining the two nuclei was not settled.



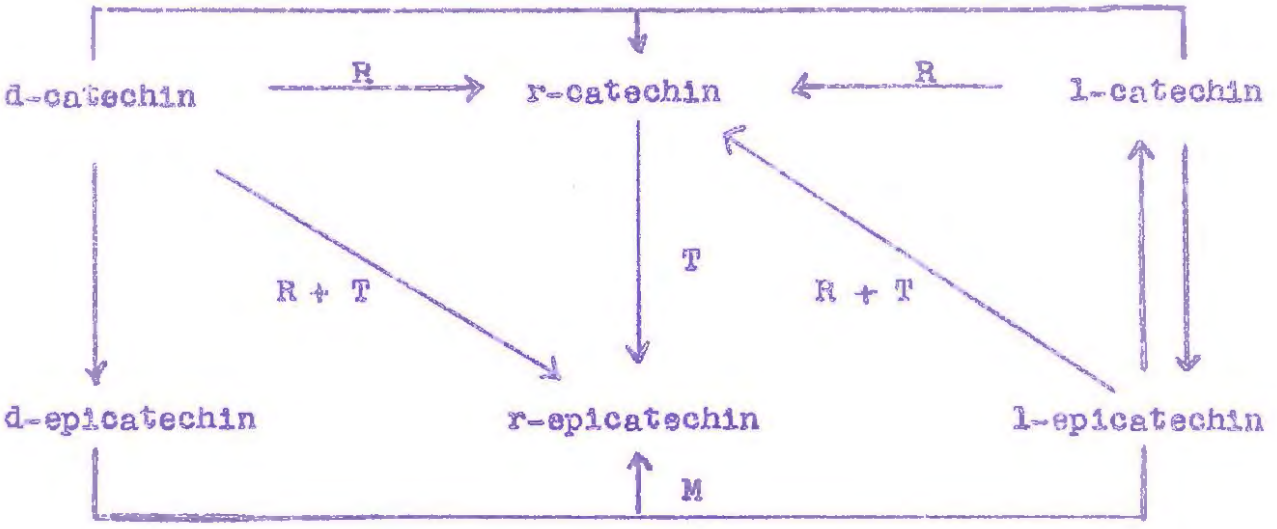

XXXVIII




XXXIX

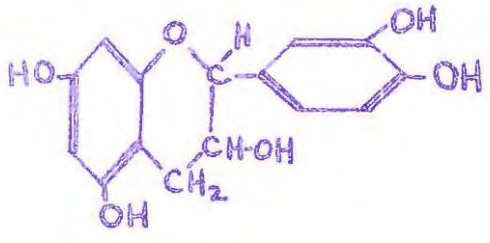
If the hydroxyl group occupies position (a) in both formulae catechin could exist only in two active and one racemic form, as only one asymmetric carbon atom would be present. If, however, the aliphatic hydroxyl occupied either of the two (b) positions in the above formulae, two asymmetric carbons must be present which would result in two racemic and four active forms. The latter was found to be the case as r-catechin could be partly transformed to r-epicatechin by boiling in sodium chloride solution, and Freudenberg and Purrman (98)(99) were able to separate

l- and r-catechins and l- and r-epicatechins from acacia catechu. The other racemic modifications were also obtainable from various sources and could be epimerised and racemised as follows (100) :

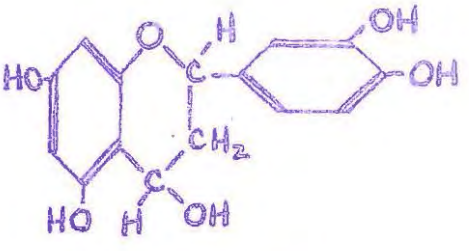



M = Mixture R = Racsmisation T = Transformation

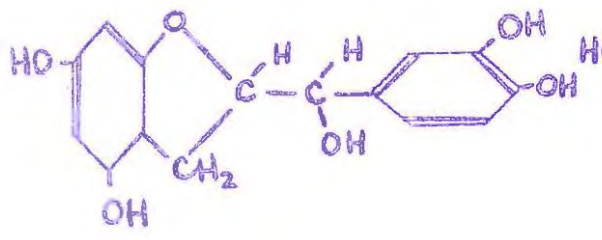
Four formulae were thus still possible for catechin (101) :



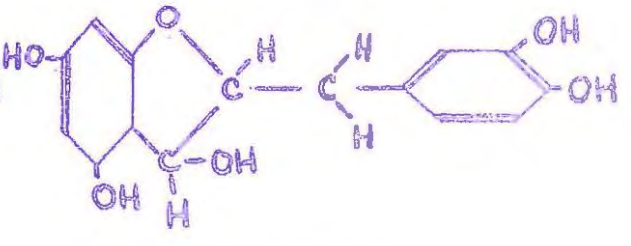
XL



XLI

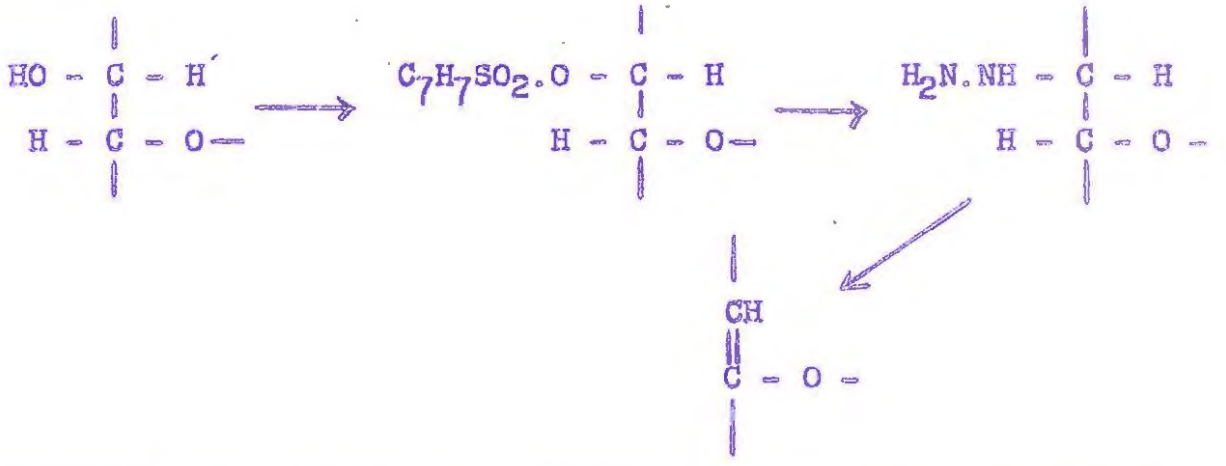


XLII



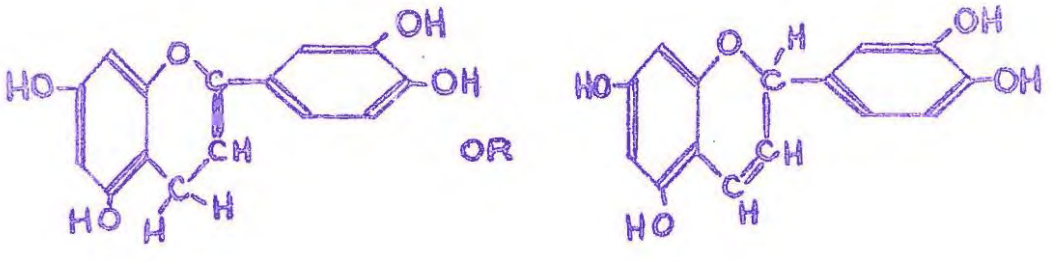
XLIII

Two courses were adopted to eliminate the incorrect formulae and to decide which was correct. The toluene sulphonyl derivative of tetramethoxy-l-epicatechin when treated with hydrazine gave the following reactions, resulting in anhydroepicatechin tetramethylether (102).

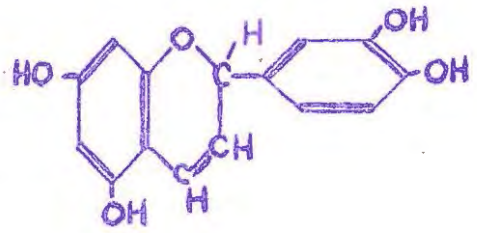


l-epicatechin thus behaves as a cis-isomeride. With tetramethyl-d-catechin a trans-configuration was indicated. Formula XLII must, therefore, be rejected as cis-trans isomerism is not possible unless the hydroxyl group is attached to the hydrogenated ring.

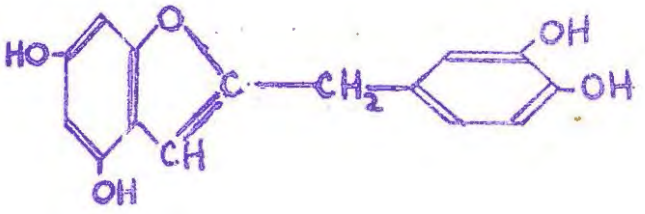
The following anhydro-derivatives based on formulae XL, XLI and XLIII could be formed in the above reaction.



XLIV

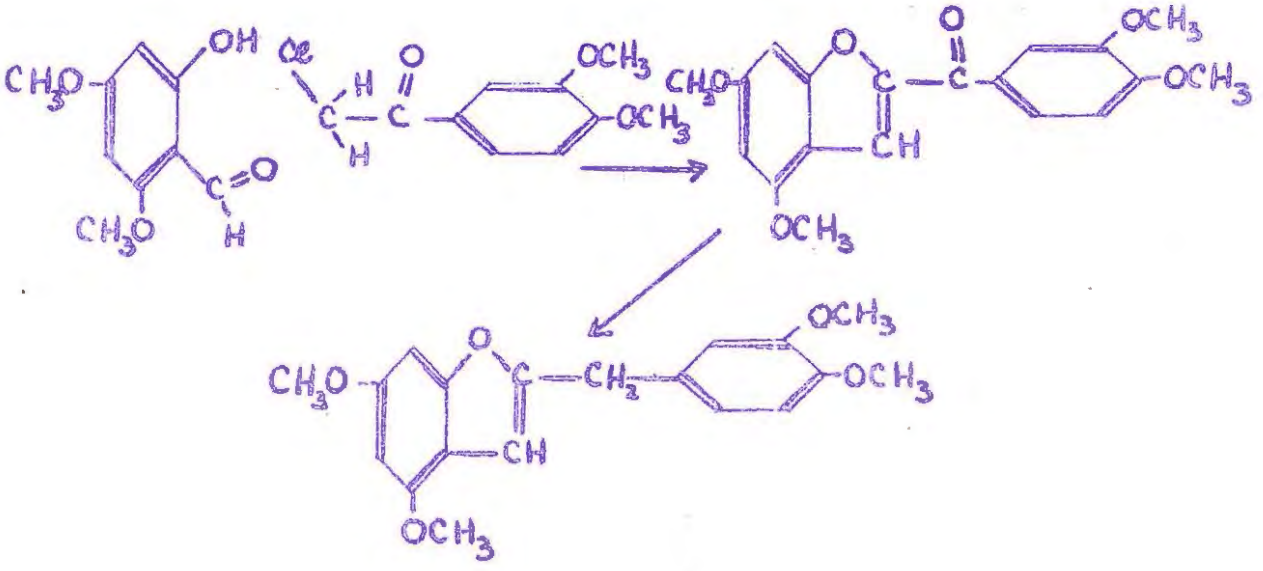


XLV



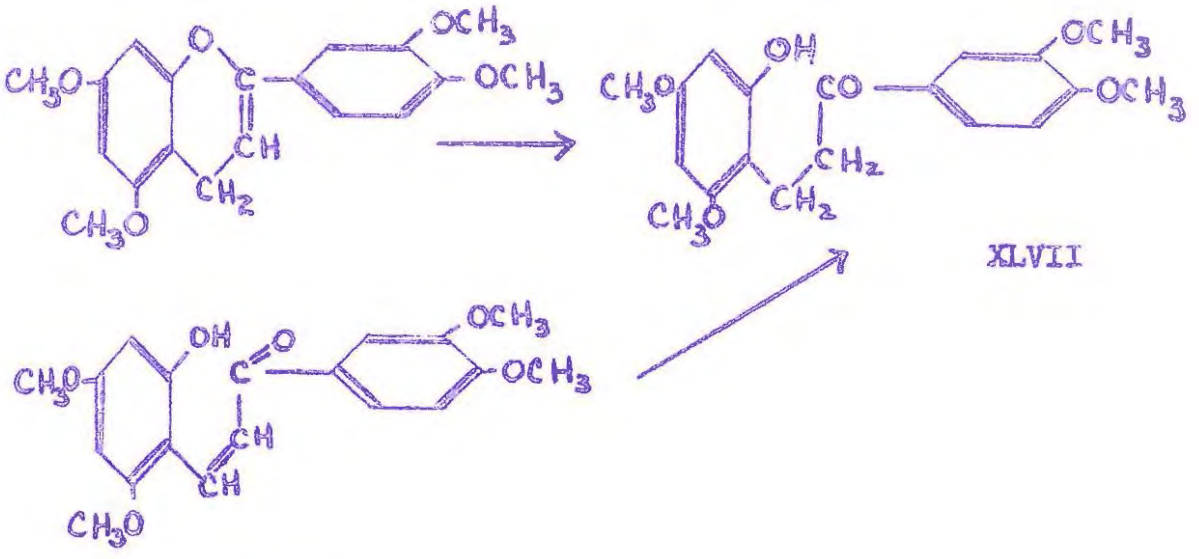
XLVI

Freudenberg and co-workers (103) synthesised XLVI by the condensation of 3,4-dimethoxyphloroglucinaldehyde with ω-bromoacetoveratrone followed by the reduction of the coumarone formed.



This substance was not identical with tetramethoxyanhydro-epicatechin prepared from the natural source and strengthened the surmise that the correct formula for catechin was XL with anhydro-derivative XLIV. Additional support for the XLIV structure for the anhydro-derivative was obtained from the fact that tetra-

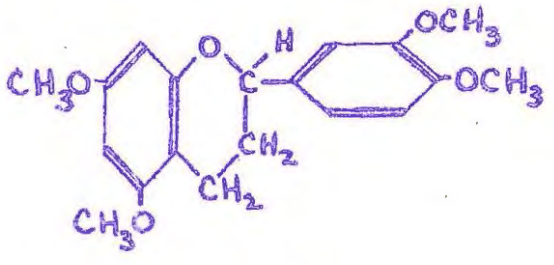
methoxyanhydroepicatechin in moist acetic acid took up one molecule of water to form 3',4'-dimethoxyphenyl-β-2-hydroxy-4,6-dimethoxyphenylethyl ketone XLVII, the constitution of which was proved by



XLVII

XLVIII

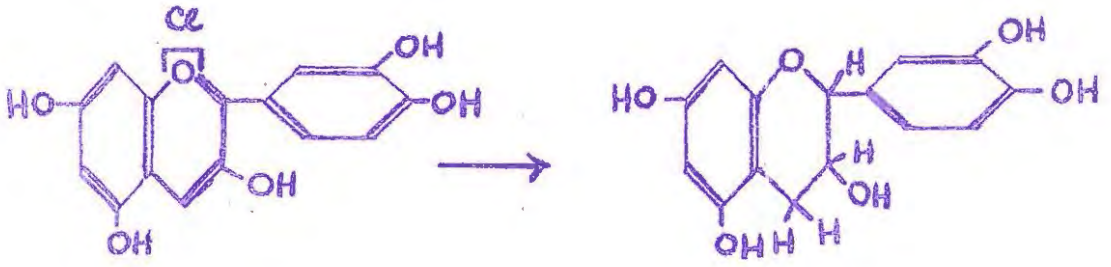
synthesis from the hydrogenation of β-2-hydroxy-4 : 6-dimethoxystyryl ketone XLVIII (103)(104). Moreover, the ketone XLVII and tetramethylanhydroepicatechin form the same hydrochloride, which yields 5,7,3',4'-tetramethoxyflavane XLIX on hydrogenation.



XLIX

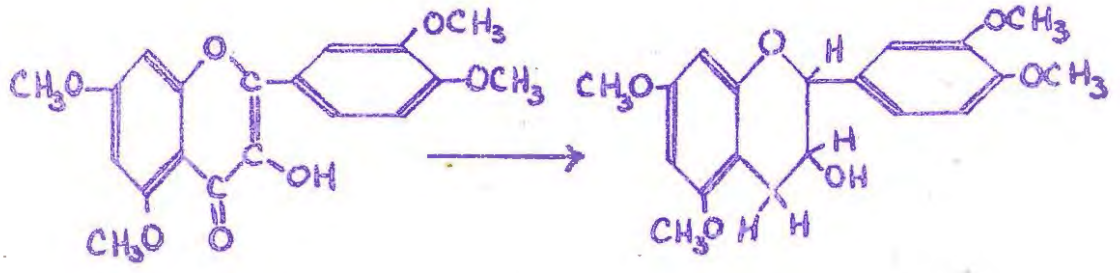
The structure assigned to catechin was finally confirmed

when Freudenberg, Fikentscher, Harder and Schmitt (105) obtained d,l-epicatechin and pentamethoxy-d,l-epicatechin by the catalytic reduction of cyanidin chloride (L) and its pentamethyl ether respectively.



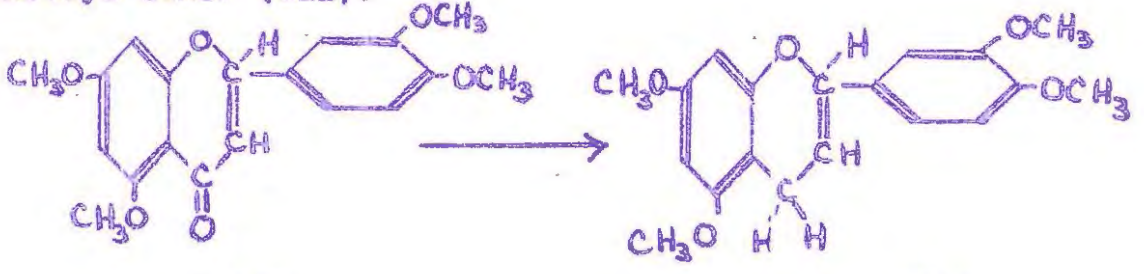
L

Similarly Freudenberg and Kammüller (106) obtained d,l-epicatechin pentamethylether by the catalytic reduction of quercetinpentamethyl ether (LI),



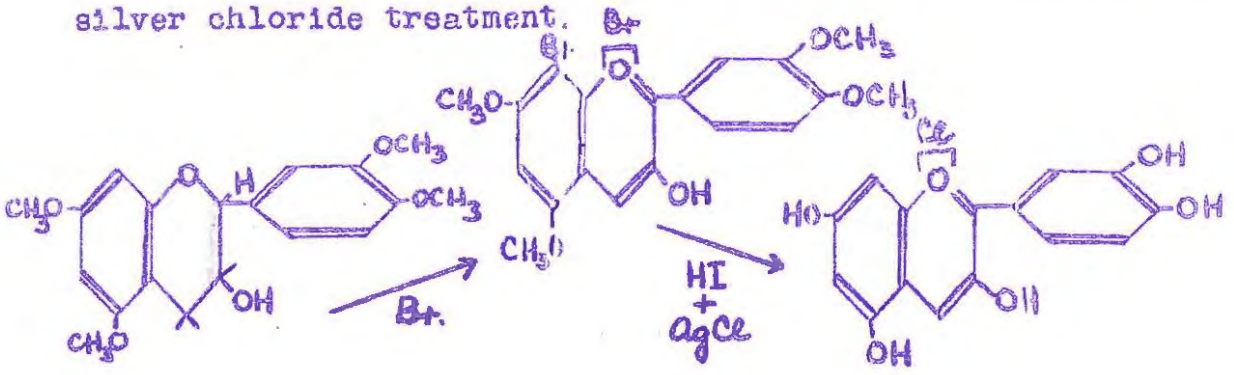
LI

and deoxyepicatechin tetramethyl ether (LIII) from luteolin tetramethyl ether (LII).



LII LIII

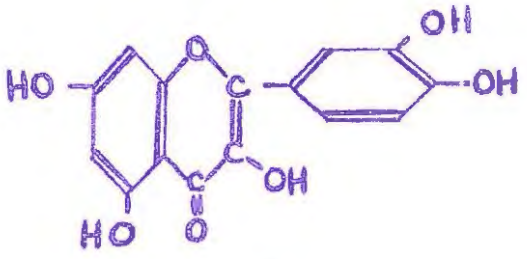
In 1935 Appel and Robinson (107) were able to reverse the above cyanidin chloride - d,l-epicatechin conversion by oxidising catechin tetra-methylether with bromine in dioxane. The intermediate bromo-derivative was demethylated with HI to cyanidin iodide which was finally converted to cyanidin chloride (L) with silver chloride treatment.




L.

Catechin forms the major proportion of the majority of catechu extracts. These are employed for tanning purposes, although this relatively small phenolic body does not precipitate gelatin and is not considered to be a true tannin. It is, however, absorbed by the hide and passes over into a tanning material gradually, possibly by oxidative condensation.

Because of their structural relationship, it is interesting to note that small quantities of quercetin LIV accompanies catechin present in catechu extracts (77)(92).



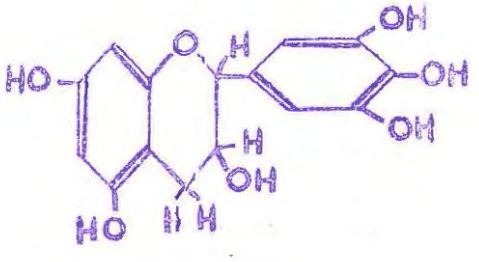

LIV

Ordinary gambier catechu contains a small amount of

tanning material, known as catechutannic acid, in addition to the catechin. In the browner varieties of cutch, a considerable amount of this tannin is present, which is insoluble in ether, gives a green colour with ferric chloride, and the phloroglucinol reaction with pine-shavings. In contrast to catechin it is a strong tanning agent, resembling other condensed tannins. Catechutannic acid is considered to be a derivative of catechin ( 368 ), and is said to be produced from catechin by heating aqueous solutions at 110°C. or by boiling alkaline solutions of the latter.

(b) GALLOCATECHIN

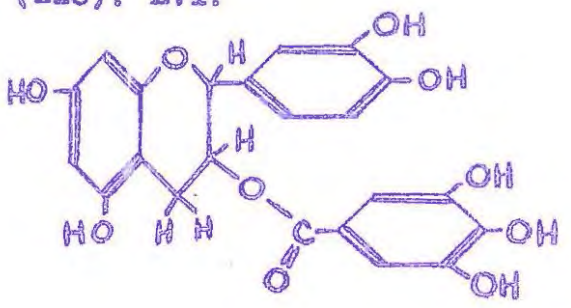
Gallocatechin LV is of special interest in the black wattle tannin problem, and its chemistry, which is similar to that of catechin, is very briefly reviewed here.




LV

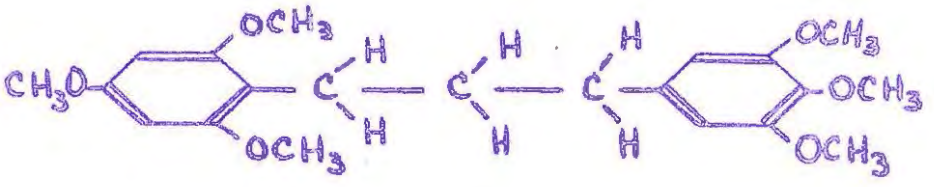
Gallocatechin $C_{15}H_{14}O_7$ was first isolated by Tsujimura (108) from Japanese green tea (Thea sinensis L.). It gives no precipitate with gelatin, a violet colour with ferric chloride and the phloroglucinol reaction with pine-shavings and HCl. l-epi-catechin is also present in low proportion (109) as well as its

galloyl ester (110). LVI.



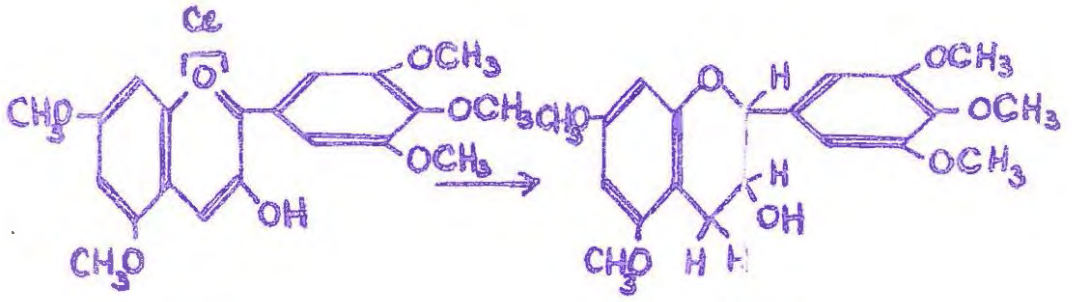
LVI

Osima also isolated (111) l-epicatechin and gallocatechin from Formosan tea. The structure of gallocatechin was proved by the reduction of pentamethoxygallocatechin with sodium in ethanol followed by methylation to give α-2,4,6-trimethoxyphenyl-β-3,4,5 trimethoxyphenyl-propane LVII.



LVII

The latter was synthesised by the condensation of ω-acetoxy-3,4,5-trimethylgalloacetophenone with phloroglucinaldehyde trimethylether, followed by catalytic reduction of the resultant product. Further proof that LV was the correct structure was obtained by reducing the pentamethyl ether of delphinidin chloride LVIII to gallocatechin pentamethyl ether. LIX.



LVIII LIX

Bradfield and co-workers (112)(113) used partition chromatography on a silica gel, according to the method of Martin and Synge (114) for the seperation of the constituents of the polyphenolic fraction of Ceylon tea. The following catechin bodies were found present in the wet ether-soluble portion of the ethyl acetate extractives : l-gallocatechin, 11.7%; dl-gallocatechin 5.8%; l-epicatechin 3.2%; dl-catechin 1.2%; l-epicatechingallate LVI 7.5% and an l-gallocatechin gallate 36.0%.

l-epicatechin gallate and l-gallocatechin gallate could be hydrolised into the catechin bodies and gallic acid by acid hydrolysis, or more efficiently by the enzyme tannase. In l-gallocatechin gallate the point of attachment of the gallic acid to the gallocatechin moiety is yet unknown.

Tea "tannin" in green tea is, therefore, a rare instance where 13% (350)of a polyphenolic fraction of the unfermented leaf consists of a mixture of low-molecular crystalline catechin bodies which may be resolved and identified by the use of partition chromatography.

(c) QUEBRACHO EXTRACT

Quebracho extract comes mainly from two species of the family Anacardiaceae (115) :

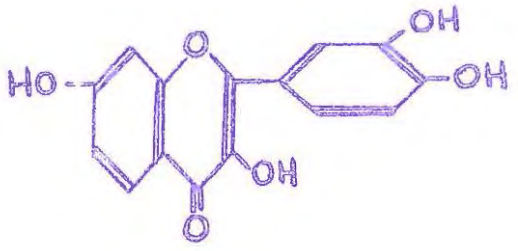
Schinopsis balansae Engler, also known as Quebracho colorado "chaqueño", the red quebracho tree, and

Schinopsis lorentzii (Gris.) Engl. or Quebracho colorado "santiagueno".

These slow-growing trees occur in large natural forests in northern Argentina and in Paraguay. The tannins are concentrated almost exclusively in the dense heart woods and are extracted on a commercial scale in the same way as black wattle extract. Quebracho extract is far less soluble in cold water than black wattle extract, and as a result the major proportion of the extracts sold are sulphited in order to increase their solubility and thus to eliminate practical difficulties during the tanning process. Quebracho extracts are easily distinguished from other vegetable extracts by their brilliant yellow-green fluorescence under ultraviolet light (116), and by the Jablonski-Pollak fluorescence test with phthalic anhydride and zinc chloride (117).

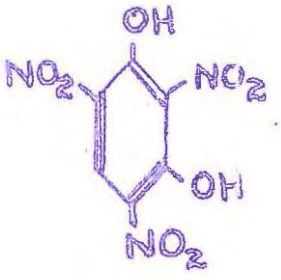
Quebracho extract contains a highly condensed tannin of average molecular weight 2400, after dialysis (118). The average molecular weight of the extract as a whole is 1160. The tannin has been examined by Arata (119) who obtained catechol from the tannin by pyrolysis, and phloroglucinol and protocatechuic acid by alkaline fusion. Perkin and Gunnel (120) isolated fisetin LX, ellagic and gallic acids from the action of strong

sulphuric acid on the extract.

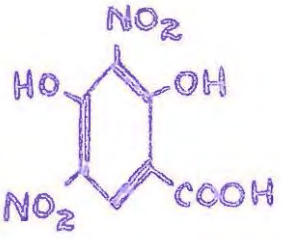



LX

Jablonski and Einbeck (121) confirmed the presence of fisetin and isolated resorcinol and protocatechuic acid, but no phloroglucinol, from alkali fusions. They also obtained styphnic acid LXI and 3,5-dinitro-$\beta$-resorcylic acid LXII from the nitric acid oxidation of the tannin (122).




LXI

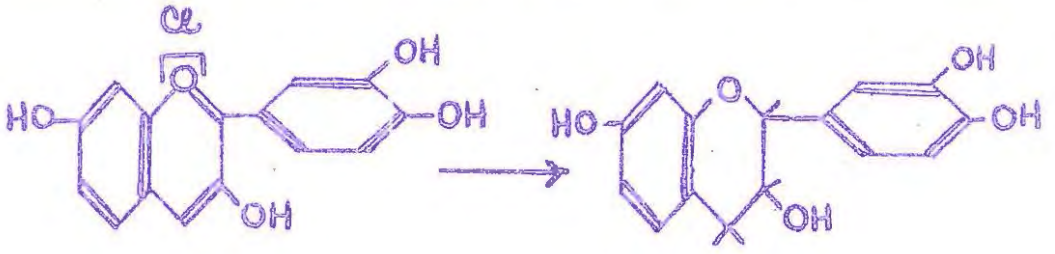



LXII

As the tannin was obviously only constituted of potential $\beta$-resorcylic and protocatechuic nuclei, and was also accompanied by fisetin, Freudenberg and Maitland (123), by analogy with catechin which is accompanied by the related flavanol quercetin, suggested that quebracho tannin might be formed by the condensation of a catechin LXIII similarly related to fisetin.

This catechin body, known as quebracho catechin was

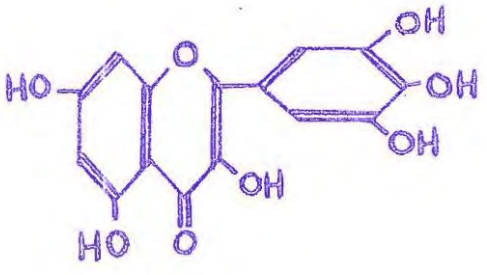
synthesised by Freudenberg and Maitland (124). The d,l-epi-quebrachocatechin obtained by the catalytic reduction of fisetinidin chloride LXIV could be epimerised to d,l-quebrachocatechin by heating with potassium bicarbonate at 115°C.



LXIV LXIII

Quebrachocatechin, like quebracho tannin, formed red insoluble phlobaphenes easily with strong acids, and gave resorcinol on alkaline fusion and veratric acid from the permanganate oxidation of the methylated derivative. In spite of many attempts no evidence of the existence of quebracho catechin in the natural extract could be found.

It is interesting to note in this connection that the flavanol myricetin LXV accompanies casuarin, a gallocatechin in the bark of Casuarina equiselifolia L. (125).



LXV

Recently White (126) conducted a physico-chemical investigation which showed the apparent complexity of quebracho extract and tended to negate much of Freudenberg and Maitland's "speculation" regarding the constitution of the extract. White employed a solubility method which gave reliable results when used by Northrop and Kunitz for testing the purity of crystalline enzymes and other proteins. (127)(128).

In order to interpret a later criticism of White's methods a brief review follows. In the simplest case a single component dissolves in a solvent at constant temperature and pressure only to a limited extent. The solubility curve of such a system is represented by Fig. III, where AB is the solubility curve of unit slope and B is the point of saturation of the solute in the solvent.

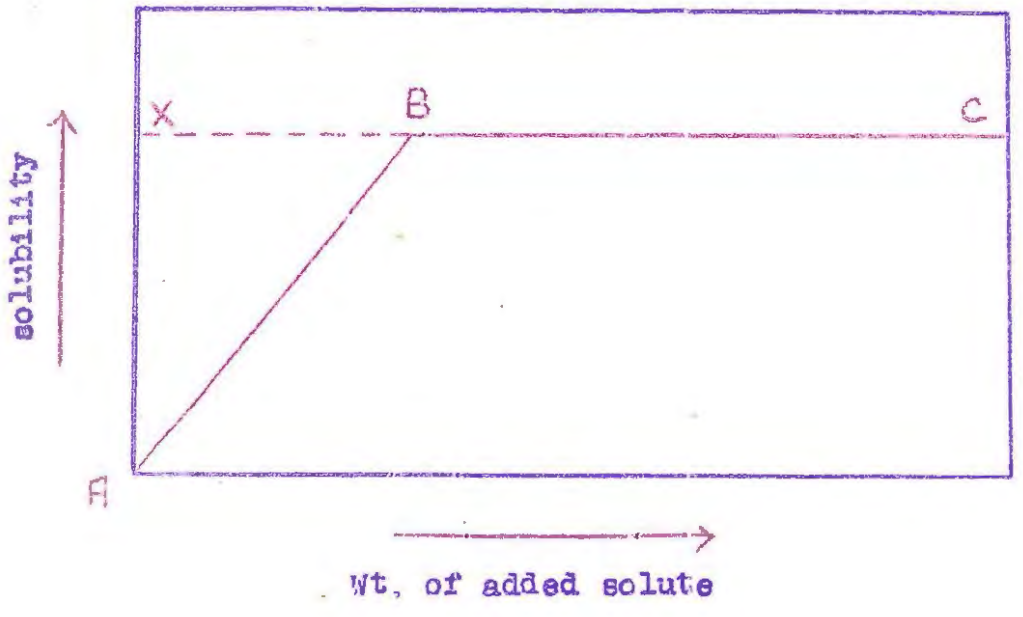



Fig. III.

The solubility curve of three solid phases in equilibrium with their standard solutions may be represented in an ideal case by Fig. IV. B, C and D where changes in slope occur each agree to points where 1, 2, and 3 reach their saturation points in the solvent used. From the back-projection of the

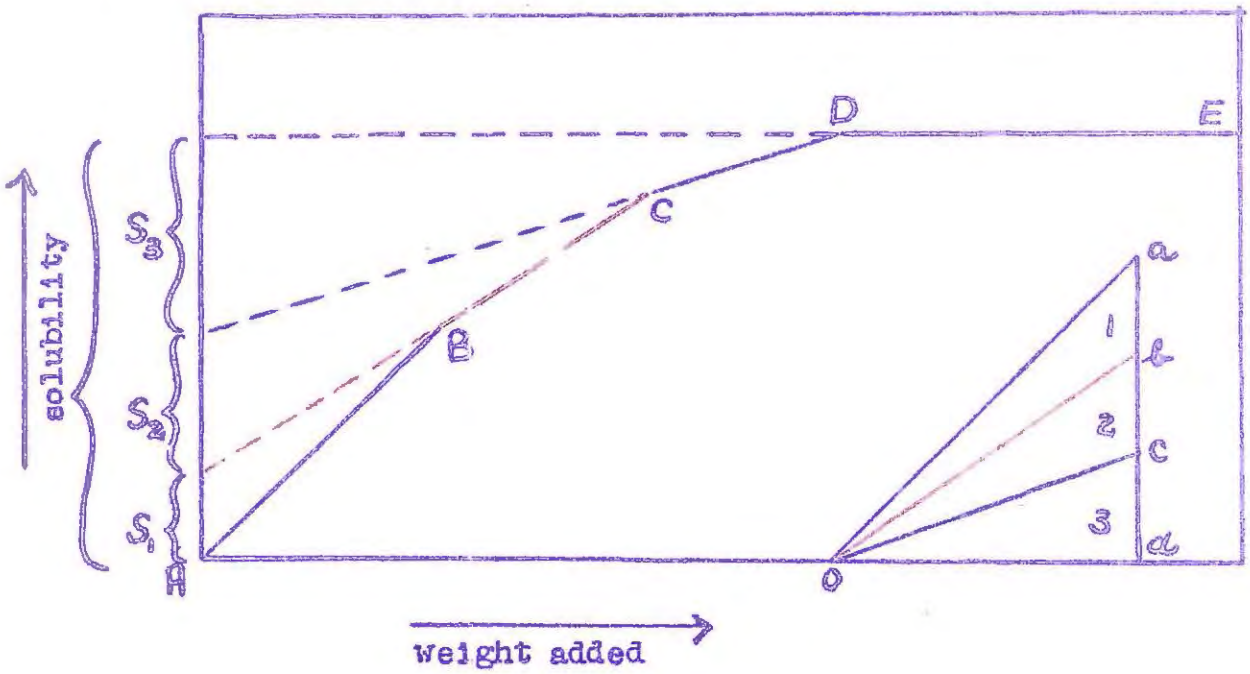
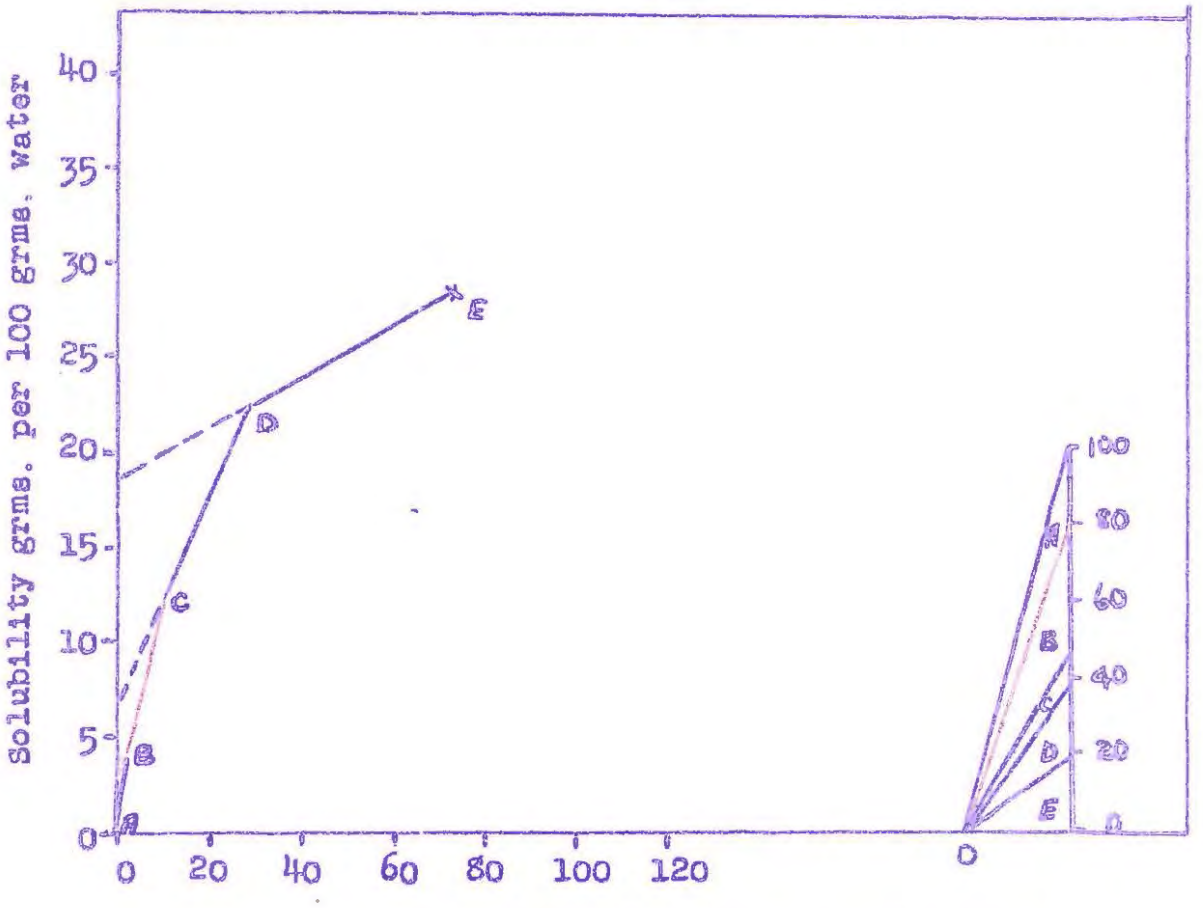



Fig. IV.

slopes the solubilities of the various components may be determined. By this method the number of components in a mixture may be determined (from the number of changes of slope) as well as their solubilities, without their actual isolation. Northrop further pointed out that each straight line section of the solubility curve corresponds to an individual component, and has a slope which is a function of the solubility of that component and the extent to which it is present in the mixture. It is thus possible to calculate the amount of each component present and

Thorp (129) provided a graphical "component diagram" for this purpose. If in a right-angled triangle of unit side parallels to AB, BC and CD are drawn through O, these cut the vertical side ad at points a, b, and c. The lengths ab, bc and cd are a measure of the proportions of components 1, 2 and 3 with which the solution becomes saturated at points B, C, and D on the solubility curve.

White applied this method to quebracho extract and obtained the following solubility curve and component diagram, Fig. V.




Grms. quebracho per 100 grms. water

Fig. V.

He was unable to construct a complete curve as above 75 grms tannin per 100 grms water separation of the insolubles by centrifugation or filtration was unsatisfactory. Changes in slope at certain concentration levels in the solubility curve corresponded to similar changes in the slope of refractive index and light absorption curves obtained from the same solutions. White regarded this as an important confirmation of the validity of the solubility curve.

From the solubility curve he constructed Thorp's (129) component diagram. From this and the solubility curve he concluded that quebracho tannin consists of at least five components present to the extent A = 20%, B = 33.6%, C = 4.85%, D = 20.5% and E = 21.05%. Component A constitutes the so-called "insolubles" of quebracho extract, which were found to be sparingly soluble, while component E represents the most soluble portion of the extract and is probably itself heterogeneous.

White subjected the twelve solutions obtained in the construction of the solubility curve to one-dimensional paper chromatography on a single sheet. A series of successive parallel yellow fluorescent spots were visible when viewed under ultra-violet light, after the paper had been developed with Partridge's (130) butanol-acetic acid-water (4 : 1 : 5) mixture. The yellow fluorescent spots which remained at the points of application of the test solutions had an equal intensity for all solutions. This spot, therefore, constituted the insolubles in the extract or component A. All the remaining yellow fluorescent

components (under ultra-violet light) increased in intensity up to solution 7 and remained constant in intensity thereafter. Solution 7 represented the point where component B attained saturation. It appeared thus, that B consisted of a complex of fluorescent substances of which fisetin was one.

White also claimed the identification of free gallic acid (previously isolated by Perkin and Gunnell (120)), glucose, arabinose and xylose in the extract.

Components D and E were known to be reddish in colour, and it was presumed that two red-brown (ordinary light) trailed-out "spots" on the chromatograms of RFs = 0 - 0.25 and 0.55 - 0.70, which increased in concentration throughout all 12 solutions, corresponded to these.

White claimed that the complexity of the extract, as shown by the combination of these methods, modify the previous ideas of a quebracho tannin based on Freudenberg's (loc. cit.) quebracho catechin. In defence of Freudenberg, however, it shoudd be pointed out that he was well aware of the complexity of the commercial extracts, and that White has failed to take advantage of known methods for separating tans from non tans prior to his experiments. Freudenberg's formula provides for a mixture of molecules due to various degrees of polymerisation and innumerable stereoisomers.

## DISCUSSION OF WHITE'S SOLUBILITY METHOD

White states ((126) p. 43) that the solubility curve, in addition to demonstrating the complexity of the vegetable

extract, also provides a quantitative basis for the fractionation of the product. As this has an important bearing on the problem of fraction of black wattle extract, a critical review of the above method is essential.

It must be noted that the solubility method of proving the purity or complexity of a substance or mixture of substances, had previously been applied only to crystalline substances. Kunitz and Northrop (127) applied it successfully to $\alpha$ - and $\beta$ - chymotrypsin, which are enztmatic and also crystalline proteins, only under conditions where no solid solutions were formed. Thorp (129) also applied it with success to dichlorodiphenyltrichloroethane, 22', 44', 66' - octachlorodiphenylurea and impurities closely related to these substances. In connection with this work Thorp states, "The principle of the method depends on the various compounds not influencing one another's solubilities. This will be approximately true of most non-ionised compounds in organic solvents provided that the solutions never become very concentrated and that the compounds form distinct crystalline solids".

None of the aforementioned conditions hold good in White's application of this method. Intermolecular effects due to hydrogen bondage must be active in all but the most dilute aqueous solutions. White's solubility curve by comparison, extends to exceedingly concentrated solutions (75 gms extract/100 grms water) and includes the solubilities of substances the great majority of which are amorphous.

The validity of White's conclusions are based entirely on two factors :

(a) the fact that light absorption and refractive index curves show changes in slope at the same concentration levels as the solubility curve and

(b) the fact that the fluorescent components which constitute component B attain apparent saturation (as judged on the paper chromatogram) corresponding to a very slight change in slope in the solubility curve.

On the other hand component A, the "so-called "insolubles", should show increasing fluorescence over solutions 1 - 4 (where saturation is almost achieved). This is not evident from the paper chromatogram. Furthermore, the only really marked break in the solubility curve occurs just before solution 9 where the concentration (about 35 grms extract/100 grms water) is already excessively high.

In any case, the presence of sugars and other non tans in the commercial extract, together with polymers of varying size each with a large number of stereoisomers, could give the results obtained by White.

Other criticisms regarding the anomalous behaviour of fluorescent compounds on paper chromatograms due to their high affinity for cellulose, are reserved for discussion with the work on the paper chromatography of black wattle extract. (See page 165).

From the above it would appear that while certainly confirming the known complexity of the commercial extract, the use of

the solubility method on a quantitative basis for the fractionation of the extract is not justified.

## A RECENT CHEMICAL INVESTIGATION OF QUEBRACHO EXTRACT.

Putnam and Gensler (131) in November 1951, published much analytical work on a fraction of quebracho extract.

The commercial extract was partitioned between water and an upper layer consisting of ethyl acetate, acetone and water. From the upper layer 62% of the extract was recovered, and the solid product was continuously ether-extracted for one week. The ether insoluble portion was treated with methyl isobutyl ketone, and the portion which dissolved (53%) was investigated.

Chromatography showed that at least an admixture of fluorescent bodies were still present. The fraction was adsorbed on to calcium oxide and again desorbed, and also passed through an anion exchange resin for its further purification. Both "purified" products agreed with each other in analysis as well as with the original fraction as a whole. The empirical formula corresponded to $C_{31}H_{28}O_{11}$, and the molecular weight by Rast determination using camphor varied from 222 to 446; and by isothermal distillation in acetone from 430 (14 days) to 746 (25 days). The theoretical value for the above formula is 576.

This fraction was thus considered to be the "main constituent" in quebracho extract and the aforementioned formula was preferred as it could accomodate more of the assembled data than any other.

The methylated derivative was prepared both by methylation with diazomethane and dimethyl-sulphate and fractionated. Four fractions investigated varied in % C from 67.4 to 65.5; in molecular weight from 543 to 665; and in the appearance of their infra-red spectrograms. Remethylations made the infra-red spectra identical and narrowed differences in carbon content, but variations in molecular weight 533 - 702 still persisted. Analytical results of the "purest" product obtained corresponded to the empirical formula $C_{31}H_{21}O_4(OCH_3)_7$. When treated with methyl iodide and silver oxide, this methylated product showed a reduction in methoxyl content and corresponded to the formula $C_{31}H_{22}O_5(OCH_3)_6 \cdot H_2O$.

Acetylation was performed with an acetic anhydride, triethylamine, zinc dust and tetraethyl ammonium bromide mixture. The product was acetylated with acetyl chloride/dimethylamine mixture, and the fully acetylated derivative found to contain 46.5% of acetyl groups. This corresponded to a formula $C_{31}H_{17}(OCOCH_3)_{11}$, to which the determined molecular weight also agreed closely. An X-ray diagram showed that the compound which melted at 153-155°C, was also "crystalline".

This derivative is important as it is the first instance in which any phloba-tannin has been substituted to such a degree, that all the oxygen was accounted for in functional derivatives. Putnam and Gensler claim that mimosa tannin might be similarly substituted.

Refluxing the acetyl derivative with potassium acetate

caused a loss of eight of the eleven acetyl groups. Those groups hydrolised were considered to be attached to phenolic hydroxyls, while three substituted non-phenolic hydroxyls remain stable.

The fully methylated derivative $C_{31}H_{21}O_4(OCH_3)_7$ could only be partially substituted under the above conditions, and corresponded to the formula $C_{31}H_{19}O_2(OCH_3)_7(OAc)_2$ after dual acetylation.

Veratric acid was obtained in 15% yield by the permanganate oxidation of the methylated derivative. Alkaline degradation gave a 1.6% yield of resorcinol.

From all the above evidence $C_{31}H_{28}$ was considered as the basic structure.

## DISCUSSION

Although it is doubtful whether Putnam and Gensler were dealing with a pure phenolic entity, especially when their efforts at showing homogeniety by chromatography and their purification methods are taken into account, their claim of the complete substitution of the tannin is of importance to the black wattle problem.

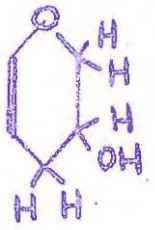
## CONDENSATION MECHANISMS IN CONDENSED TANNINS

Catechin, synthetic quebracho catechin and gallotannin, as previously stated, do not give those characteristic precipitates with gelatin-salt reagent normally associated with condensed tannins. Catechin and gallocatechin are, nevertheless, usually associated with condensed tannins in nature, and are easily converted by boiling in aqueous solution to amorphous compounds which tan and precipitate gelatin. They further resemble condensed tannins by giving similar degradation products and produce insoluble red phlobaphenes when treated with hot mineral acids.

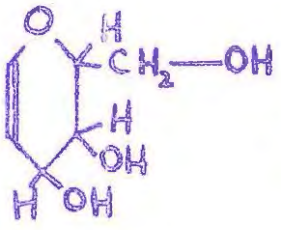
## BERGMANN AND POLJARLIEFF'S THEORY

As catechins, therefore, show many of the quantitative reactions of condensed tannins, their condensation mechanism was examined by Bergmann and Poljarlieff (132). In the so-called "phlobaphene reaction" condensation appears to occur without the elimination of water, and Bergmann and Poljarlieff tried to establish which part of the catechin molecule was responsible or functional in this condensation. They stated that the simple phenolic bodies of which the catechins are constituted were not particularly sensitive to condensation under conditions of high acidity. Catechin-tetramethylether on the other hand, when heated with concentrated HCl in ethanol at 105°C formed a red mass (C = 67.85% H = 6.02%). When compared with catechin-tetramethyl-ether (C = 65.9% H = 6.4%), it appeared that some water had been

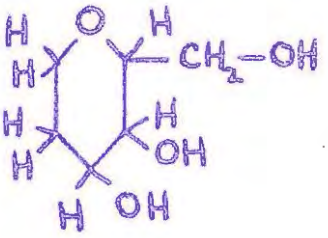
eliminated, and as the phenolic hydroxyls were fully substituted, they concluded that a pyrane ring structure containing at least one hydroxyl group and one double bond LXVI was responsible for condensation.



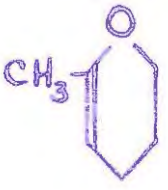
LXVI



LXVII



LXVIII



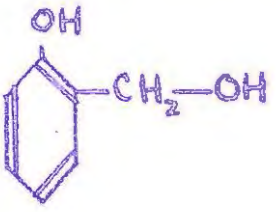
LXIX

Bergmann and Poljarlieff obtained further positive evidence for their theory by the examination of glucal LXVII, hydroglucal LXVIII and anhydroacetobutyl alcohol LXIX. Whereas glucal was found to be extremely sensitive to mineral acids and readily formed coloured condensation products, the remaining pyrane structures appeared stable. The acetylation of catechin in the 3-position furthermore, tended to exert a stabilising effect towards its conversion into amorphous material on standing in aqueous solution (133). Bergmann and Poljarlieff finally assumed that condensed tannins similarly contained the pyrane structure LXVI which they concluded was responsible for phlobaphene formation.

Their work, based purely on analogy, is open to the following criticism :

(a) Many phenolic compounds e.g. salicylalcohol LXX possess

the tendency towards self-condensation, while others e.g. resorcinol (134) and phloroglucinol (135) condense under conditions of high

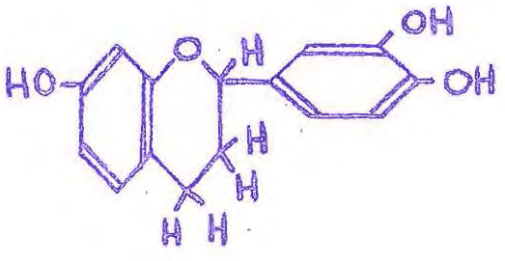


LXX

acidity.

(b) The formation of a phlobaphene may be judged only by the insolubility of the product in hot water, its red colour and its amorphous nature. As catechin-tetramethylether is already insoluble in water the analogy is not a good one.

(c) Freudenberg and Maitland while engaged in their constitutional work on quebrachocatechin (123) put Bergmann and Poljarlieff's theory to the test by synthesising 7,3',4'-trihydroxy flavane LXXI from 3,4-diacetylphoracetophenone and β-resorcylaldehyde.



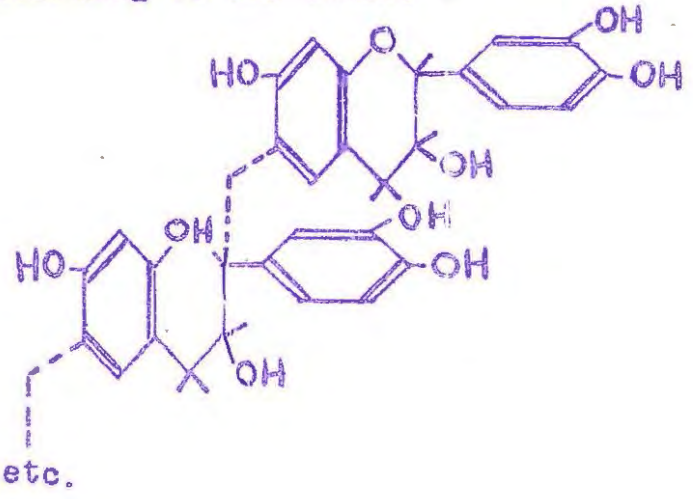
LXXI

This compound contains no hydroxyl, but possesses a double bond in the pyrane ring. It was found to be more sensitive to alkalis than catechins and gave a phlobaphene on boiling with dilute acids.

Freudenberg and Maitland (124) concluded that the presence of an hydroxyl group in the pyrane ring was not an essential requisite for phlobaphene formation.

## FREUDENBERG AND MAITLAND'S THEORY

Freudenberg and Maitland also showed that when their synthetic quebracho catechin LXIII was heated for a few hours with dilute acid, an insoluble amorphous product resulted, the elementary analysis of which did not differ appreciably from that of the original material. Methylation of the condensed material followed by subsequent oxidation, produced veratric acid only, and acetylation gave an amorphous product of slightly higher acetyl content than the starting material . From this evidence they concluded that condensation in the initial stages was not accompanied by water elimination, and that neither the catechin group nor the aliphatic hydroxyl group in the 3'-position took any active part. In order to explain the higher acetyl content they assumed that in the presence of acid at elevated temperatures at least some of the pyrane rings open, followed by simultaneous condensation with a second unit, according to the scheme :



This condensation product still contains an oxygen bridge, and the principle of continued condensation is possible with this mechanism. Such condensates consisting of 5 - 6 catechin units would correspond to the condensed tannins.

When heating occurs for a longer period, secondary splitting off of water takes place, probably involving the secondary hydroxyl groups. This stage of condensation corresponds to the red phlobaphenes formed from condensed tannins and catechins.

To obtain further support for their theory, Freudenberg and Maitland examined the leaves and wood exhaustively for evidence of the quebracho catechin "precursor" corresponding to their synthetic product. As this was not achieved, they concluded that its absence must be due to its greater sensitivity towards self-condensation than d-catechin, for example. They next divided quebracho extract into a cold water-soluble fraction A, a cold water insoluble but hot-water soluble fraction B, and a hot-water insoluble fraction C. A was considered to be the small-, B the middle-, and C the high-molecular condensation product of synthetic quebracho catechin. 1 grm of each was treated with 50 ml. of 2% HCl on a waterbath. The following analyses resulted (Table VII).

TABLE VII

| Substance | Heating Period (Hrs) | % C | % H | % Acetyl Content of Acetylated Product Found | % Acetyl Content of Acetylated Product Reqd. |
|---|---|---|---|---|---|
| Synthetic Quebracho Catechin | 0 | 65.7 | 5.1 | 39.6 | 38.9 |
| Hypothetical condensate of Quebr. catechin | - | 65.7 | 5.1 | - | 41.7 |
| Condensation-product of Synthetic Quebracho Catechin | 4 | 65.5 | 5.3 | 41.2 | - |
| | 12 | 67.1 | 5.2 | - | - |
| Quebracho Catechin - $H_2O$ | - | - | - | - | 33.8 |
| Fraction A | 0 | 63.6 | 5.0 | 40.2 | - |
| " " | 4 | 64.4 | 4.7 | - | - |
| Fraction B | 0 | 63.2 | 5.0 | 38.9 | - |
| " " | 4 | 64.3 | 4.9 | - | - |
| " " | 12 | 64.3 | 4.7 | - | - |
| Fraction C | 0 | 63.2 | 4.9 | 38.0 | - |
| d-Catechin | 0 | 62.1 | 4.9 | 43.2 | 43.0 |
| Condensation-product of d-Catechin | 1 | 62.6 | 5.0 | 40.5 | - |
| | 4 | 64.3 | 4.7 | 37.7 | - |
| d-Catechin - $H_2O$ | - | - | - | - | 39.1 |

In criticism of Freudenberg and Maitland's work the following are to be noted :-

(a) Condensations of synthetic quebracho catechin were carried out under the influence of 2% HCl at elevated temperatures.

Such conditions are hardly likely to occur in plants and the assumption that the resulting condensation-mechanisms are similar to those occurring in the presence of enzymes in plants seems unjustified.

(b) Although there is good agreement between the analytical values of condensed synthetic quebracho catechin and those required by theory, the condensation of d-catechin does not follow a similar trend.

(c) Analytical values of the condensed quebracho catechin and the three fractions of quebracho tannin do not agree well.

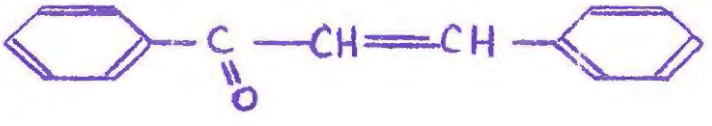
(d) Their failure in obtaining degradation-products larger than veratric acid and resorcinol, casts doubt on the mode of linkage of the condensed product.

## RUSSEL'S THEORY

Opposing views to the above were advanced at the same time (in 1934) by Russel (134)(135). Catechin was then the only 3-hydroxyflavane known to occur naturally, and although Russel considered that the condensed tannins were constituted from catechin-like bodies, he held the view that the aliphatic hydroxyl group was more likely located in position 4 than in the 3-position.

These ideas were based on the following considerations :

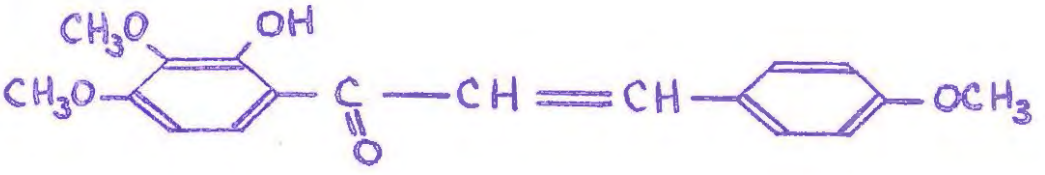
(1) Chalkone or benzylideneacetophenone LXXII, when reduced



LXXII

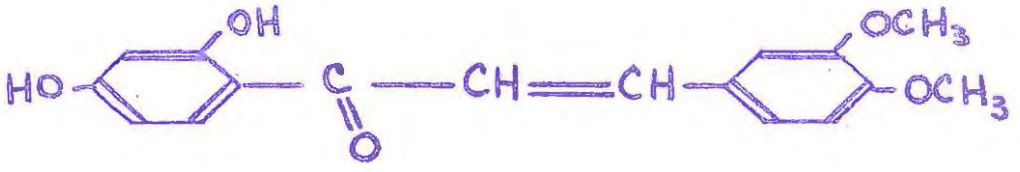
with zinc dust and dilute acetic acid forms a crystalline bi-molecular reduction-product.

(2) 2-hydroxypolymethoxychalkones LXXIII on reduction give amorphous alkali-insoluble reduction products, while 2,4-



LXXIII

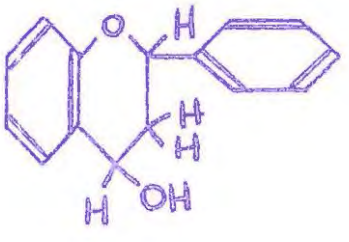
dihydroxy-3':4'-dimethoxy chalkone LXXIV gives an amorphous alkali-soluble reduction product



LXXIV

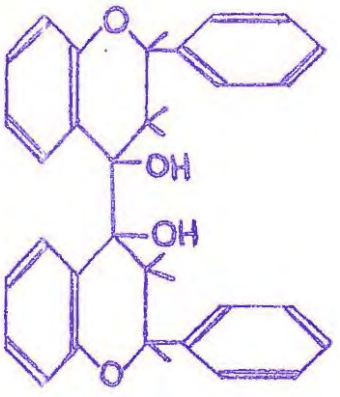
(3) 4-hydroxychalkones on the other hand give crystalline reduction-products like chalkone itself.

From the above evidence Russel concluded that the 2-hydroxyl takes part in ring formation during the reduction process, and that the amorphous reduction products produced from 2-hydroxy-chalkones were either 4-hydroxyflavanes LXXV, or else the corres-



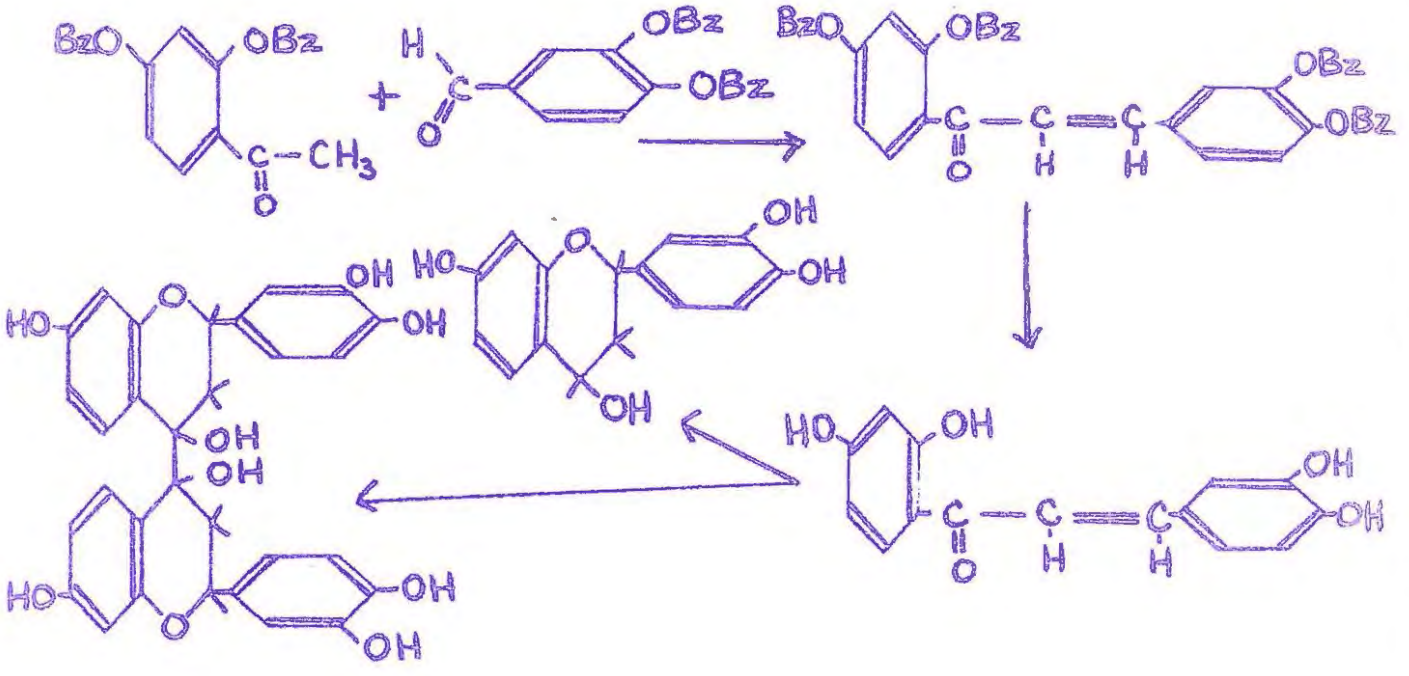
LXXV

ponding bi-molecular compounds called flavpinacols LXXVI (presumably because chalkone itself and also benzophenone (136) form bimplecular compounds).



LXXVI

Russel synthesised what he considered to be 4,7,3',4'-tetrahydroxyflavane or the corresponding flavpinacol from resacetaphenone dibenzoate and protocatechualdehyde dibenzoate :



Qualitatively the properties of the product agreed with that of hemlock and mimosa "tannin".

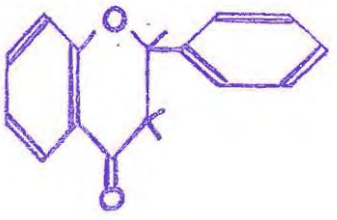
Russel and Todd (135) measured the amount of hydrogen absorbed by the chalkones during the reduction process. The formation of a 4-hydroxyflavane requires 2 hydrogen atoms per chalkone unit, whereas a pinacol structure requires only one. A reduction of a number of chalkones revealed that only one hydrogen atom per chalkone unit was absorbed, and this was used as further evidence of the formation of the pinacol structure.

Russel synthesised a number of these flavpinacols and compared the amorphous products with natural condensed tannins. Bis-(7,8,3',4'-tetrahydroxy)-flavpinacol was found to resemble Hemlock tannin (137) in qualitative reactions, while others resembled mimosa, quebracho and other condensed tannins. Russel, Todd and Wilson (138) compared the ultra-violet absorption spectra of some of these flavpinacols with mimosa, quebracho, ellagic acid and gallotannin. The absorption spectra of the synthetic pinacols were indistinguishable from those of the natural phlobatannins, but differed from those of ellagic acid and gallotannin.

Russel's views gained wide acceptance at one time, and his synthetic methods were even used by Osima (364) to synthesise bis-(5:7:3':4'-pentahydroxy)-flavpinacol which was considered to be identical with the amorphous tannins in Formosan tea leaves.

He was also severely criticised by Freudenberg, Karimullah and Steinbrunn (139). They declared that the reduction products of the polyhydroxychalkones could not be 4-hydroxyflavanes

as the latter would undoubtedly be crystalline. The alternative pinacol structure they regarded as unproved, as the synthesis of pinacol LXXVI occurred only with extreme difficulty from flavanone LXXVII (140).

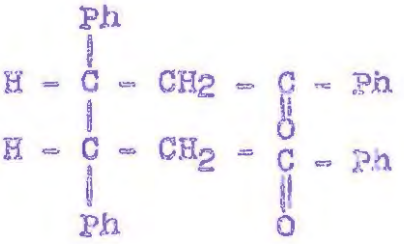


LXXVII

The formation of pinacol, which is crystalline and is one of many stereoisomers, is accompanied by the formation of much amorphous material. Catechin pentamethylether and luteotinidol tetramethyl-ether also for example, when formed by the reduction of the corresponding quercetin and luteolin methyl ethers give rise to amorphous products which are obviously more than dimolecular. Russel did not start from flavanones, and the polyhydroxychalkones he used were even more susceptible to self-condensation. From a comparative study Freudenberg concluded that structures containing phenolic nuclei separated by a carbon chain e.g. catechins and gallotannin have similar ultra-violet absorption spectra to the nuclei themselves.

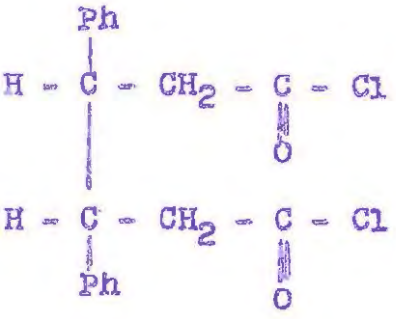
More criticism of Russel's "flavpinacols" has recently come from Finch and White (141). They have shown that Russel's dimolecular reduction product formed when chalkone is treated with zinc dust and acetic acid is not the pinacol (presumably LXXVIII) but (±) -1,3,4,6-tetraphenyl hexane -1,6-dione LXXIX.

LXXVIII



LXXIX

The structure of LXXIX was proved by the formation of a dioxime and its synthesis from (±) - β, β'-diphenyladipoyl chloride LXXX and diphenyl cadmium.



LXXX.

This finding, although not extended to the 2-hydroxychalkones where the possibility of ring-formation does exist, tends to cast more serious doubt on Russel's hypothesis.

## DISCUSSION

In conclusion it may be said that Russel's comparison of the ultra-violet absorption spectra of different phenolic compounds and tannins is not justified as absorption in the ultra-violet region is governed by the chromophoric constitution of the

compounds under investigation. In the tannins the phenolic nuclei constitute the chromophoric groups, and their spectra will therefore have the general resonance-characteristics of such nuclei.

None of the above condensation theories concerning condensed tannins, appear free from much criticism. Ghosh (142) however, states that the pyrane ring appears to play a great part in the catechin-tannin transformation.

## CONCLUSIONS

From this brief review of the considerable amount of past work on the structures of the tannins, the following points emerge :-

(a) Although structures have been elucidated for the crystalline breakdown products of some of the hydrolysable tannins, the amorphous nature of the condensed tannins has hitherto created a baffling problem.

(b) All the natural tannins studied appear to be fundamentally composed of polyhydroxy benzene units, either condensed by ester formation with sugars, or condensed with each other, or both.

(c) In addition to the hydrogen bonding function of the fundamental polyhydroxy benzene units, a minimum size appears to be necessary for tanning properties.

(d) The common denominator of most of the tannins is the presence of ortho dihydroxy or trihydroxy benzene units, and the

formation of an insoluble lead salt by such units provides an obvious method for eliminating those non tans which are chemically different, such as sugars.

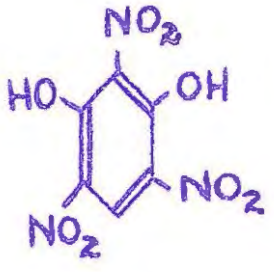
# CHAPTER III

## A DISCUSSION AND SOME CRITICISM OF PREVIOUS CHEMICAL INVESTIGATIONS OF BLACK WATTLE EXTRACT

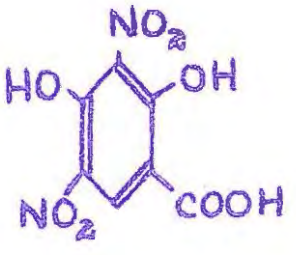
For the earliest constitutional work on the extracts of commercially-important acacias, the so-called "mimosa extract" (the nomenclature applied particularly in European countries) was used. From the preceding historical survey it is evident that except for a period during the last war when a low admixture of green wattle tannin (Acacia decurrens Willd.) was present, the commercial extract consisted entirely of tannin from the bark of Acacia mollissima Willd. Comparison of earlier work with that now in progress is, therefore, justified.

### H. EINBECK AND L. JABLONSKI

Einbeck and Jablonski in 1921, oxidised mimosa extract with nitric acid and obtained both styphnic acid LXXXI (143) and 3,5-dinitro-$\beta$-resorcylic acid LXXXII (121). Von Hemmelmayr (144)

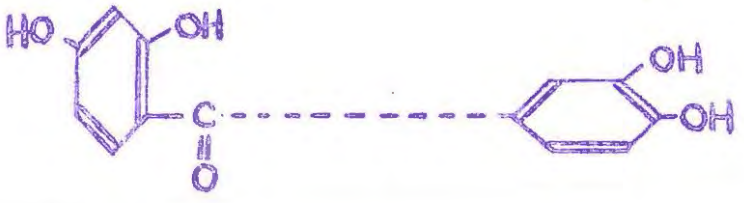



LXXXI




LXXXII

from his studies on the nitration of β-resorcylic acid found that when 3,5-dinitro-β-resorcylic acid was heated in the presence of nitric acid it was quantitatively converted to styphnic acid. Einbeck and Jablonski thus postulated that 3,5-dinitro-β-resorcylic acid was the primary and styphnic acid the secondary product of the reaction of concentrated nitric acid on mimosa extract. Identical products were also obtained from quebracho extract. According to them, both quebracho and mimosa extracts gave the fluorescein-reaction with phthalic anhydride and zinc chloride; a reaction not given by flavanol-like ring systems. They considered that the resorcinol nucleus was attached to the tannin unit as a metadihydroxybenzyl radicle, and that in both tannins a chalkone-type residue LXXXIII existed.



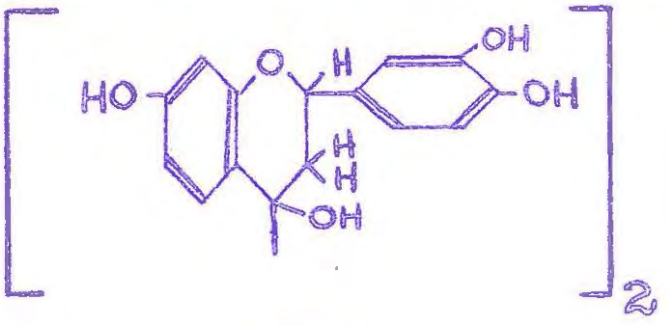

LXXXIII

W. EGGERT

Eggert (145) oxidised methylated mimosa tannin with alkaline permanganate. He was unable to separate the mixture of acids which resulted, but suggested that it might consist of veratric and O-trimethylgallic acids.

A. RUSSEL

Russel (135) obtained phloroglucinol, catechol and protocatechuic acid from the alkaline fusion of tannin from South African wattle bark. He recorded that the tannin was converted to a phlobaphene on acid treatment, gave a blue-violet coloration with either alcoholic or aqueous ferric chloride and contains C = 60 - 62% and H = 4.5 - 4.9%. These figures were not altered by lead salt purification. Russel compared the ultra-violet absorption spectrum and qualitative reactions of 2,4,3',4'-tetrahydroxychalkone with mimosa tannin, but in view of the criticism levelled at his synthetic work as well as his comparison of the ultra-violet absorption spectra (see pp. 84 - 86) these results must now be regarded with doubt. His isolation of phloroglucinol and catechol nuclei only, is surprising in the light of more recent work, casting doubt on the authenticity of his material. Even the comparison of his synthetic bis-(7,3',4-trihydroxy) flavpinacol LXXXIV with mimosa extract appears to rest on confused reasoning. This compound gives a green colour with ferric chloride (compared with the blue of mimosa extract) and contains a resorcinol nucleus, whereas Russel actually isolated a phloroglucinol nucleus from mimosa extract. The ultra-violet absorption curve of mimosa tannin (138)




LXXXIV

showed absorption peaks at 270 mμ (minor peak) and 286 (major peak); and the methylated derivative similarly at 270 mμ (minor peak) and 281 mμ (major peak). These curves differed from those of the parent chalcones but proved identical with their reduction-products, the so-called flavpinacols.

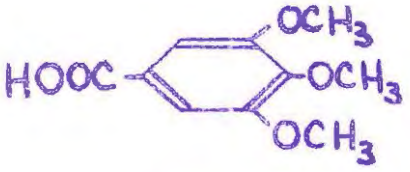
In 1937 Douglas and Humphreys (118) attempted the "molecular weight" determination of mimosa and other tannins using the conventional cryoscopic method and water as solvent. The non-tannins were almost completely removed by electrodialysis, and the tannin content of the material increased from 70.3% before dialysis to 95.41% afterwards. Values obtained varied from 432 to 603 before dialysis to 1570 - 1704 after dialysis. Mimosa tannin showed a much higher molecular weight than gambier, (324 before, and 520 after dialysis) which contains catechin predominantly, and a lower molecular weight than quebracho (1159 and 2421 respectively). Criticism of this work is recorded in Chapter IX.

J. H. CORBETT

Constitutional work on the commercial wattle extract started in 1940 at Rhodes University College with the work of Corbett (146). Corbett was the first to make a serious attempt at "purifying" the phenolic fraction of non-tannins for constitutional work, by using Russel's (136) salting-out and acetone extraction method, Browne's (147) ultrafiltration method, Grassman's (148) chromatographic technique, as well as a "lead salt" purification method. The ultrafiltration method, although the most

satisfactory, gave very low yields of pure tannin. Elementary analysis of the tannin "purified" by various methods varied only slightly : C = 60.8 - 61.7% and H = 4.53 - 4.61%. Averages of these values corresponded to an empirical formula of $C_{15}H_{13.4}O_{6.3}$, with a theoretical molecular weight of 294. Molecular weight estimations of the purified samples, by Beckman's cryoscopic method in aqueous solution, varied from 620 for the lead-salt purified material, to 1010 for tannin separated by ultrafiltration. Corbett also prepared tannin derivatives aimed at obtaining "some compound which could be more easily purified than tannin itself". The acetylated derivative was prepared from the crude tannin by the action of acetic anhydride and sodium acetate. It contained 40.0% acetyl groups (gravimetric method) corresponding to 4.6 hydroxy groups per $C_{15}$ unit. Dimethyl sulphate and KOH in methanol was found to be the most satisfactory methylating agent, yielding a derivative which gave the analysis : C = 64.3%, H = 6.25%, $-OCH_3$ = 34.6%, molecular weight (Rast) = 600 - 650. A low proportion of hydroxyl groups, which could be acetylated, remained unmethylated in the methoxyl derivative. Acetylation of the methylated derivative also caused the substitution of methoxy by acetyl groups to a slight degree. Oxidative degradation completely disrupted the tannin and its acetylated derivative, while the methylated derivative yielded 5% of an acidic mixture with properties intermediate between a di- and tri-methoxybenzoic acid. Separation of the acids was effected by the fractional crystallisation of their silver salts. One of these was positively

identified as O-trimethyl-gallic acid LXXXV and the other corresponded to veratric LXXXVI on analysis and in properties.




LXXXV


LXXXVI

A. M. STEPHEN

Stephen (149) continued this constitutional work at the Leather Industries Research Institute. He paid less attention to the complete elimination of non-tannins from the tannins and was content to perform the bulk of his work on material which had been salted-out once and then acetone extracted, according to Russel's directions. This contained 86% tannin and he stated "results of analyses and chemical degradations have, therefore, to be recognised as produced from mixtures of tannins and non-tannins in varying proportions". He also concluded that "...no straightforward method, i.e. one not involving chemical change in tannin, has yet been devised to produce a material with greater than 86% tannin content ....", and thus overlooked some of the work by Corbett.

Elementary analysis on an acetone-extract gave the values C = 57.4%, H = 5.01%. By corrections for gain in weight during weighing these were increased to C = 59.2%, H = 4.95%, and finally the figures C = 62.6%, H = 4.56 were arrived at by making

further corrections for ash content (0.37%) and moisture content (6.7%). After desiccation in an Abderhalden gun (toluene) the same values varied from C = 57.5%, H = 4.37% (3 days' drying) to C = 60.0%, H = 4.9% (10 day's drying). From these figures and from subsequent work it was obvious that Stephens' acetone-salt extract contained larger admixtures of sugary non-tannins than Corbett's "purified" preparations. Mean figures of six analyses on the dry tannin corresponded to C = 60.0%, H = 4.92% or an empirical formula of $C_{15}H_{14.8}O_{6.6}$.

Acetylated tannin was prepared using both acetic anhydride/pyridine or acetic anhydride/sodium acetate mixtures. The former gave a product of 39.3%, and the latter 39.0% acetyl groups. Because of the instability of the tannin, saponification methods of acetyl determination gave erroneous results. The trans-esterification method of Matchett and Levine (150) proved satisfactory. Acetylations of larger quantities of materials gave low acetyl values varying from 29.3% to 34%, which could be raised by reacetylation to 37.0%.

Fractional precipitation of this partly acetylated (29.3%) material (from acetone extracted tannin) was effected by the addition first of 6 N acetic acid, and then water, to a glacial acetic acid solution. Analysis of various fractions obtained showed some variation :

| | % Acetyl | % C. | % H. |
|---|---|---|---|
| Fraction A | 29.3 | 57.6 | 4.58 |
| " B | 29.3 | 58.4 | 4.65 |
| " C | 31.5 | 57.3 | 4.60 |
| Original Sample | 29.3 | 58.9 | 4.64 |

The above fractions were reacetylated twice. Found :

Fraction A = 35.6% acetyl

" B = 33.5% acetyl

" C = 35.0% acetyl

As all these were below that obtained on a single acetylation of the tannin (39%), some change was suspected to have occurred in the reactive hydroxy groups.

Similar fractionations were repeated on material of 20% acetyl content obtained from the acetylation of commercial extract. After full acetylation the acetyl content varied from 36.2% to 38.0% indicating little separation. Finally a product of 23.8 - 24.6% acetyl content was fractionated into four fractions, the acetyl content of which varied as follows on complete acetylation :

| | % Acetyl after Fractionation. | % Acetyl after complete Substitution |
|---|---|---|
| Insoluble Fraction | 23.0 | 34.5 |
| Sparingly Soluble Fraction | 24.5 | 37.0 |
| Mod. Sol. Fraction | 24.5 | 39.0 |
| Soluble Fraction | 22.5 | 40.5 |

The soluble fraction apparently contains one acetyl group (per $C_{15}$ unit) more than the insoluble fraction. Stephen claimed that the latter fractionation was evidence of the non-homogeneity of the tannin, but the result must be considered in the light of the previously-mentioned difficulty in obtaining high values, and the low average acetyl values obtained after two acetylations in the first fractionation discussed. The acetic anhydride/sodium acetate mixture used by Stephen was evidently only partly effective in obtaining "maximum" values, and the effect of a known admixture of chemically dissimilar non-tannins (14%) also requires consideration.

Direct acetone extraction of the bark furnished tannins which could be acetylated to the maximum values of 39.6, 40.1, 40.1, 38.6 and 41.0. These compared favourably with the maximum acetyl values of tannin similarly obtained from commercial sources.

Three fractions of acetyl content 38.7%, 38.4% and 39.4% were obtained from acetylated (38.0%) fresh-bark acetone-extracted tannin. The least soluble of these had a molecular weight (cryoscopic : dioxane) of 3000, while that of the remaining two varied from 1500 to 2000. To determine if such molecular variations could be produced by extractions at different temperatures, 7-day leachings of the bark at temperatures ranging from 6° to 80°C. were effected. An increase of acylatable groups and molecular weight occurred with increasing temperature. The significance of this result will be discussed in the light of the more recent findings of the present investigation.

The most effective methylations of the tannin (60 grms) were carried out with the use of dimethyl sulphate (1.5 moles) and KOH (1.6 moles) in methanol as recommended by Corbett. The product (80% recovery) contained on an average 31.6% methoxyl groups. More drastic methylations with larger proportions of reagents increased the methoxyl content to 33.6 - 35.8%. This material could be further methylated with silver oxide and methyl iodide (151) to a maximum of 36.5% methoxyl.

Stephen also methylated black wattle tannin with diazomethane, but found the reagent unsatisfactory as reaction appeared to occur with extreme difficulty. 18% methoxyl content was achieved only by repeated reaction, while that of already 33 - 34% methoxyl value (obtained with dimethyl sulphate) was unreactive. Acetylated tannin containing 4.2 acetyl groups/$C_{15}$ unit reacted with diazomethane causing the hydrolysis of 2.4 acetyl groups and the replacement with methoxyl groups of the remainder (1.8).

Elementary analysis of the methylated tannin (32.0% methoxyl) corresponded to C = 63.3%, H = 5.61%.

Stephen compared the elementary analyses of incompletely methylated (32% methoxyl) and acetylated (29.3% acetyl) derivatives with that of the original tannin. The empirical formula of the tannin varied from $C_{15}H_{14}O_{5.9}$ (corrected formula) to $C_{15}H_{14}O_{6.6}$, and that of the derivatives corresponded to $C_{15}H_{10}(OCH_3)_{3.5}O_{2.9}$ and $C_{15}H_{10}(OAc)_{2.83}O_{3.67}$. Apart from the fact that agreement in oxygen content was observed in the two derivatives and the latter tannin formula, the analyses of the incompletely substituted

material were meaningless.

Oxidation of the methylated tannin with permanganate in various solvents gave varying amounts of veratric and O-trimethyl gallic acid, separated and identified by Corbett's method. The best yield of mixed acids (7.7%) was obtained by oxidising first in acetone and then in aqueous solution. Oxidations of the tannin, the acetylated and brominated derivatives gave no identifiable end-products with a variety of oxidising agents.

Alkaline fusion of the tannin under various conditions produced resorcinol (1 - 2% yield) and a trace of gallic acid. Stephen concluded that the low yields of degradation products from all degradations confirmed the complexity of the tannin structure.

Stephen studied the action of acids on tannin. By boiling with 5N HCl he produced a water-insoluble amorphous phlobaphene which could be acetylated to the same degree as the original tannin. Water-elimination during phlobaphene formation therefore appears unlikely. Red precipitates of phlobaphenes produced under more drastic conditions (e.g. HCl in glacial acetic acid) could not be substituted to the same degree.

Nitric acid oxidations of the tannin itself, and the acetyl and methoxyl derivatives all yield styphnic acid LXXXI (previously isolated by Einbeck and Jablonski) and oxalic acid.

Stephen attempted the formation of anthocyanin-type structures from the tannin by the bromination technique which Appel and Robinson (107) (See page 58) used for the conversion of d,l-epicatechin to cyanidin chloride. No identifiable products

and none containing ionic chlorine could be isolated, and it is extremely doubtful whether an anthocyanidin residue is produced by the bromination procedure. No conclusion was thus possible regarding the presence of catechin units in mimosa tannin.

The bromination of the tannin and its derivatives occurs easily. A portion of the bromine content of the product could be removed by silver nitrate, and the degree of bromination varied with conditions. In general it appeared that a maximum of 4 to 5 bromine atoms could be substituted in each $C_{15}$ tannin unit.

Stephen subjected the tannin to drastic hydrogenation, using copper chromite and Raney nickel catalysts for varying periods at various pressures. Low proportions of oils were formed and much charring was evident in most reactions. The oil was fractionated but no end-products could be identified.

The main feature of Stephen's work was the isolation of resorcinol and gallic acid in low yield on alkaline fusion. In addition he developed general methods of forming derivatives as well as their analyses, and investigated many possible lines of approach, although most of these gave negative reactions. Much confusion was, however, caused by the use of impure starting-materials as well as the presentation of an unsifted mass of analytical results mostly from partly substituted derivatives.

M. H. SILK.

Silk (152) continued the hydrogenation study of black wattle tannin along the lines successfully applied by Adkins, Frank

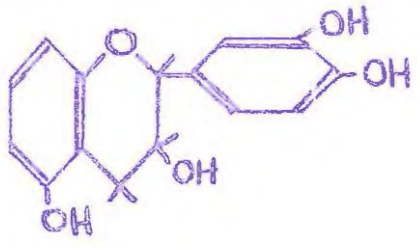
and Bloom (153) for the isolation of 4-n-propyl cyclohexanol-1, 4-n-propylcyclohexanediol-1,2, and 3-(4-hydroxy cyclohexyl)-propanol-1 from lignin (154)(155)(156), but could not identify any products resulting from either hydrogenation or hydrogenolysis. Partly hydrogenated tannin from these reactions also yielded an oil on destructive distillation. This was re-hydrogenated and the product, which resembled cyclohexanol in odour and appearance, was fractionated. The fractions varied in molecular weight from 113 to 214; in their elementary analyses (C = 68.2 - 71.9%; H = 8.7 - 11.4%); and also in acetyl value (23.4 - 25.4%). No empirical formula would fit the above figures, and the various fractions were, therefore, still mixtures of hydrogenated structures of low molecular weight due to inefficient fractionation. More work employing both Raney nickel and copper chromite as catalysts and a variety of organic solvents, similarly gave no identifiable hydrogenation products.

J. M. WILLIAMS

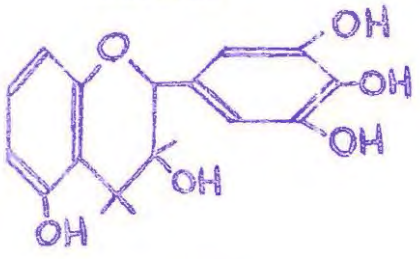
Williams (157) claimed the preparation of 95% "pure" tannin by the extraction of the fresh bark with ethyl acetate and with methanol (158). Acetylation of such fresh-bark extracts gave a product of the following composition : Acetyl = 41.5%, C = 58.94% and H = 4.93%, Molecular weight (ebulliometric : acetone) = 433 - 470. Acetylation of the tannin with acetic anhydride/sodium carbonate gave a product of 35.5% acetyl content.

Williams confirmed Stephen's finding that resorcinol

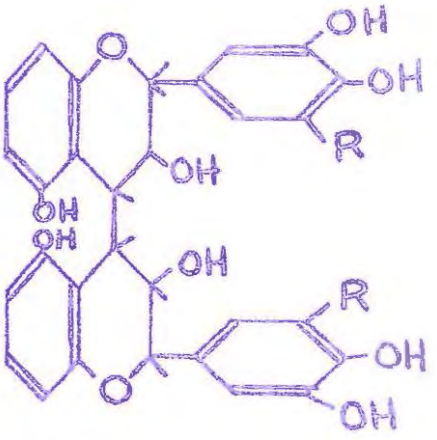
only, and no phloroglucinol was obtained on alkaline fusion of the tannin. He repeated the bromine oxidation of the tannin according to Appel and Robinson's (107) method, and considered that the resultant red gum represented an "anthocyanin" pigment. He represented the fresh-bark extract as a mixture of monomers LXXXVII and LXXXVIII, whereas the commercial extract could be



LXXXVII



LXXXVIII



LXXXIX R = H

XC R = OH

represented by the corresponding dimeric structures LXXXIX and XC.

These structures were presented with no experimental support other than that listed above and Stephen's work, and must be considered as purely speculative. Williams was obviously unaware of Einbeck and Jablonski's earlier isolation of a β-resorcylic acid derivative.

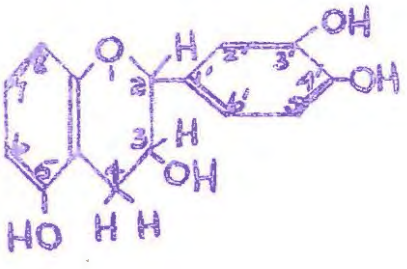
H. LOWITT.

Lowitt (159) made an investigation of various methylation and acetylation procedures for phenolic compounds and compared the

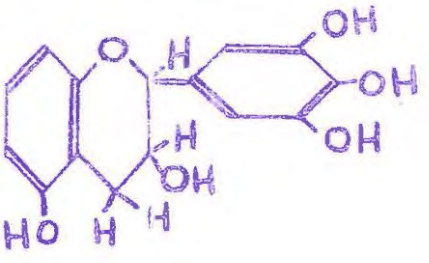
results with that obtained from black wattle tannin. Acetic anhydride/sodium acetate and acetyl chloride/pyridine used successively, were regarded as the most effective acetylating agents. The following average results were recorded :

| Extraction Method of Tannin | % Acetyl | % C | % H |
|---|---|---|---|
| Ethanol-extract | 41.0% | 58.4 | 4.7 |
| Acetone-extract | 40.1% | 58.6 | 4.7 |

Lowitt studied the replacement of acetyl by methoxyl groups with diazomethane using Nierenstein's method (160) with piperidine as base. From a comparative study he concluded that acetylated ortho-hydroxy groups are slowly, and acetylated aliphatic-hydroxy groups even more slowly "replaced" by methyl groups. In the acetylated tannin two groups were easily replaced and the remaining two slowly replaced under Nierenstein's conditions. He thus concluded, without further evidence, that in a postulated catechin unit those groups replaced with difficulty could be represented by a sterically hindered acetyl in position 5, and an acetylated aliphatic hydroxyl in position 3.



XCI



XCII

The fresh-bark extract was postulated to be a mixture of the catechin monomers XCI and XCII, whereas the commercial extract could be represented by a mixture of dimers of the Russel type. No experimental evidence was given to substantiate these claims.

Lowitt was obviously influenced by the erroneous ideas of Williams and was likewise oblivious of Einbeck and Jablonski's work. In addition he overlooked the fact that the ortho-di- and ortho-trihydroxy structures, known to be present, would provide as much if not more steric hindrance as the 5-position in his postulated structure. Furthermore, little is known of the "replacement" mechanism of Nierenstein's reaction. From Lowitt's data it is exceedingly obvious that the "replacement" was accompanied or even preceded by the hydrolysis of the acetyl groups, in the basic medium. It was, for example, necessary to reacetylate the tannin after methylation with diazomethane/piperidine in order to obtain a fully substituted derivative. The "replacement" mechanism of Nierenstein's method is open to serious doubt.

R.A. HEUGH.

In spite of much previous work Heugh (161) was the first to prove the identity of veratric acid as one of the oxidation products of methylated tannin conclusively. He synthesised both O-dimethyl- $\gamma$ -resorcylic acid and veratric acid which have identical elementary analyses, equivalent weights and melting points. The identity of the oxidation product and the latter acid was further established by the formation and comparison of their methyl esters.

Heugh showed that the conditions of oxidation of the methylated tannin influenced the proportions of the two acids produced. Drastic oxidations with alkaline permanganate decreased the proportion of O-trimethylgallic acid isolated, and under extreme conditions veratric acid only resulted.

He also nitrated methylated black wattle tannin in the presence of urea, and thus introduced two nitro groups per $C_{15}$ unit. Alkaline permanganate oxidation of this product afforded veratric acid and a yellow strongly acidic gum which corresponded in elementary and equivalent weight analysis to a mononitrodimethoxybenzoic acid. This failed to recrystallise and further purification was not attempted.

Heugh attempted the reduction of the methylated tannin with sodium in ethanol according to Kostanecki and Lampe's (162) method. This reduction is capable of opening the pyrane ring in d-catechin tetramethylether to form a $\alpha$ , $\gamma$ ,-diphenylpropane derivative. Reduction of methylated tannin in ethanol, n-propanol, n-butanol and n-pentanol resulted in the partial removal of methoxyl groups, as even after drastic remethylation a 5 - 9% reduction in methoxyl content was observed. (Molecular weight of product (Rast) = 410 - 424). Such a reduced and remethylated product was finally oxidised with permanganate. In addition to veratric and O-trimethylgallic acids, an acidic gum was isolated. This could be obtained only in a semi-crystalline form, and corresponded to a dimethoxyphenylproprionic acid on analysis. No further separation was attempted but oxidation of

the gum gave veratric acid only.

Alkaline fusion of the methylated tannin produced a small quantity of yellow oil which corresponded in odour and analysis to resorcinol monomethyl ether.

S. G. SHUTTLEWORTH

Shuttleworth (369) carried out a conductimetric study of 16 phenols and concluded that (a) the dihydroxyphenols were stronger acids than monohydroxyphenols and titrate as monobasic acids up to pH = 11; (b) trihydroxy phenols give partial titration of the second hydroxyl group below pH = 11; (c) in catechins and other units where phenolic nuclei are well separated by a carbon chain, the constituent phenols titrate as independent units; (d) if it is to be assumed that the black wattle tannins contain an average of two phenolic nuclei per $C_{15}$ unit separated by a carbon chain, the tannins show abnormally low acidity.

More recently Shuttleworth (232) examined the increased acidity obtained from black wattle tannins (observed by Woodhead (370) during bisulphiting and by Stephen during refluxing with alkali), by potentiometric titrations. By comparing the results obtained from the tannins with various phenols on an equivalent basis, he concluded that the tannins closely resemble pyrogallol in behaviour when refluxed in alkaline medium in the presence or absence of air, and also when oxidised under alkaline conditions. Oxidation and alkaline hydrolysis caused an increase in the ultra-violet absorption-peak at 280 mμ. The former caused a considerable

change in the shape of the curve, whilst the latter only effected an increase in the height of the curve over the whole range 240 - 320 mμ.

K. S. KIRBY

Kirby (163)(164) conducted an investigation on the acetone-soluble portion of the commercial extract, which constitutes about 66% of the original extract. Oxidation of this with nitric acid produced styphnic and oxalic acids.

Methylation was effected with dimethyl sulphate in methanol and with aqueous KOH. This process served as the basis for the fractionation of the methylated derivative, and was incompletely described in his thesis (163) and incorrectly represented in a subsequent publication (164), thereby causing much confusion. Towards the end of the above reaction a brown resin separated together with insoluble potassium sulphate.

The portion still soluble in aqueous methanol was filtered off, and the solvent removed. (Fraction A : 20% of starting-material). The ethanol-soluble portion of this fraction was adsorbed on an alumina column from benzene and eluted with benzene containing 2% ethanol. The main fraction (Analysis : C = 66.45%, H = 6.54%, $-OCH_3$ = 34.8%) from the column gave veratric acid only on permanganate oxidation.

The brown resin was dissolved in a water/chloroform mixture. The chloroform extracts yielded a dark brown powder, the major portion of which was benzene-soluble (Fraction B : 60%

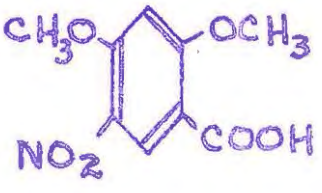
of starting material), and a minor proportion benzene-insoluble (Fraction C : 20%).

Fraction B was chromatographed as above on an alumina column and the major portion eluted with benzene/ethanol (100 : 3). Analysis of the product corresponded to C = 66.03%, H = 6.08%, -OMe = 35.9% and oxidation gave trimethoxygallic acid only. Fraction C appeared similar to Fraction B.

These oxidations indicated that a true chemical separation had been achieved, but the analyses of the two fractions did not differ appreciably and certainly did not account for the apparent replacement of a pyrogallol nucleus in Fractions B and C for the catechol nucleus in Fraction A.

As the yields of these acids (8 - 12%) were abnormally low, Kirby considered that they may represent an end-group estimation. The fully methylated tannin was found to react with acid chlorides, and Kirby thought that this was indicative of the presence of aliphatic hydroxyl groups. The attempted oxidation of secondary hydroxyl groups to ketones by Oppenauer's method proved unsatisfactory.

The oxidation of Fractions A and B with nitric acid gave styphnic acid, and also 2,4-dimethoxy-5-nitrobenzoic acid XCIII in 1% yield. The isolation of this acid showed that after

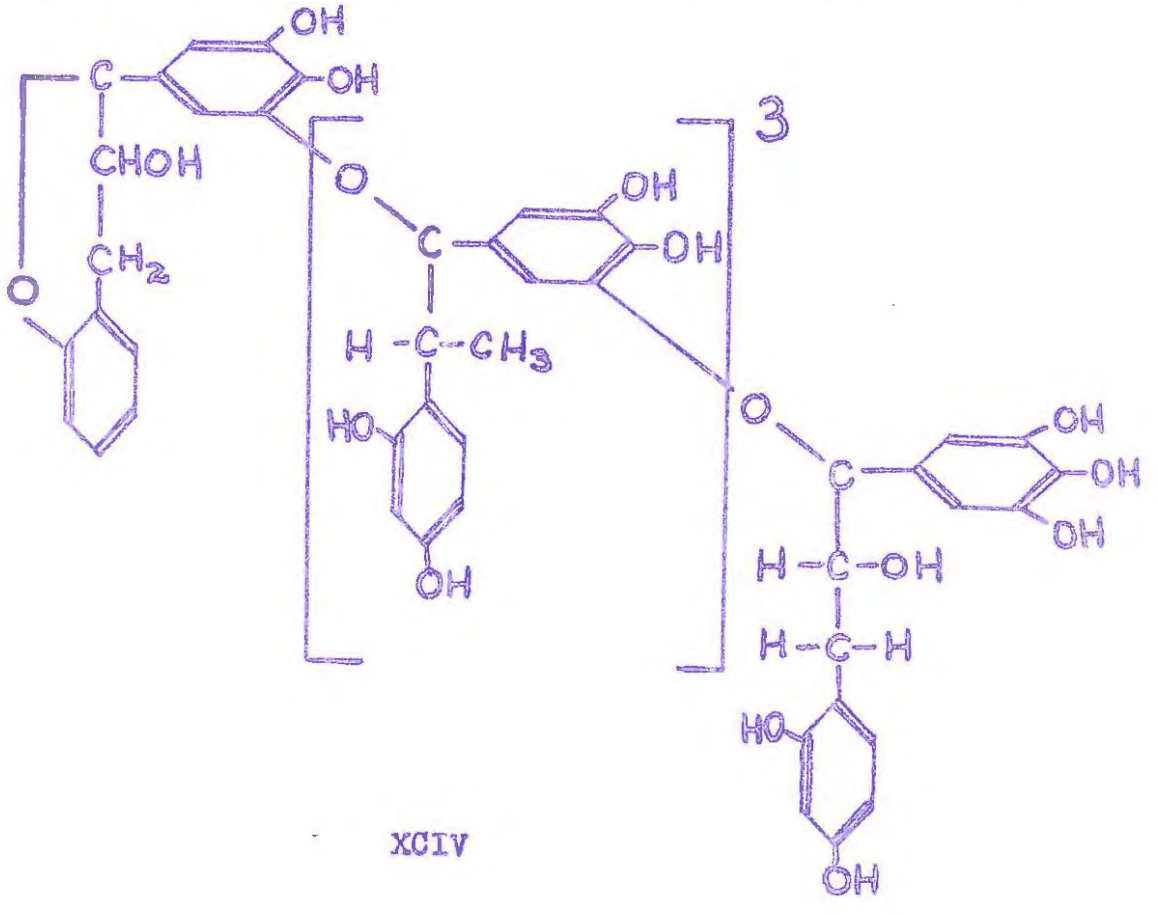



XCIII

drastic methylation, some resorcinol nuclei do not take part in benzopyrane-ring formation.

Analysis of Fraction B was in good agreement with the formula $C_{19}H_{22}O_6$ containing four methoxyl groups. As both pyrogallol and resorcinol nuclei are present in this fraction, representing a total of 5 hydroxyl groups, at least one phenolic hydroxyl group must be involved in an ether linkage. A Kuhn-Roth determination on Fraction B indicated 2.1% terminal methyl groups.

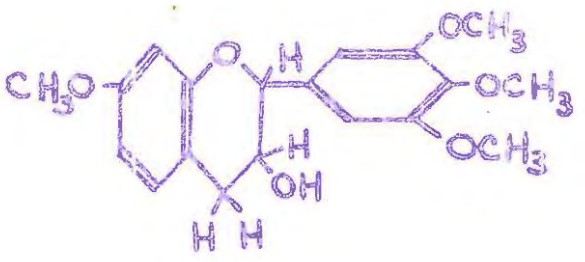
Taking into account all the above data Kirby considered the tannin to be represented by the formulation XCIV, in which the pyrogallol end-group is replaced by a catechol unit in some




XCIV

instances.

Kirby was incorrect in assuming that a methylated catechin unit of the type XCV gave a theoretical (60%) yield of O-trimethyl



XCV

gallic acid. In actual fact d-catechin tetramethylether gives half the theoretical yield of veratric acid (165) which is more stable to permanganate oxidation than O-trimethyl gallic acid (166). He was also incorrect in assuming that because the fully-substituted tannin still acylates, a proportion of aliphatic hydroxyl groups were present. Corbett had already shown that a degree of replacement of methoxyl by acetyl groups occurred under such conditions. These facts have important structural significance and they certainly modify his formulation.

KIRBY, KNOWLES AND WHITE and PUTNAM AND GENSLER.

During the writing of this script, two papers by Kirby, Knowles and White (167)(168) on the chromatography of black wattle and other extracts, and one by Putnam and Gensler (131) dealing inter alia with the acetylation of black wattle and quebracho extracts, have been published. These will be discussed under appropriate headings in this thesis.

## SUMMARY

Synopses and discussions, dealing with the various aspects of the accumulated evidence which resulted from the previous work of all these investigators, are presented as introductory sections in the various chapters which follow.

# THE EXTRACT OF THE BARK OF ACACIA MOLLISSIMA WILLD

(Black Wattle Extract)

## CHAPTER IV

## THE FRACTIONATION OF BLACK WATTLE EXTRACT

### 1. INTRODUCTION

The elucidation of the structures of various phlobatannins presents many difficulties. Chief of these has been the quantitative isolation in the pure state of those commercially important polyhydroxy compounds which constitute the major fraction of the extracts and which actually combine with the hide or skin in the tanning process.

Vegetable extracts contain variable amounts of sugars, gums, salts and glucosides associated with the polyhydroxy tannin units. When determining the molecular structure or composition of the tannin, the separation and identification of these so-called non-tans is also of fundamental importance.

Black wattle extract produced by three commercial firms (7) averaged on a dry basis 72.85% tannin, 23.10% non-tans and 4.05% insolubles, whilst a cold aqueous extraction of the fresh bark in this laboratory contained 80.19% tannin and 19.81% non-tans on a similar basis.

Of all the workers who have in the past studied the

composition of black wattle extract, not one has so far effected the quantitative isolation of the tannins or complete separation of non-tans from the polyhydroxy tannins, with the result that no satisfactory agreement could be obtained between analyses on the tannin itself, its methoxy and its acetyl derivatives. This is probably due to the fact that the 20% non-tans consists chiefly of sugars and allied compounds, which acetylate and methylate to varying degrees. Their presence also precludes the possibility of arriving at any definite conclusion regarding the composition of the tannins or the significance and yields of degradation-products obtained.

The literature contains mention of dialysis (170) and solvent extraction (171) methods to increase the tannin content of extracts. Trimble (172) while investigating phlobatannins, introduced the use of acetone for removal of sugars. This solvent has been shown to be only partially effective for black wattle. Russel (136) in his paper on flavpinacols, used a method of salting out the commercial "Mimosa" extract once with sodium chloride to free it from sugars, extracting the dried material with acetone to free it from salt, and finally washing the crushed product with ether to remove simple organic impurities. He makes no claim as to the "purity" of the product, however, stating that the method is empirical, and that the tannin probably contains large amounts of impurities. He also "purified" (ibid. p. 169) the tannins through their lead salts, but gave no details of experimental procedure. This "purification" he found did not

alter the elementary analyses in any way.

Corbett (146) found that ultrafiltration gave effective "purification" but the method was relatively unsatisfactory due to its tediousness and the low yields obtained.

Stephen (149) did not improve on Russel's work. He followed the same procedure, as described above, utilising a South African commercial wattle extract which contains 73% tannin. After "purification" he obtained a product which on a dry basis contained 87.5% tannin, 12.2% non-tannin and 0.3% solids. On this he based the bulk of his work. He also attempted a mixed solvent "purification", but he abandoned this method, on failing to separate off the precipitate cleanly.

Williams (157) used successively acetone, ethyl acetate and amyl acetate in that order, and also ethyl acetate alone, on freshly cut bark. He claimed a high degree of "purity" (94 - 95.8%) by these methods, but his results have since been found to be erroneous and abnormally high for the methods employed. 95% "purity" was also claimed (unpublished) for the product obtained from the cold methanol extraction of fresh bark. Such extracts have been shown by the author to contain only about 80% tannin. As a result Heugh (161) based his work on this fresh-bark methanol-leached product.

Electrodialysis has been considered a useful method for obtaining black wattle tannin of high "purity". Douglas and Humphreys (118) when preparing tannin samples for molecular weight determinations, produced a product of 95% "purity". This was

repeated by Rich (173) to obtain a product of molecular weight 1800 and 91.6% "purity". The method is very slow, yielding only small quantities of a dark high molecular weight product. It is therefore obviously unsuitable for a qualitative and quantitative investigation of the whole tannin fraction, and the use of such oxidised material is in any case undesirable. Kirby (163) investigated only the acetone-soluble portion of "Mimosa" extract.

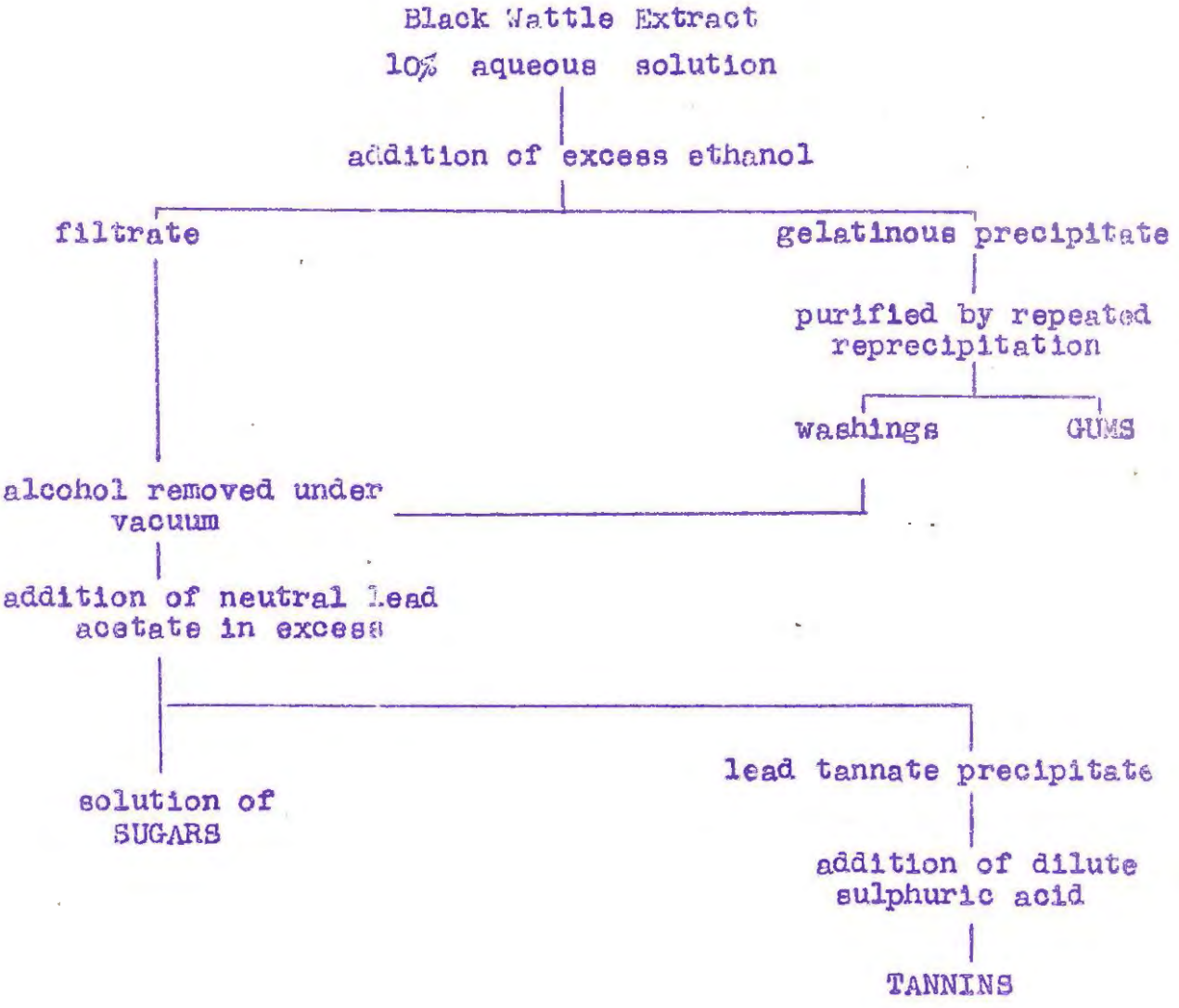
From the above discussion it would seem that hitherto no satisfactory method for the large scale quantitative separation of wattle extract had been devised, and it appeared necessary to re-investigate some of these methods. The problem was then to devise an easy direct method capable of quantitative fractionation of the extract without causing condensation or oxidation of the tannins.

## 2. THE SEPARATION OF TANNINS AND SUGARS FROM THE GUMS.

Black wattle extract was found to consist of (a) gums (b) sugars and (c) polyphenolic tannins. The extract was fractionated according to two alternative schemes :

(see over)

Fractionation Scheme I.




The gum was precipitated from a dilute aqueous solution (500 ml. of 10%) of the extract by the slow addition of excess ethanol (500 ml.) during vigorous stirring. The gelatinous precipitate was centrifuged (3500 r.p.m. for 15 min.) and then sucked to semi-dryness over 24 hours on a Buchner funnel. The product appeared dark-brown and obviously still contained tannins. It was accordingly redissolved with vigorous stirring in a minimum

of cold water, and the precipitation repeated. This process required 4 - 5 repetitions for the complete elimination of tannins. Yield of gum = 2.45 grm. or 4.9% of the starting-material.

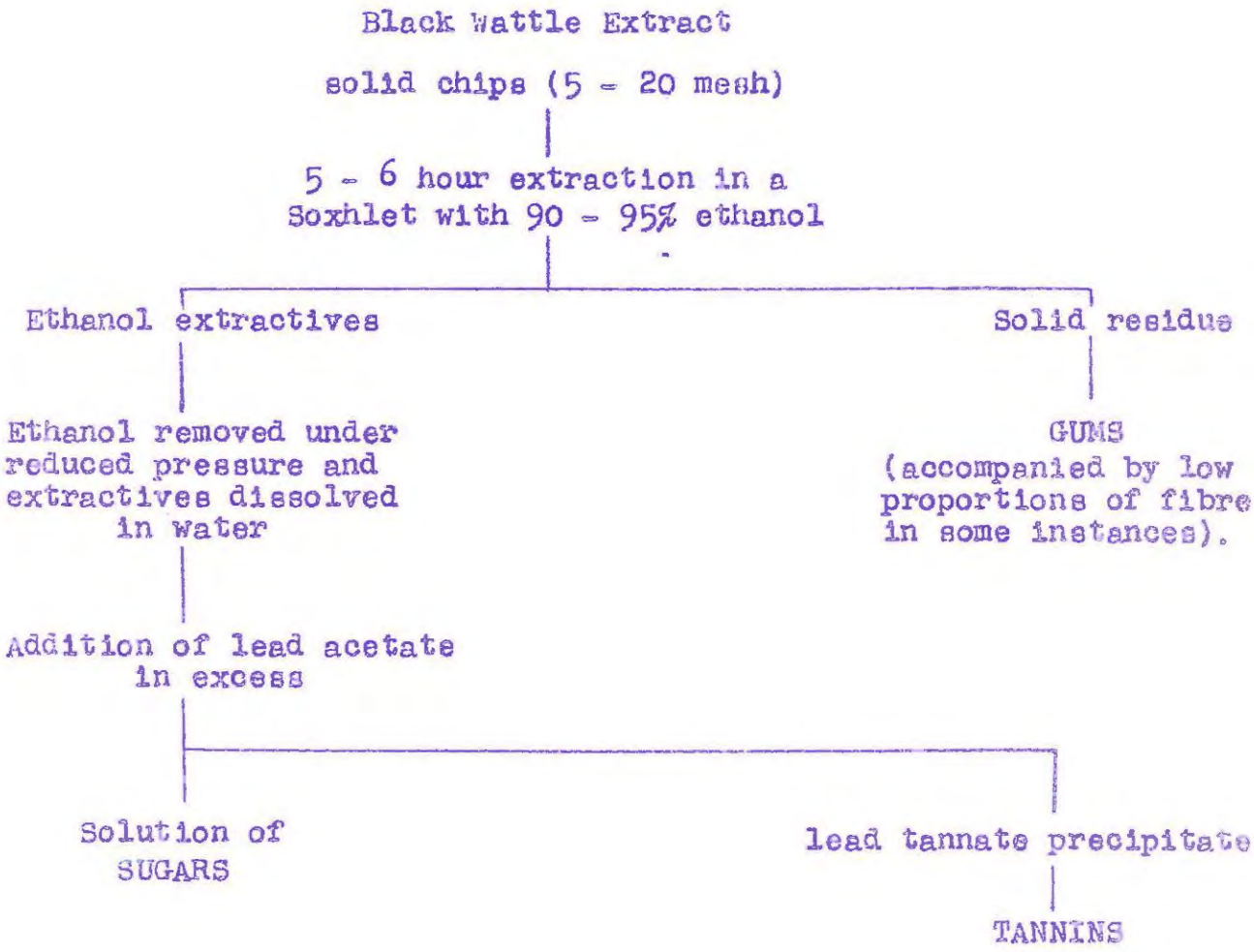
All supernatant solutions from the precipitations were collected and the alcohol removed under diminished pressure with the exclusion of oxygen. The tannin was precipitated by the addition of a large excess of lead acetate, and the lead tannate removed by centrifugation.

An aliquot of the supernatant solution was evaporated to dryness under reduced pressure and repeatedly extracted with boiling methanol. The residue after removal of the methanol contained traces of phenols ($FeCl_3$ reaction and one-dimensional paper chromatogram). These were removed by shaking with pre-chromed hide-powder.

The tannins were liberated from the lead tannate according to the lead-salt purification method discussed later.

Fractionation Scheme II.

An alternative method is to remove the sugars and tannins from the gums which remain as a residue, by the extraction of the commercial extract chips (5 - 20 mesh) with 90% ethanol in a soxhlet (169). After the removal of ethanol from the extractives, the sugars and tannins may be separated by the lead-salt technique :




The solid commercial extract (10 grm) in 5 - 20 mesh particles was continuously extracted in a Soxhlet for 5 - 6 hours with boiling 90% ethanol. This extraction effected the complete removal of both tannins and sugary non-tannins from the gum which remains in the thimble in the same shape as the original chips. Yield of gum = 0.69 grm. or 6.9% of starting-material. The sugars and tannins extracted may be separated as below by the lead-salt precipitation, after removal of ethanol under reduced pressure. This method suffers the disadvantage that the tannins are boiled in ethanol-water (9 : 1) solution for a considerable period thus

possibly contributing to additional condensation reactions. On the other hand it offers a superior and less laborious separation method for the gum when compared with Scheme I.

## 3. THE SEPARATION OF SUGARS FROM BLACK WATTLE TANNINS

Four possible methods were investigated in connection with the above separation, although it was realised at the outset that not all of these give a complete recovery of tannins. Some methods, for example, are known to cause the fractionation of the polyphenols, and have actually been used for this purpose recently. The compositions of the products and residues in each instance were assessed by the hide-powder method of tannin analysis.

### (a) Criteria of Purity

For assessing the degree of purity of tannin samples, the Society specifications (174) for the hide powder method were adhered to rigidly. Since the method is empirical its efficiency when dealing with extracts of abnormally high purity calls for thorough investigation.

The purified tannin samples prepared by the most effective methods discussed in this paper, contained less than 5% non-tans by the official method. In such estimations the non-tan solutions obtained in the usual way were colourless, gave no precipitate with the gelatin-salt reagent, but a strong blue colour with ferric alum solution. On concentration in the course of the estimation,

the solution (50 ml.) slowly assumed a light yellow-brown colour leaving 5 - 10 mgm. of brownish residue. This redissolved readily. When diluted to 5 ml. it gave a heavy precipitate, and to 50 ml. (the original volume) a mere turbidity with the gelatin-salt reagent. Reaction with ferric alum still produced a blue colour in both solutions. When re-shaken with hide powder the non-tan solution loses its brown colour, does not darken on concentration, and so causes a 40% reduction in weight of non-tans.

When estimating tannins in products of high purity the escape of a small but vital amount of phenolic hydroxy compound occurs. Page (175)(176) shows that water is capable of causing slight but continuous hydrolysis of tannin from leather. The tannin estimation is therefore never completely quantitative. In the estimation, where a standard proportion of water is used, an equilibrium between the tannin and the protein of the hide powder is set up. Even if the escaping units include a proportion of small polyhydroxy compounds which do not tan, they probably bear a simple structural relationship with the bulk of the tannin, since they apparently polymerise with ease, and react with hide-powder on concentration.

Values of 95.5 - 96.0% by the official method are thus at least equivalent to 97 - 98% purity. Nevertheless, the results recorded here are those obtained by the official method.

(b) Preparation of Material

For the separation of commercial tannins and non-tannins

the aqueous solution which resulted from Scheme I after the removal of gums, was used.

Cold methanol-extracts of fresh bark, which were also investigated, were prepared as follows. The bark was stripped of all oxidised and discoloured areas, cut into fine slices with a stainless steel knife, and immediately dropped into methanol. This solvent causes virtually complete leaching of tannins and sugars from the bark by repeated extraction, giving a light amber-coloured solution. Gums remain behind. The methanol-tannin solution was rapidly filtered, then concentrated and taken to dryness under vacuum. The product was light-pink, containing about 80% tannins.

Methanol was used as solvent wherever possible, as wattle tannins are known to darken as the result of atmospheric oxidation when dissolved in water. In organic solvents, particularly methanol, this effect is minimised.

(c) Methods of Separating Tannins and Carbohydrate Non-tannins

The four methods discussed, arranged in order of efficiency are :

1. Lead Salt Method
2. Hide Powder Method
3. Salting-out Method
4. Mixed Solvent Methods

Of these the first is the most effective, while the last gives only partial separation.

(1) Method A. The Lead Salt Technique

Arata (177) and Russel (136) claimed to have carried out purification of tannins by formation of a lead salt, but gave no details. The method was also tried by Temple (178), using hydrogen sulphide to liberate the tannin from its lead derivative. He was however unable to free the tannin solution from the lead sulphide formed. The author attempted the latter procedure but found only small quantities of tannin liberated by hydrogen sulphide treatment. In addition, in the presence of tannin the lead sulphide failed to centrifuge or filter off satisfactorily.

(i) Experimental

10 gm. methanol fresh-bark extracted tannins of 80% tannin content was dissolved in 500 ml. distilled water. The tannin solution was vigorously stirred and a strong solution of neutral lead acetate (10%) slowly added in excess. A clean white precipitate of lead tannate formed instantaneously. The lead tannate was centrifuged down and washed four or five times with small quantities of water to remove non-tans and excess lead acetate. The supernatant solution and washings were kept for a non-tannin investigation. Due to the semi-colloidal nature of the product, the lead tannate was carefully macerated with the water on each occasion to ensure efficient washing. Under the above conditions the lead tannate appeared quite stable and showed no darkening in colour. The final product was suspended in water, and dilute sulphuric acid slowly added. The pH decreased from 4.8 to 2.0, at

about which point the aqueous solution assumed a light tannin-like colour, and lead sulphate of low solubility, precipitated. The precipitates were easily centrifuged or filtered off. There now remained a tannin solution contaminated by small quantities of free sulphuric acid, and traces of salts. With very vigorous stirring and careful addition of dilute N/5 NaOH the pH was raised to about 4.0; well below the natural pH of black wattle extract (4.6 - 5.0). At this pH all the $H_2SO_4$ should have been neutralised, and the solutions were taken to complete dryness under reduced pressure. The product was repeatedly extracted with hot absolute methanol, ethanol or acetone, which left a residue of the salts. The latter was separated off by centrifuging or filtering under anhydrous conditions, and the filtrate concentrated under reduced pressure.

Analysis : Tannins = 94.51% and 94.85%

Non-tannins = 5.49% and 5.15%

Repetition of Process :

The fractionation was repeated on a larger scale yielding 25 - 30 grms. purified tannin.

Analysis : Tannins = 94.36% and 93.71%

Non-tannins = 5.64% and 6.29%

The larger amounts of material apparently only slightly decreased the efficiency of washing.

The above product was reprecipitated with lead acetate and the purification process repeated.

Analysis : Tannins = 95.05% and 94.82%

Non-tannins = 4.95% and 5.18%

Duplication of the method does not effect much improvement in "purity" as determined by the official method.

(ii) Discussion

Purification via the lead salt affords an excellent method of obtaining quantitative separation of pure tannins, provided the use of a large high-speed centrifuge is available. The lead tannate is fairly stable and does not oxidise easily, and oxidation may be avoided by concentrating the aqueous solution under reduced pressure in an inert atmosphere. The resulting purified tannins are thus very light in colour.

The chief advantage is that the yield is almost quantitative, so that the product should contain both the small and large molecular units. No fractionation is thus obtained as with Methods B, C and D. The supernatant solutions and washings could be concentrated for the examination of the carbohydrate non-tannins.

Care must be exercised in the final stage, when neutralising the slight excess of acid present. Vigorous stirring should accompany the addition of the dilute alkali, or else the more astringent phenolic groups tend to form a small amount of their sodium derivative. The recovery of tannins from commercial extracts of known analysis was 88%, without accounting for manipulative losses.

(2) Method B. The Hide Powder Technique.

(i) Experimental

75 grms. hide powder was lightly chromed and then washed with distilled water as in the Official Method of Tannin Analysis.

The powder was suspended in 800 ml. water and vigorously stirred. 10 grms. of the methanol-extracted tannin in 200 ml. water was rapidly added, and the stirring continued for 10 minutes. The partially-tanned hide powder was dried by squeezing in a linen cloth and again suspended with agitation in 800 ml. distilled water to wash away non-tans. These washings lasting 15 minutes each, were repeated 4 times with successive quantities of distilled water. The original solution which was detanned by the hide-powder was reserved for investigating the carbohydrate non-tannins.

After completing the washing, the tanned powder was squeezed to dryness in a linen cloth, and suspended in a mixture of 300 ml. acetone and 300 ml. water. The solution immediately assumed a light tannin colour, but was left standing overnight. The aqueous acetone solution was filtered off and concentrated under reduced pressure. On removal of the acetone which vaporises first, the aqueous tannin solution remaining became colloidal in appearance. Small amounts of hide powder, apparently soluble in the acetone-water mixture, but insoluble in water only, settled on the glass wall. These colloidal impurities were removed by shaking up with kaolin and filtering through a No. 11 Whatman filter paper. The concentration of the clarified tannin solution was now continued, and it remained clear to dryness. Two more extractions of the same hide powder with 600 ml. lots of a 50% acetone-water mixture followed.

TABLE VIII

| | Time of Soaking (hrs.) | Yield (grms.) | % Yield |
|---|---|---|---|
| 1st Extraction | 15 | 2.80 | 28 |
| 2nd Extraction | 8 | 2.10 | 21 |
| 3rd Extraction | 12 | 1.23 | 12.3 |
| | | Total | 61.3 |

A total of 61.3% tannin is thus removed by three consecutive extractions, leaving about 15.-20% tannin combined with the hide powder. This falls in line with the findings of Innes (179) who observed the stripping of leather by acetone and other solvents. Using 70% acetone he found that 6 - 20% tannin remained in leather on stripping.

The product from the first extraction was very light in colour and gave, on analysis :

Tannins = 95.62%

Non-tannins = 4.38%

Treatment of the above non-tans with fresh hide powder, and making allowance for dilution, reduced the value to :

Non-tannins = 2.90%

(ii) Discussion of Method

This furnishes an easy, direct method of separating the non-tannins from the tannins without oxidising the latter.

Innes (179) found that when stripping leather with acetone more tannin was removed from the middle than from the grain layer.

In the above estimations also it was observed that the first stripping yielded the lightest tannin, and that subsequent strippings with acetone tended to yield darker products. It thus seems probable that the residual tannin retained in the hide powder tends to be the more astringent higher molecular weight fraction.

Separation by this method, therefore, tends to remove the highest molecular tannin fraction from the smaller tannin units. Should the astringent fraction retained be a separate chemical entity, this method would not be entirely satisfactory.

According to Innes the stripped hide, still containing 6 - 20% tannin is not putrescible, and the hide powder once tanned in the above process may be used repeatedly. Successive usage of the same hide powder should furnish higher yields of tannin on stripping and this should partly overcome the above difficulty of partial fractionation.

Suitable anionic resins may be more effective than hide-powder for this purpose, but these have not yet been tried, although used by Morris et Al (197) for the purification of morin.

(3) Method C. The Salting-Out Method.

As shown by Page (180), and applied by Russel (136), Stephen (149) and Nunn (181), the salting out of a dilute tannin solution with sodium chloride, brings about a fair amount of "purification" of the tannins. Combined with acetone extraction it increased the tannin content from 74% to 86%, when using commercial "Mimosa" extract. These investigators only applied the method once.

Owing to the gummy nature of the salted-out tannins, it was felt that a fair amount of occlusion of non-tans took place, and that repetition of the method would yield better results.

(i) Experimental

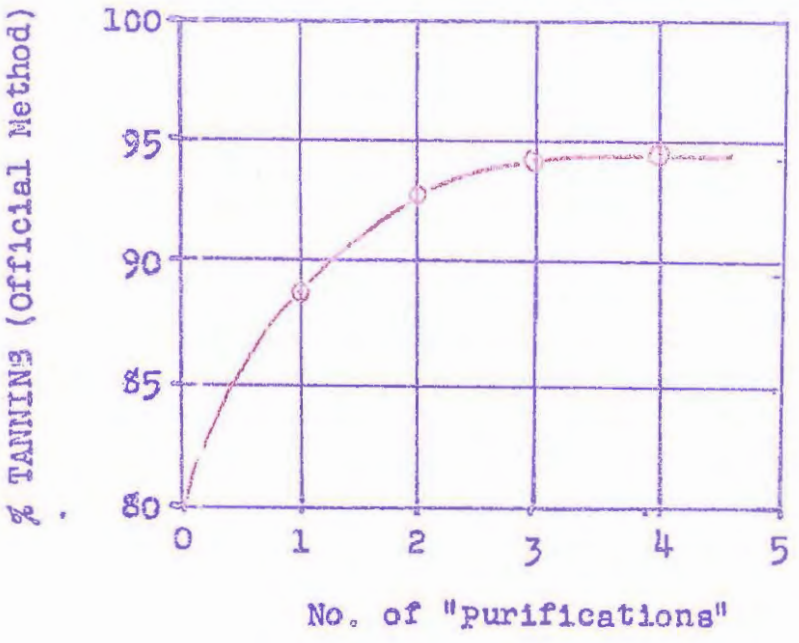
Fresh bark was cut into thin slices and extracted with distilled water in the cold for 18 hours. The 20 - 30 Bk. solution obtained after filtering, was lighter than a commercial extract of equal strength, and gave a light-coloured solid, containing 80 - 81% tannin, on concentration. The tannins separated out as a gummy mass on the slow addition of solid salt to the vigorously stirred solution. This was dried in vacuum, extracted with acetone, and analysed. Using this product the process was repeated four times, when no further increase in tannin content was found (Table IX and Fig. III).

Due probably to occlusion of non-tans, one "purification" does not give the same degree of separation as the two previous methods.

TABLE IX

| | % Tans | % Non-tans |
|---|---|---|
| Fresh Bark Extract | 80.19 | 19.81 |
| 1st "Purification" | 88.44<br>89.20 | 11.56<br>10.80 |
| 2nd "Purification" | 92.70 | 7.30 |
| 3rd "Purification" | 94.10 | 5.90 |
| 4th "Purification" | 94.22<br>94.06 | 5.78<br>5.94 |

Fig. III.




The method thus reaches its maximum effectiveness after three applications.

(ii) Discussion

When coupled with further solvent separations (see later) the above method gives a product of 95.2% tannin content.

Chief disadvantage is the atmospheric oxidation which can only be excluded with the greatest difficulty. The salted-out product is always much darker than that obtained by any other method discussed. In addition no sharp separation of non-tannins occurs.

As it is known that black wattle tannin consists of a gradation of molecular sizes (180), fractional separation of the tannin according to molecular size is inclined to occur, and only

partial recovery of the tannin fraction is obtained. A part of the total tannin mixture of different chemical constitution might be discarded in this way.

This possibility, together with the oxidation caused, renders the method less suitable than either of the previous ones. The salting-out technique on the other hand, might be useful for fractionating the tannins.

(4) Method D. Mixed Solvent Separations.

(i) Previous Applications of Solvent Methods.

Purifications of tannins by usage of solvents have been attempted on numerous occasions.

Moeller (171) found that when tannins were extracted with water the extracts contained a much larger proportion of non-tans than when unspecified organic solvents were used. Acetone was used by Russel (136), Stephen (149) and Nunn (181), chiefly for the removal of contaminating salt from black wattle tannins. Williams (157), as previously mentioned, claimed erroneously high purities when using acetone, ethyl acetate and amyl acetate successively. Also in a recent Journal of the American Leather Chemists' Association (182) mention is made of Quebracho tannin of 93% purity obtained through "mixed solvents".

(ii) Solubility of Black Wattle Tannins

Since anhydrous media were used as much as possible, the conditions of solution of black wattle tannins were examined.

Under strictly anhydrous conditions only members of the lower alcohols such as methanol and ethanol are capable of effecting solution. This limited solubility of "Mimosa" tannin in anhydrous pure solvents, was also recorded by Chesire (183).

As propounded by Shuttleworth and Cunningham (14) in their discussion on stripping of tannins from leather, both these solvents and water, in addition to being of small molecular size, exhibit strong hydrogen-bonding ability. Dry tannins are probably very strongly hydrogen-bonded intermolecularly, and the units possess a variety of functional groups, some of which are predominantly proton donating and others chiefly proton accepting. Solvents capable of opening up these intermolecular bonds and also preventing their reassociation, will cause solution. Water is unique in that it can function as donator and acceptor simultaneously, but permits reassociation because water molecules can act as bridges. The lower alcohols such as methanol and ethanol, although more proton accepting in nature than water, are still closely related to water as regards size and function. Due to the presence of the aliphatic radicle there is less tendency to form bridges, and their solvent power relative to water is thus enhanced.

Other organic solvents such as ketones, esters and higher alcohols which are known to play a part in hydrogen bonding, do not cause solution of the dry tannins, probably because of their more exclusive anionoid (proton accepting) character.

Methanol and ethanol were thus employed for dissolving the dry tannins in this mixed solvent method.

Where 6 - 10% of moisture is associated with the tannin, other solvents may be employed to obtain solution e.g. amyl acetate (commercial), acetone (commercial purified and redistilled), and dioxane (commercial). The presence of moisture appears to supply the cationoid character required, thus enhancing the dissolving powers of these solvents.

(iii) Separations using a Single Solvent

The dried non-tannins from an analysis of the 80% methanol-extracted product were tested with various solvents, and the solubility of the non-tans compared with that of fresh bark tannin extract. (See Table X).

TABLE X.

| Solvent | Tannins | Non-tannins |
|---|---|---|
| Ethyl ether (pure) | Insoluble | Insoluble |
| Chloroform (pure) | " | " |
| Carbon tetrachloride (tech.) | " | " |
| Methyl ethyl ketone (pure) | " | " |
| Butyl alcohol (tech.) | " | " |
| Ethyl acetate (pure) | V. sparingly sol. | " |
| Dioxane (tech.) | " " " | V. sparingly sol. |
| Amyl acetate (tech.) | Sparingly sol. | Sparingly sol. |
| Ethyl acetate (tech.) | Soluble | V. sparingly sol. |
| Ethanol (absolute) | " | Moderately sol. |
| Acetone (tech.) | " | " " |
| Methanol (pure) | Easily sol. | Easily sol. |

The above terms are relative and based on observation only. No quantitative estimations were carried out, and a host of higher molecular weight solvents giving no solution to either, are not listed.

From the above, commercial ethyl acetate, and to a lesser degree ethanol and acetone, seem to be the only ones capable of preferentially dissolving the tannin fraction.

Accordingly 10 grms. of dry powdered tannins (cold methanol extract) was rapidly shaken with 200 ml. re-distilled commercial ethyl acetate. After about 75% of the tannins had dissolved, the ethyl acetate solution was rapidly filtered, the filtrate concentrated under reduced pressure, and the solid product analysed :

Tannin = 90.13% and 89.48%

Non-tannins = 9.87% and 10.52%

This procedure was repeated twice again on the above product :

Tannin = 91.03% and 89.87%

Non-tannins = 8.97% and 10.13%

Three such solvent extractions were also repeated on a different sample :

Tannin = 89.63% and 89.81%

Non-tannins = 10.37% and 10.19%

The residues from these extractions (about 40% of the starting material), gave on analysis :

Tannin = 79.08%

Non-tannins = 20.92%

One extraction effects the optimum separation, and a

value of close on 90% seems the maximum obtainable. The method was thus abandoned and a mixed solvent separation attempted.

Locally available commercial ethyl acetate contains a low percentage of ethanol. Since the boiling-points of pure ethanol and pure ethyl acetate differ by 1°C, distillation of the commercial sample gives little fractionation. The presence of ethanol is probably largely responsible for the ease with which dry commercial ethyl acetate dissolves dry tannin.

(iv) Purification using Mixed Solvents.

As previously discussed, the method involves the solution of dry tannins in anhydrous methanol or ethanol. To these were added purified ether or pure ethyl acetate in which the tannins are very sparingly soluble or insoluble. A flocculant precipitate rich in non-tans results which is removed by filtration, and yields a filtrate richer in tannins.

In this investigation two combinations, methanol-ether and ethanol-ethyl acetate, were used. They are similar in principle and similar results were recorded.

(a) Methanol-Ethyl Ether Method

The fresh-bark extracted tannin (13 grms.) was dissolved in 50 ml. pure methanol. On the addition of dry ether, a white flocculant precipitate formed which settled slowly. This was filtered but after initial rapid filtration, the precipitate appeared to assume a gummy nature, and the rate of percolation

slowed down completely. The moist precipitate now appeared to drip through into the filtrate. This was also observed by Stephen (149) as a result of which he abandoned the method.

It was surmised that the above phenomenon is due to the condensation of atmospheric water vapour on the tannin, caused by the rapid vaporisation of the volatile ether from the filter-paper. The receiving flask, funnel and filter-paper were accordingly placed in a desiccator (calcium chloride) and allowed to stand for 10 minutes. The solution with partially settled precipitate was rapidly poured in through a second funnel in the lid of the desiccator. The ethereal-methanol solution ran through rapidly at an undiminished rate, leaving a white gelatinous precipitate (1.5 grms) on the filter-paper.

This was analysed :

Tannins = 64.75%

Non-tannins = 32.25%

The filtrate was concentrated under reduced pressure in the usual way. Analysis :

Tannins = 90.29% and 90.24%

Non-tannins = 9.71% and 9.76%

In this precipitation, the particles formed originally appeared finely dispersed, but they soon joined together to form a flock. After continued addition of ether, a stage is reached when no more precipitate is formed, and only milkiness results. At this stage the process was usually discontinued.

Addition of Excess Ethyl Ether

The above ether-purified material (90.3% tannin) was redissolved in a minimum methanol to give a very concentrated solution. On addition of dry ether, only a milky suspension formed, which rapidly settled into a gum on the walls of the corked container. After most of the milkiness had disappeared on standing, the solution was poured off and filtered. A clear filtrate resulted, yielding a light tannin on concentration. Analysis :

Tannin = 91.23%

Non-tannins = 8.77%

The gummy precipitate on the walls of the flask was washed once with ether and the solvents removed under reduced pressure. Analysis :

Tannin = 91.18% and 90.08%

Non-tannins = 8.82% and 9.92%

This shows that once milkiness forms on addition of excess ether, the tannin precipitates together with a constant proportion of non-tans, and no further fractionation results.

(b) Ethanol-Ethyl Acetate Method.

The exact principles applied in the methanol-ether method were repeated here. Behaviour of the solutions and the tannin was exactly the same, with the exception that after obtaining the usual precipitate, no milkiness was observed on the addition of excess pure ethyl acetate to the ethanol-tannin solution.

Results :

Precipitate

Tannins = 59.42%

Non-tannins = 40.58%

Filtrate

Tannins = 90.48%

Non-tannins = 9.52%

Tannin which had previously been thrice purified by commercial ethyl acetate (89.8%), still gave a precipitate by the above method. Analysis :

Tannin = 91.89%

Non-tannins = 8.11%

It was found that when a very concentrated solution of tannin in ethanol was made, the addition of pure ethyl acetate still caused a small amount of precipitation. The above tannin (91.89%) was thus treated and the filtrate gave :

Tannin = 91.38% and 91.19%

Non-tannins = 8.62% and 8.81%

(v) Discussion of Solvent Separations

The mixed solvent method appears very slightly superior to the use of one solvent only, but both are still unsatisfactory.

The precipitate formed contains a high proportion of non-tannins initially, but the ratio probably decreases to reach a constant value on repetition of the process. Some evidence for this trend is obtained from the treatment of green wattle tannin (Acacia decurrens Willd.) by the ethanol-ethyl acetate method :

Precipitate from 1st Purification (0.96 grms.)

Tannin = 57.59%

Non-tannins = 42.41%

Precipitates from 2nd, 3rd and 4th Purifications combined (0.65 grms.)

Tannin = 67.27%

Non-tannins = 33.73%

The establishment of a constant ratio of tannins to non-tannins in the precipitate could thus be responsible for the limitations of the method.

In spite of exhaustive tests and successive precipitations, the solvent method was found at its best to give only partial separation of black or green wattle tannins and non-tannins. During fractionations a proportion of the tannins is removed with the non-tannins in the precipitate, and these methods could thus also be used for fractionating the tannins. As water is excluded all fractions may be concentrated with the minimum amount of oxidation.

Theoretically, solvent purification may be explained on the hydrogen-bond concept. In the impure tannins the hydroxy unit as a whole is far stronger proton donating than accepting, although both types of groups are possibly present. The non-tans e.g. sugars, pentose etc., on the other hand are predominantly proton accepting (anionoid) in character. Both are soluble in solvents which can act as donors and acceptors simultaneously e.g. the lower alcohols. By the addition of a large excess of solvents such as ether and ethyl acetate, which are exclusively anionoid in

character, most of the non-tannins are precipitated since they themselves are anionoid in character. The tannins are chiefly retained as they can still hydrogen bond to the second solvent added, whilst their proton-accepting groups are satisfied internally or by the ethanol or methanol present. For the same reason a small proportion of the proton-accepting non-tans are still retained in the solution when ethanol or methanol is present. This explains why no single hydrogen-bonding solvent appears capable of separating tans and non-tans. The commercial ethyl acetate used as such is a mixture of ethanol and ethyl acetate, and its function is precisely the same as the mixed solvent method discussed.

(d) Combination of Separation Methods

In an effort to effect the even more complete separation of non-tannins from the tannins, combinations of the above methods were applied : the results are summarised in Table XI.

The 4.91% non-tans from one of the above determinations was reshaken with hide powder, causing a 40% reduction in weight, and reducing this value to 2.93%

(e) Combustion Analyses on Tannins freed from Carbohydrate Non-tannins.

Combustion analyses on black wattle tannins have been performed by Russel (136), Nunn (181) and Stephen (149). All three used the same method of "purification", e.g. of salting out once, extracting the sludge with acetone, and washing the tannins

TABLE XI

| Method Applied | Sample already purified by | Purity % Before Treatment | Purity % After Treatment | Colour of Product |
|---|---|---|---|---|
| Hide Powder | Lead Acetate | 95.05<br>94.82 | 95.73<br>95.09 | Light |
| Lead Acetate | Salting-out solvents | 95.20 | 97.28<br>96.92 | Dark |
| Mixed Solvents Ethyl Acetate | Salting-out | 93.70 | 95.21<br>95.01 | Dark |
| Mixed Solvents Methanol-ether | Salting-out | 93.70 | 95.20<br>95.22 | Dark |

with ether after removal of acetone. Stephen claimed 86% tannin content by this method. The "purification" was repeated by the writer on commercial extract, and the dark brown product analysed. A comparison of results obtained is given in Table XII.

TABLE XII

| Investigator | % C | % H | Drying time at 110°C in vacuo. |
|---|---|---|---|
| Russel | 60 - 62 | 4.5 - 4.9 | 24 hours |
| Nunn | 58.6 - 59.3 | 4.7 - 4.9 | 24 hours |
| Stephen | 57.4 | 4.94 | 24 hours |
| Roux | 57.75 | 4.54 | 2 hours |
| Roux | 57.61 | 4.66 | 4 hours |
| Roux | 57.48 | 4.54 | 9 hours |

Russel's results are high compared with other investigators. By various corrections of doubtful validity, Stephen also increased his figures to those given by Russel.

Under the above conditions two hours' drying appears to be quite sufficient for black wattle tannin. The dark nature of the product suggests that a fair amount of oxidation had already taken place.

Comparison of fresh-bark extracted tannin before and after separation ;

(a) A typical analysis of methanol-extracted fresh-bark tannins is the following :

C = 58.15% and H = 4.92%

C = 57.89% and H = 4.55%

This product was very light in colour, contained 80% tannin, and was dried for 2 hours in an Abderhalden gun at 110°C. The ash was negligible.

(b) "Purified" Samples. The analyses of various tannin preparations separated by different methods are shown in Table XIII.

TABLE XIII

| Tannin Content of Sample (Official Method) | How Separated | Colour | % Ash | % C | % H |
|---|---|---|---|---|---|
| 95.6 | Hide Powder once | Very Light | 0.0 | 61.02 | 4.83 |
| 95.2 | Salting-out thrice and mixed solvents once | Brown | 0.3 | 60.90<br>61.04<br>60.90 | 4.93<br>4.94<br>4.85 |
| 95.7 | Lead Acetate once and Hide Powder once | Light Brown | 0.5 | 60.11 | 4.80 |
| 96.1 | Salting-out thrice and Lead Acetate once. | Dark Brown | 0.5 | 60.59<br>60.73 | 5.08<br>4.96 |
| 95.0 | Lead Acetate twice | Light | 1.3 | 60.57<br>60.84<br>60.50 | 4.76<br>4.78<br>4.27 |

In all the above combustions the product was dried at 110°C. for 2 hours under vacuum, and redried again just before combustion for 30 minutes. The carbon and hydrogen values were corrected for ash only when present.

The presence of more than a trace of ash seems to cause slightly low combustion figures, even though corrections were applied. A trace of carbon is retained and is actually visible in the boat after combustion of the lead acetate purified samples. Taking this into account the method of preparation seems to have little effect on combustion figures. The very astringent and also the low molecular-weight units discarded in some preparations, are therefore possibly closely related in chemical character to the bulk of the tannins.

Tannin of 95 - 96% tannin content as judged by the Official Method thus contains :

C = 60.90 - 61.04%

H = 4.83 - 4.94%

taking only the analyses which contain the least ash.

When compared with incompletely separated material previously used, the carbon values have increased by 5%. This is to be expected as the non-tans have been found to consist of sugars, and gums which have carbon values of 40 - 45% and the combustion of a rich non-tan fraction (74% non-tans) by Stephen (149) gave C = 47.6% and H = 6.07.

(f) Discussion

In the past constitutional research on black wattle

tannins was carried out on samples still containing relatively large admixtures of sugary non-tannins, and also representing only a proportion of the polyphenolic extract. Various methods have now been investigated which yield tannins from which the carbohydrate non-tannins have been almost completely eliminated.

Of these the lead-salt method permits the almost quantitative separation of tannins and non-tannins. The hide powder method gives a clear separation of non-tannins but the most astringent tannins are unfortunately irreversibly fixed on the hide-powder and cannot be recovered. Repeated use of the same hide-powder should to a large extent eliminate this difficulty. The salting-out and mixed-solvent methods are less effective but could be used for fractionating the tannin when investigating its homogeneity.

The elimination of carbohydrate non-tannins from the tannin is reflected in the elementary analysis of "purified" tannin samples when compared with those in which a known admixture of non-tannins is still present. Analyses of tannins separated by various methods show little variation.

These separation schemes for the extract as a whole, should also be applicable to other vegetable extracts. They will be used for determining the effect of various non-tannins on the commercial tanning process as recently carried out by Kuntzel and Zissel (193) for oak bark tannin, and also for determining the influence of extraction conditions on the non-tannins, the polyphenols, and ultimately on the resultant leather. Haglund (194) has shown that spruce bark tannin also contains an alcohol-insoluble

portion of high viscosity which is removed last (195) during the fractional extraction of the bark. The commercial advantages of cold-leached bark extracts, compared with commercial "mimosa" extract thus also requires reinvestigation.

(g) Summary

a) In the past all constitutional research on black wattle was carried out on samples contaminated by non-tans, and representing only a portion of the polyphenolic extract.

b) Two schemes have been devised for the first time for fractionating the extract into chemically dissimilar groups of compounds e.g. gums, sugars and polyphenolic tannins.

c) Various techniques of separating the carbohydrate non-tannins from the polyphenolic tannins have been investigated. One of these, the lead-salt method, appears satisfactory while the others may be used for fractionating the tannin.

d) Two methods are available for separating the gums from the rest of the extract.

e) These fractionation and "purification" methods in addition to providing an interesting basis for the fractionation of the extract and the tannins, also furnish methods for determining the effects of the presence of the groups of non-tannins on the tannins during the practical tanning process. These schemes may also be effective for other vegetable extracts.

# CHAPTER V.

## PAPER CHROMATOGRAPHY OF BLACK WATTLE TANNIN

### A. INTRODUCTION

The previously-described fractionation methods will separate the extract qualitatively or almost quantitatively into sugars, gums and tannins. The homogeneity of each must be determined in order to establish what significance may be attached to degradation products or analytical figures obtained from any one of these fractions. The main interest in this study lies with the polyphenolic or true tannin fraction which, due to its amorphous nature and reputed high molecular weight, presents a complex problem.

The concept of purity or homogeneity when dealing with highly-condensed macromolecules must naturally be regarded in its widest sense. The investigation of what is obviously an amorphous heterogeneous mixture of compounds is also complicated by their similarity in chemical and physical properties, and the additional lack of criteria of purity. For this reason little headway has been made with the chemistry of the condensed tannins in the past.

Newer methods which may be suitable are :

(i) Solubility methods.

(ii) Adsorption chromatography.

(iii) Paper partition chromatography.

(iv) Partition chromatography on columns, and

(v) Countercurrent distribution methods.

The solubility methods of Thorp (129) and Kunitz and Northrop (127)(128)(184) have already been applied by White (126) to quebracho extract. A discussion on this given (see page 69) in Chapter III in connection with quebracho extract, and its application to black wattle tannins was considered unsuitable for the same reasons.

One-dimensional paper chromatography was first used by White (126) in connection with the above solubility study on quebracho extract, and this has been followed by a number of publications which will be discussed below.

Kirby, Knowles and White (168) have also very recently (1952) used the counter-current distribution method to a very limited degree in conjunction with chromatography for showing the complexity of "mimosa" extract.

In this investigation one- and two-dimensional chromatography as well as ordinary chemical methods were used for investigating the complex polyphenols of the tannin fraction.

## B. ONE-DIMENSIONAL PAPER CHROMATOGRAPHY

### (i) A Review of the Application of One-Dimensional Paper Chromatography to Polyphenols and Plant Extracts.

In addition to the above-mentioned work of White on quebracho, Bate-Smith (185) also demonstrated the usefulness of paper chromatography for the identification of polyphenolic bodies in plant extracts. Bate-Smith and Westall (186) used a variety of $C_6$ and naturally-occuring $C_{15}$ phenolic compounds in order to

study the relation between chromatographic behaviour and chemical structure. Under carefully controlled conditions, RF values accurate and reproducible to ± 0.02 could be obtained using butanol-acetic acid-water (4 : 1 : 5) and m-cresol-acetic acid-water (50 : 2 : 48) solvent mixtures. Apart from expected departures due to well-known anomalous effects e.g. the "ortho-effect", the RF values were found to be related to the nature and number of substituent groups in $C_{15}$ and $C_6$ skeletons in such a way that in many instances a straight line was obtained when log $\left(\frac{1}{RF} - 1\right)$ was plotted against the number of substituent groups of any one kind (e.g. hydroxyl groups). Such a relationship between constitution and chemical structure also exists in amino-acids (188) and the symbol $R_M$ has been suggested for the above function.

Bradfield and Bate-Smith (187) similarly examined catechins, gallocatechins and gallocatechingallate esters from green tea leaves. It was established that the introduction of a third hydroxyl group in a vicinal position in a benzene ring to ortho-hydroxy groups already present, diminishes the RF value but increases the $R_M$ value. The numerical value of $\Delta R_M$ was, however, dependent on the constitution of various molecules as a whole.

Bate-Smith (189) used the same solvent mixtures for examining the behaviour of flavanones, chalcones, flavanols, flavones, flavonols and flavylium salts. He found that the RF values obtained show the following regularities.

(a) The above classes of $C_{15}$ compounds, having the same number of hydroxyl groups have approximately the same RF values,

and the RF values are reduced with each additional hydroxyl group.

(b) With the exception of rhamnose, the glycosidic combination of sugars with phenols causes approximately the same fall in RF value as the addition of an hydroxyl group. With rhamnose either a slight rise or fall in RF value is observed.

(c) Methylation of an hydroxyl group causes a rise in RF value, as a rule between one-third and two-thirds of the rise which would result from the loss of the hydroxyl group.

Hillis (190)(191) investigated Eucalypt Kinos and various tannins one-dimensionally using phenol-2 N acetic acid (1 : 1) to which 0.3% NaCl had been added, phenol-2 N acetic acid plus 2 N HCl (1 : 1), and ethanol-benzene-water (40 : 20 : 40) mixtures, as well as the cresol-acetic acid-water mixture of Bate-Smith and Westall, and the butanol-acetic acid-water (4 : 1 : 5) mixture of Partridge (192). Hillis found that all the tannins were complex mixtures but although he obtained good resolution with these solvents he did not identify any of the constituents. As a result of the work he concluded that current theories which suggest that tannins consist mainly of series of related polymers based on a catechin unit were improbable.

Very recently Kirby, Knowles and White (167) have examined a number of tannins one-dimensionally using butanol-acetic acid-water (4 : 1 : 5) and water-saturated sec-butanol. They criticised Hillis' use of m-cresol : acetic acid : water, and phenol : 2 N acetic and hydrochloric acids mixtures for separating polyphenolic tannins. They claimed that their work again illus-

trated the complexity of the extract, and that no single chemical type predominated.

(ii) Preparation of Material

As the lead-salt method was the only one giving a quantitative precipitation of the phenolic fraction, this method was first used for the preparation of "purified" tannin samples from which the gums had previously been removed. The presence of a low proportion of sugars, was later found not to affect the appearance of the chromatograms to any degree, but the removal of gums was considered essential due to their colloidal nature and their marked ability to retain tannins. The tannins were dissolved in methanol (20 - 30% solution) and 5 - 10 µl. spotted on to the paper in small quantities at a time with drying between each, to build up small spots 0,5 cm. in diameter.

(iii) Experimental Conditions

According to Consden, Gordon and Martin (196) and Pate-Smith (185), a number of precautions are essential for the accurate reproduction of RF values. These include

(a) accurate temperature control, as equilibria in ternary mixtures are sensitive to temperature fluctuations,

(b) the thorough equilibration of the solvent mixture at the temperature at which it is to be used,

(c) the exposure of the paper, with spots applied, to the aqueous phase of the solvent-water mixture for 24 hours before the

commencement of irrigation,

(d) the irrigation of the paper continued only to a fixed distance from the starting-line.

(e) variations in the paper and alterations in the solvent mixture (e.g. esterification in alcohol-acid mixtures) controlled by running a control compound of known RF value with each chromatogram.

In the present work (a), (b), and (d) were observed, and when acid-alcohol mixtures were used these were discarded and renewed after 7 days' use. The chromatogram was developed in each instance to a distance 11 inches from the starting-line, and where the paper was continuously irrigated for the identification of sugars (see Chapter X), reference compounds were used. All experiments were run at 22°C. using Whatmans No. 11 paper which is thin and less absorbent than others in current use. The ultra-violet source used was a 125 watt "Osira" lamp with emission in the visible violet.

Downward migration was preferred as this allowed for the continuous irrigation of the paper when necessary. The apparatus used was very simple (Fig. IV). It consisted of a photographic dish A supported on a wooden frame B which was heavily waxed. The whole was covered with a narrow bell-jar C which ensures a small air-space. A flask D containing the same solvent mixture as in A assisted in maintaining a saturated atmosphere in the lower region of the enclosed space. Airtightness was ensured by silicone grease on ground-glass surfaces. The appara-

tus was maintained and operated in a constant-temperature room.

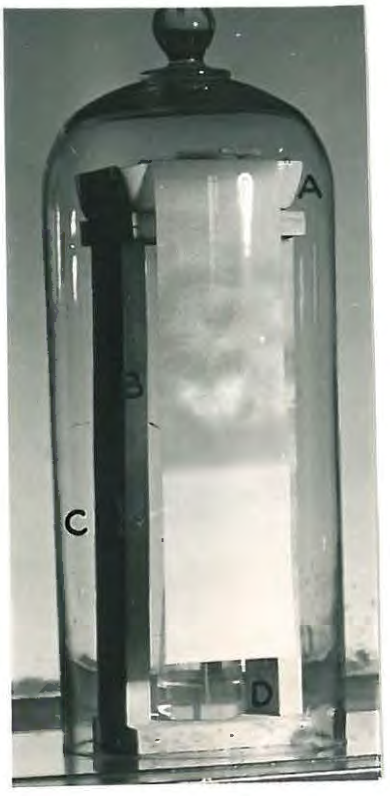



Fig. IV.

Apparatus for One-Dimensional Chromatography

(iv) Appearance of a One-Dimensional Paper Chromatogram of Black Wattle Tanning.

Butanol-acetic acid-water (4 : 1 : 5) was used in a preliminary investigation as Bate-Smith (loc. cit.) had shown that this solvent mixture was excellent for the separation and accurate reproduction of RF values of the polyphenols.

The developed paper chromatogram of fresh-bark and commercial methanol extracts showed the presence of a dull brown relatively uniform streak from the point of application to about RF =

0.45 in ordinary light. The streak was far more pronounced and much darker brown in colour than the fresh-bark extract, due to the oxidation of the former.

Under ultra-violet light the presence of at least four different areas was visible. These were represented by (a) a dull brown streak RF = 0 - 0.47 which appears to constitute the major portion of the extract and represents the condensed tannins. (b) a weak yellow fluorescent spot RF = 0.53 (c) a bright yellowish-green fluorescent spot which was suspected to be fisetin RF = 0.72 and (d) a blue fluorescent spot RF = 0.90 which was intensified with ammonia vapour. (Fig. V).

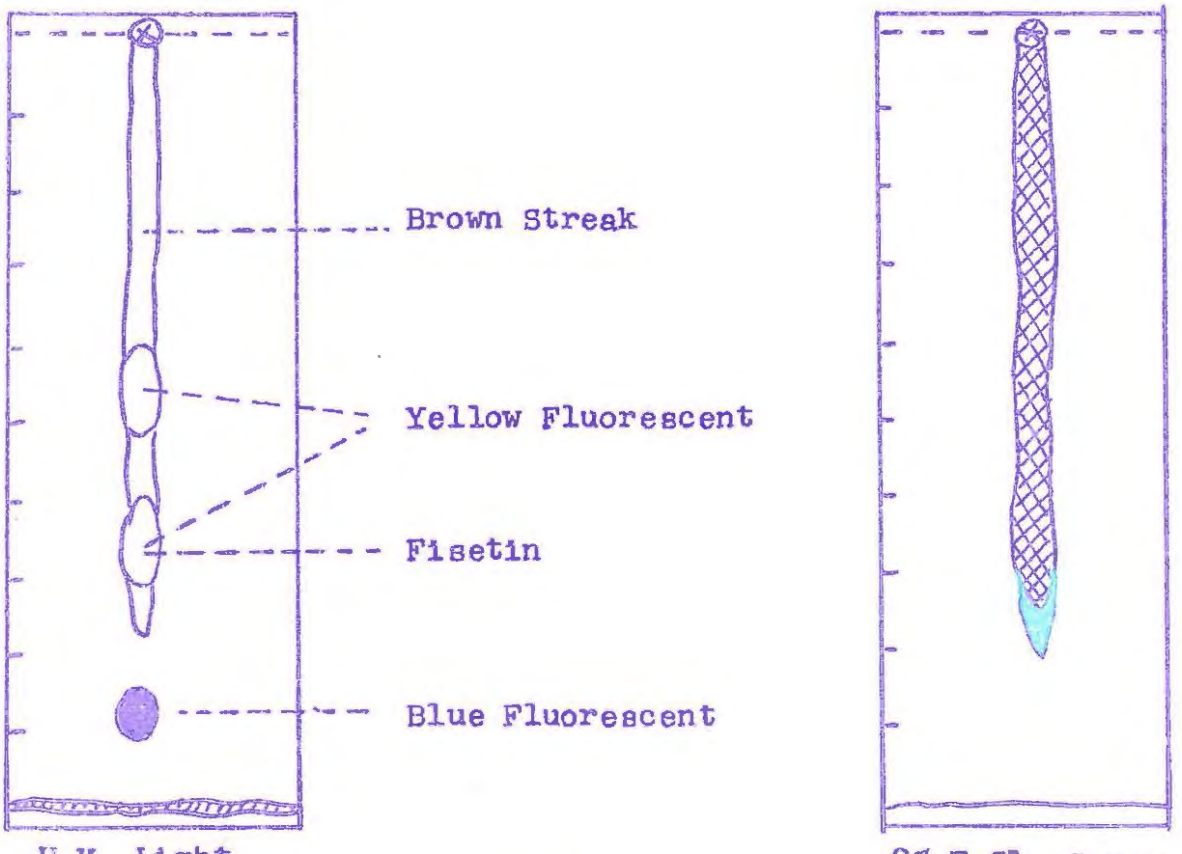



Fig. V.

Chromatogram of Methanol-extracted Commercial Black Wattle Tannin.

From the above it was considered that the bulk of the condensed tannin was accompanied by a low proportion of fluorescent bodies, as the bright fluorescence under ultra-violet light was known to be a very sensitive property of such a compound as fisetin.

(v) Identification of the Associated Fluorescent Polyphenols.

The separation of the compound of RF = 0.72, suspected to be fisetin was attempted by Perkin and Gunnel's method (120) of separating fisetin from quebracho with concentrated sulphuric acid. 9 kg. of black wattle extract was treated by this method.

A one-way chromatogram of the ethereal extract of the resulting solution appeared complex (U.V. light) consisting of (a) a bright yellow fluorescent spot RF = 0.53, (b) a bluish spot (0.68), (c) a second larger and more brilliant yellow fluorescent spot (0.72) followed by a long blue zone, the latter part of which fluoresced blue in ammonia vapour (0.90). (Fig. VI).

Sprayed with Br-cresol-green (201) only the spot RF = 0.68 showed up yellow against the green background, and also exhibited a deep blue colour with 2% $FeCl_3$ spray reagent. It was thus acidic and possibly a pyrogallol derivative. Separated by bicarbonate treatment and recrystallized from water it proved to be GALLIC ACID. MPt. and mixed mpt. 253°C. (decomp.)

Found ; Equiv. wt. = 170 %C = 49.2 %H = 4.0

Calc. for $C_7H_6O_5$ : Equiv. wt. = 170 %C = 49.4 %H = 3.5

Gallic acid does not appear to be present in the extract

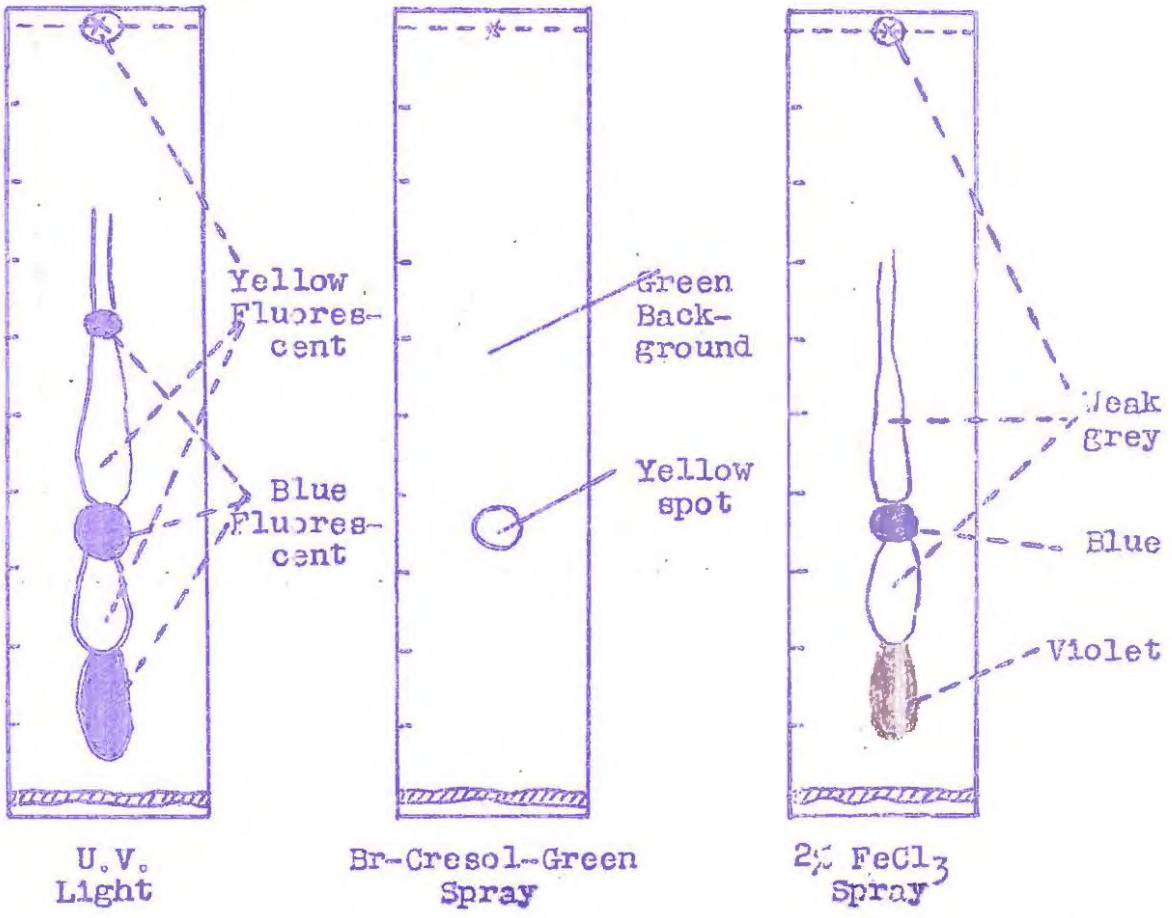



Fig. VI.

Chromatogram of Ethereal Extract of Sulphuric Acid treated commercial black Wattle Extract.

and is probably formed by the decomposition or hydrolysis of some constituent in the commercial extract. Gallic acid was also obtained by the same method from quebracho extract by Perkin and Gunnel (loc. cit).

The phenolic residue was dissolved in water, the o-hydroxy phenols precipitated with neutral PbAc, centrifuged, the lead salts washed and recentrifuged. Ether extraction of the mother liquor

and washings gave the meta-hydroxy phenolics which on vacuum sublimation (100° - 120°C) gave RESORCINOL. Recryst. from benzol. Mpt and mixed mpt 108°C.

Found : %C = 65.06 %H = 5.63

Calc. for $C_6H_6O_2$ : %C = 65.45 %H = 5.45

Purple colour with $FeCl_3$. The residue was examined by chromatography. In addition to the blue spot RF 0.92, traces of one of the yellow fluorescent principles (0.76) existed as impurity. The PbAc pption. was thus repeated to remove this o-hydroxy phenolic body. The purified product gave a yellowish-red colour with vanillin-HCl reagent (Lindt (198)), a weak purple with $FeCl_3$ spray, a light blue fluorescence under U.V. light in the presence of ammonia (Bate-Smith (186)) and was obviously a meta- or monohydroxy phenol. Phloroglucinol has the above properties but RF = 0.76. It is most likely a phloroglucinol derivative and will be investigated at a later stage.

The o-hydroxy phenolic fraction was liberated from the lead salt with dil. $H_2SO_4$ and ether extracted. A chromatogram showed the predominance of two bright yellow fluorescent substances but traces of the phloroglucinol derivative were still present. The dried product was dissolved in hot ethanol, treated with excess boiling water, and the yellow brown powder which separated (0.1 gm) examined by chromatography. The fluorescent principle (0.53) was still present, and the fisetin could not be entirely freed of this impurity even after a second precipitation, due to the small amount

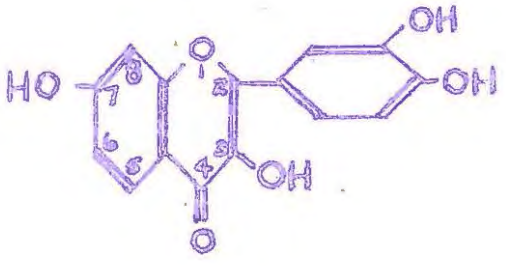
of material available. Mpt. 318 - 324°C. (decomp). RF = 0.72. Olive green colour with alcoholic $FeCl_3$. Fisetin (RF = 0.72) was also isolated from quebracho by the method of Perkin and Gunnel (120) and showed a minor impurity RF = 0.85 recorded by Bate-Smith (186). Mpt. 320 - 325°C. (decomp.) Mixed mpt. with fisetin from black wattle extract ; 318 - 325°C. (decomp).

Found for Fisetin from black wattle extract : %C = 63.01, 62.85 %H = 4.9, 4.8 (dried 110°C. Abderhalden)

Calc. for $C_{15}H_{10}O_6$ : %C = 62.94 %H = 3.50

Chromatographic comparison with fisetin from quebracho colorado and the hydrolised extract of Cotinus coggygria Scop. (Syn. Rhus cotinus) left no doubt that the brilliantly green-yellow fluorescent spot was FISETIN.

Bate-Smith (186) suggests that the abnormal fluorescence of fisetin under ultra-violet light is due to the unusual freedom from substitution in position 5. On account of its abnormally




bright fluorescence the compound gives the impression, from visual examination of paper chromatograms, of being present in the commercial extract in far greater proportion than is actually the case.

Although much fisetin was probably occluded or condensed during the above drastic sulphuric acid treatment, the yield of 0.001% gives some indication of the very low proportion in which these associated phenolic bodies occur in black wattle extract.

The other yellow fluorescent principle (RF = 0.53) is apparently present in even lower concentration. The yellow fluorescence matches that of fisetin and it is possible that these compounds may be related. From the preceding discussion of Bate-Smith's work (189) on related $C_{15}$ compounds two possibilities might account for its lower RF value :

(a) If both are agluconeS, the more highly hydroxylated have lower RF values than the less hydroxylated.

or (b) glycosidation of the hydroxyl groups with glucose, in whatever position, usually results in a large decrease in RF value.

As both resorcinol and gallic acid nuclei are known to predominate in black wattle tannin (see alkali fusions pp. 192-196) the above isolation of these compounds suggests that strong sulphuric acid is capable of degrading the tannin to a limited extent.

All the fluorescent bodies are ether-soluble and could also be separated from the remainder of the tannins by the repeated extraction of strong aqueous solutions with ether (see p. 404).

(vi) Choice of Suitable Solvent Mixtures for the Paper Chromatography of the Polyphenolic Tannins

The butanol - acetic acid - water mixture, while giving excellent separations of the associated fluorescent bodies, did

not appear equally effective for the predominant tannin fraction. The developed one-dimensional chromatograms of the tannins which were spotted on at different concentrations, showed trials or streaks, and little partial resolution was visible in ordinary or ultra-violet light, or when sprayed with ammoniacal silver nitrate or ferric chloride.

It was suspected that this trailing was either the result of the high affinity of the tannin for cellulose or caused by the use of incorrect solvent systems. It was also possible that such a complex mixture of compounds of overlapping RF values was present, that no effective separation could be achieved by one-dimensional chromatography.

To clear up some of these possibilities and to obtain information suitable for subsequent two-dimensional work, a study of various solvent systems was embarked on.

## Alcohol - Water Mixtures

Water-miscible and water-immiscible alcohols have been used by Rockland, Blatt and Dunn (199) for the separation of amino-acids. The presence of some acids (e.g. HCl) in completely miscible mixtures appeared undesirable as two widely-separated boundaries were formed, and in upward chromatography the amino-acids did not migrate above the lower boundary. In the absence of acids satisfactory resolution was obtained. From the previous work of Consden, Gordon and Martin (196) it appears that success in resolving amino-acids with water-miscible solvents depends on

the relatively low water content of papers not subjected to preliminary equilibration with aqueous solvents. Both of these groups of workers used various propyl, butyl and amyl alcohols mixed with varying proportions of water. With water-miscible alcohols the ratio alcohol/water = 7/3 appeared most satisfactory, while with water-miscible alcohols the water-saturated solution of the alcohol was usually employed.

These mixtures were applied for a study of the tannins and the effect of varying proportions of acids also determined. The chromatograms were run one-dimensionally and the efficiency of the solvent mixtures assessed by examining the developed paper chromatogram of the tannin as well as determining the efficiency with which two catechin bodies were separated. Casuarin from the bark of Casuarina equisetifolia Lin. was used for this purpose.

Casuarin was identified by Osima (200) as a d-gallocatechin by its reduction with metallic sodium to a hexamethoxydiphenyl-propane, which was identical with that obtained synthetically and by the reduction of authentic gallocatechin. Casuarin of the same melting-point (182°C) and giving the same blue-green colouration, was isolated by Osima's method from the bark of Casuarina equisetifolia grown in Zululand. One-dimensional chromatograms (Fig. VII) showed that in addition to gallocatechin, catechin was also present in high proportion. The RF values of these compounds in both solvent mixtures agreed with that in the literature. (Bate-Smith (186)).

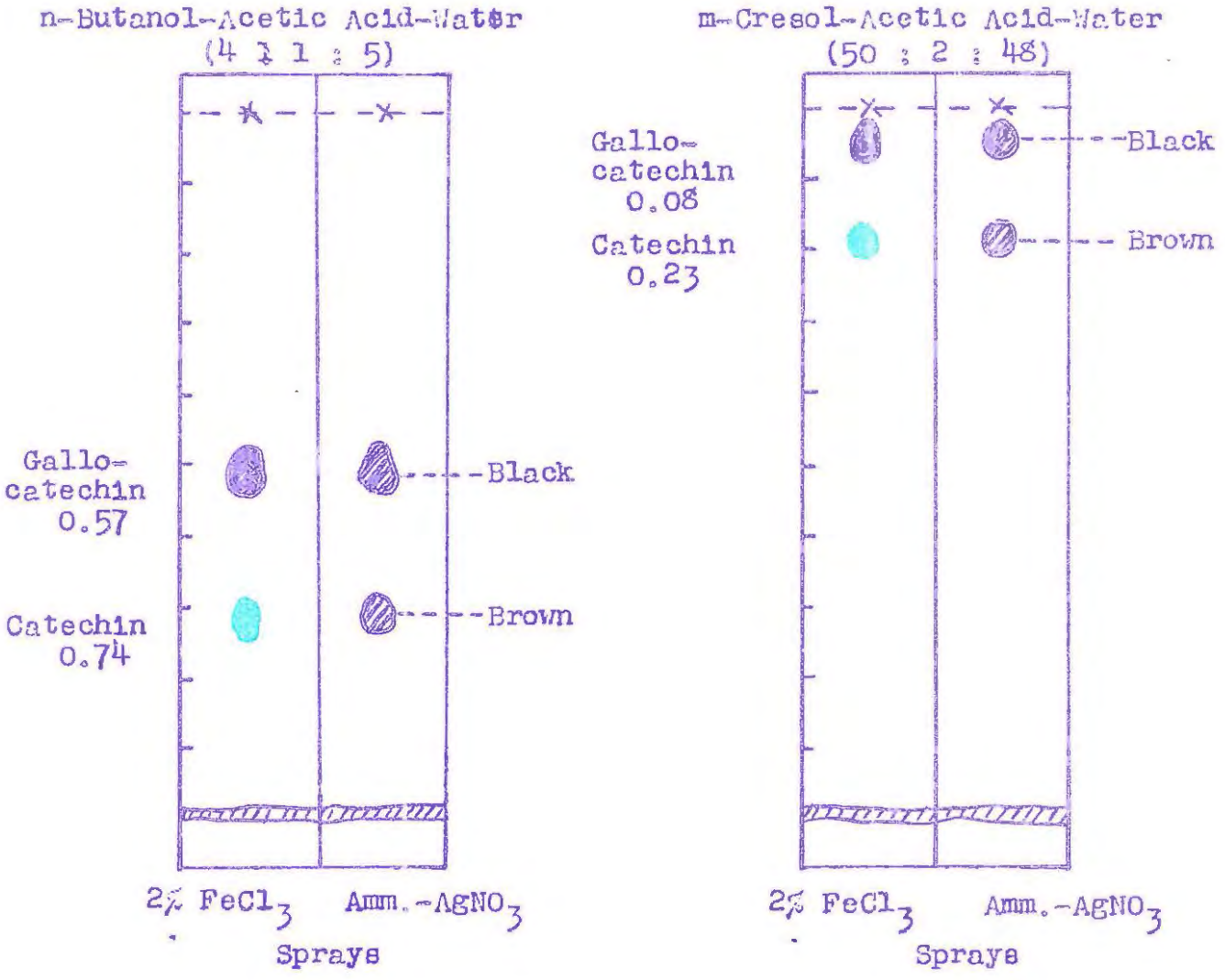



Fig. VII.

Chromatograms of Casuarin in n-Butanol - acetic acid - water and m - Cresol - acetic acid - water.

The following conclusions were drawn from a careful study of the solvent mixtures :

(a) Alcohol-water (7 : 3) mixtures and water-saturated alcohols were excellent for separating phenols on one-dimensional chromatograms. The degree of separation varied with the various alcohols. Water-saturated sec-butanol and tert-amyl alcohol, and tert-butanol-water (7 : 3) mixtures were most efficient. n-propanol-water, iso-propanol-water, ethanol-water and methanol-water (all 7 : 3) were less efficient but still gave good separation of catechin bodies. Diacetone alcohol was inefficient. These mixtures separated the fresh bark extract into areas of higher concentration and appeared to diminish much of the trailing in the lower RF region. Water-saturated n-butanol gave much the same tannin chromatogram as with the presence of acetic acid in the developing solvent mixture; the only exception being that the fluorescent spots appeared somewhat more diffuse.

(b) Water-alcohol mixtures in which the water predominates generally gave poor or no separation of catechin and gallocatechin, and it is presumed that the tannin constituents are also less satisfactorily separated. With black wattle tannins all of the yellow fluorescent material is left at the origin. This effect was also observed on cellulose columns. These fluorescent constituents appear to have a very high affinity for cellulose in the presence of predominantly aqueous solutions.

(c) The presence of acetic and other acids in solvent mixtures used for chromatography is superfluous where only phenols are examined. Phenols of the $C_6$ and $C_{15}$ classes of compounds

travel as discrete well-separated spots in alcohol-water mixtures. Where phenolic acids are present in the mixture under investigation alcohol-water mixtures alone are not satisfactory, and the presence of an acid is essential to prevent trailing. The stronger acid in the solvent mixture suppresses the ionisation of weaker organic acid, and thus enables it to move as a single unionised spot. In the gallic, β -resorcylic and protocatechuic acid mixture studied the trails probably represent the ionised areas. (Fig. VIII).

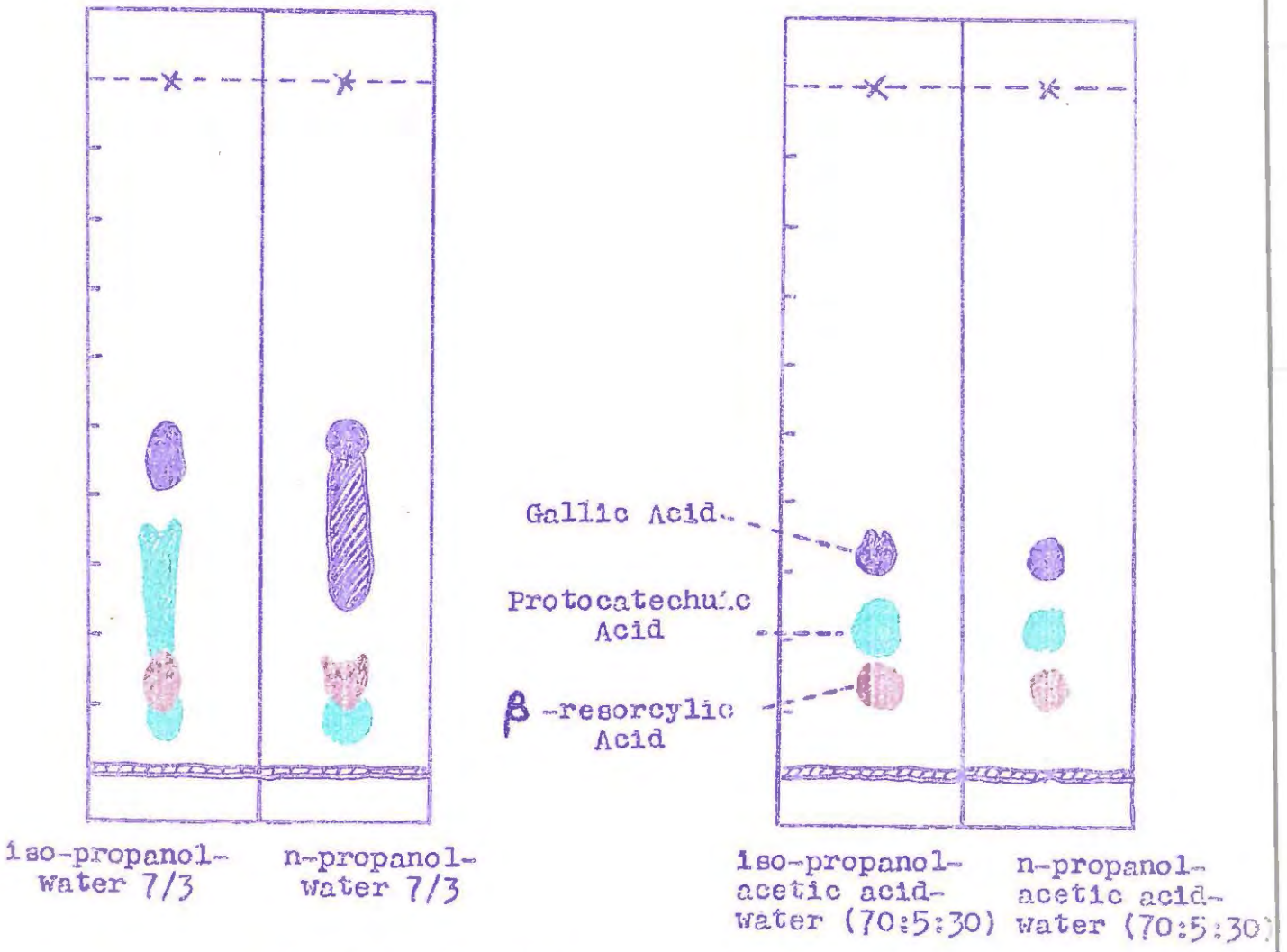



Fig. VIII. The Effect of Acids in Alcohol-Water Mixtures for the Chromatography of Phenolic Acids (2% $FeCl_3$ Spray)

This phenomenon was previously also observed by Lugg and Overell (201) in a study of carboxylic acids obtained from plant tissues.

(d) The yellow fluorescent constituents have a high affinity for cellulose even in the presence of predominantly alcohol solvent mixtures. Once adsorbed on cellulose columns they cannot be desorbed even after prolonged treatment with solutions of strongly hydrogen-bonding ability (14) such as methanol and acetone-water (1 : 1) mixtures. The significance of fluorescent areas at the origins of one- and two-dimensional chromatograms must thus be interpreted with caution, and it is most unlikely that these are chemically different from substances present in the mixture under investigation. Air-drying of spots on chromatograms at any stage appears to cause a more pronounced fixation of a low proportion of these compounds to cellulose.

(e) The Whatmans No. 11 paper was acetylated with acetic anhydride and perchloric acid in order to reduce the affinity of what was suspected to be the most highly condensed portion of the tannin. After acetylation the paper was thoroughly washed and finally irrigated with water in a chromatographic apparatus for 24 hours. After thorough air-drying the acetylated paper was used in exactly the same way as the original chromatographic paper.

Although the dry acetylated strips are less water-absorbent than the cellulose itself it behaved similarly with different solvent mixtures. The streak of low RF value was still

present on developed chromatograms of commercial black wattle tannins.

## Phenol - Water Mixtures

Phenol - acetic acid - water was recently used by Asquith (202) for separating the constituents of synthetic tannins without trailing. Rockland, Blatt and Dunn (199) also used various cresol and phenol mixtures for their work on amino-acids, while Bate-Smith and Westall (186) showed that $C_{15}$ polyphenols were effectively separated by m-cresol - acetic acid - water (50 : 2 : 48) mixtures.

In this investigation the cresol - water mixtures tried, were observed to move the tannin constituents too slowly, and low RF values appeared to be characteristic of all phenolic - water mixtures. Water-saturated phenol gave excellent separation of both tannin constituents and catechin bodies; the latter move more slowly than in alcohol-water mixtures but are more widely separated and the spots are far more discrete. The difficulty of low RF values was overcome by irrigating the paper continuously with the fast-moving water-saturated phenol mixture. Under these conditions it appears superior to the majority of alcohol-water developing mixtures.

## Other Developing Mixtures

The effects of the following solvent systems were also studied :

Formic acid - water (8 : 2) (203)

Acetic acid - water (8 : 2) (203)

n-Butanol - formic acid - water (4 : 1 : 5)

n-Propanol - formic acid - water (7 : 1 : 3)

n-Propanol - acetic acid - water (70 : 5 : 30)

Iso-Propanol - acetic acid - water (70 : 5 : 30)

m-Cresol - water (1 : 1)

Pyridine - water (7 : 3)

Of the above the pyridine/water mixture was totally unsuitable as the phenols and tannins moved with the solvent front and little separation was achieved.

80% acetic acid was remarkable in that it gave good separation of catechin and gallocatechin, and three discrete yellow fluorescent spots (under U.V. light) with the tannin as compared with two obtained with the majority of other solvents.

None of the above mixtures separated the tannin constituents completely although areas of higher concentration were indicated when chromatograms were developed with almost all the alcohol - water and with the water-saturated phenol mixtures. This one-dimensional study was now used as basis for two-dimensional work.

(viii) Discussion of Recent Publications

The publications of Hillis (191) and Kirby, Knowles and White (167) appeared after the completion of the above work.

and the comparison of their results is thus of interest.

Although Hillis performed his work by the upward migration technique of Williams and Kirby (204), his description of the fully developed chromatogram (see his Fig. 5, D) in butanol-acetic acid - water corresponded exactly to the above findings. Kirby, Knowles and White omitted recording the blue fluorescent spot RF = 0.90, and their weaker yellow fluorescent spot is indicated as having a much lower RF value (0.40 as compared with 0.50 - 0.53) in the same solvent system. (see their Fig. I).

Kirby, Knowles and White noted that the yellow fluorescent spot associated with fisetin in quebracho, is also present in mimosa extract, thus confirming our findings and identification. They also recorded the effect of acids in solvent mixtures on phenolic carboxylic acids. Kirby, Knowles and White appear to have omitted investigating phenols as developing mixtures, but regarded wet sec-butanol, tert-butanol - water (7 : 3), wet tert-amyl alcohol and n-butanol - acetic acid - water (4 : 1 : 5) as most satisfactory. With the exception of the last-mentioned, the above findings are in agreement with their statements.

They furthermore claimed that the speed of travel of the solvent and the type of paper affected the degree of resolution obtained. These effects were not noticed here as in the present work Whatman No. 11 paper was used exclusively. Their claim that the RF values of the constituents vary considerably with concentration, was also not noticed and certainly does not appear to apply to the fluorescent constituents to any marked degree.

## C. TWO-DIMENSIONAL CHROMATOGRAPHY

Kirby, Knowles and White (168) in their most recent publication, examined the extract as a whole two-dimensionally using water saturated with tert-amyl alcohol and containing 0.1% acetic acid for the first direction and wet sec-butanol for the second. They fractionated the extract mainly by fractional precipitation from an acetone solution, by the addition of successive quantities of ether. This fractionation gave excellent separations and they claimed the presence of no less than 37 different substances in black wattle extract.

### (i) Choice of Solvent Mixtures

From the previous one-dimensional study water-saturated phenol, sec-butanol and tert-amyl alcohols as well as tert-butanol-n-propanol- and isopropanol - water mixtures were shown to be superior developing solvent mixtures. The two first-mentioned were therefore selected for the two dimensional study.

### (ii) Apparatus

The apparatus (Figs. IX and X) was very similar to that used for one-dimensional work. The shallow glazed earthenware troughs used were much longer, 10" x 4½" x ½" and the cover for the whole apparatus much larger.

Fig. IX.

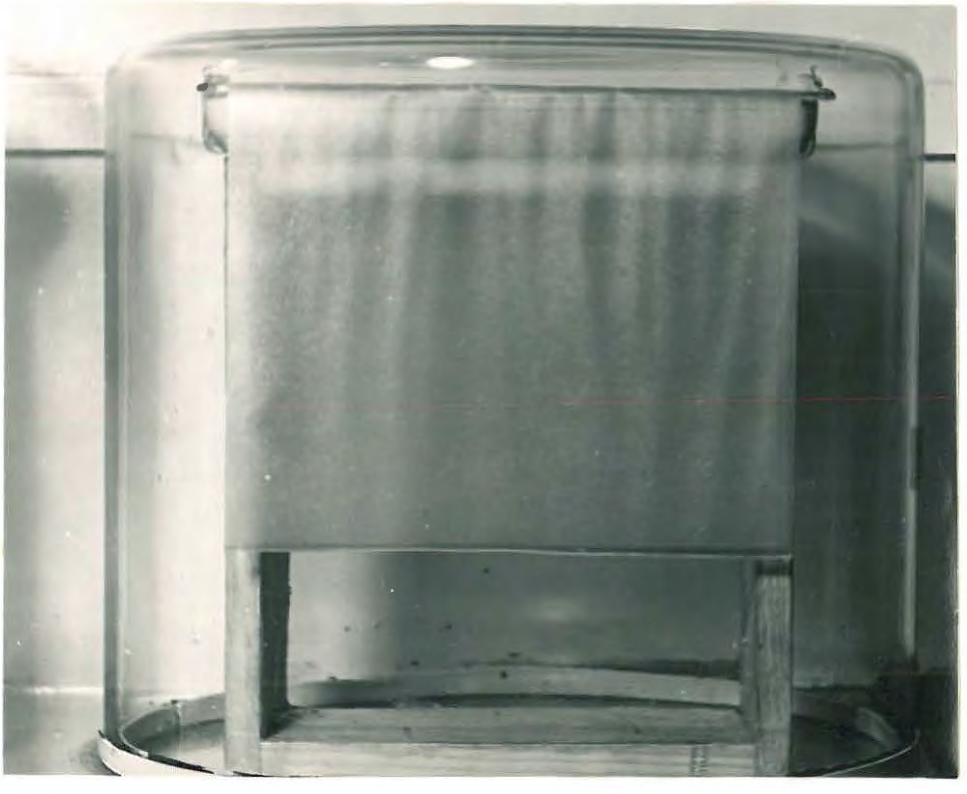


Fig. X.

A single concentrated spot 0.5 cm. in diameter was applied at a point 1½ and 3 inches respectively from the two edges of a sheet of No. 11 Whatman Paper (14" x 9"). (Fig. XI).

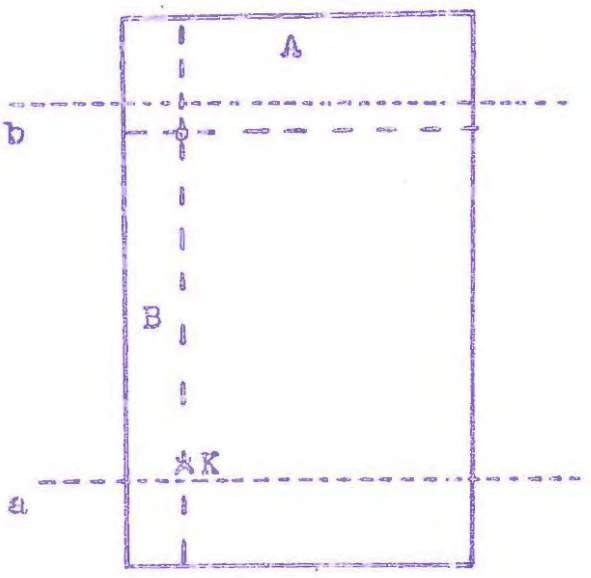



Fig. XI.

Appearance of Chromatographic Sheet Prior to 2-way Irrigation

The end A was now placed in the trough containing water-saturated sec-butanol and irrigation continued until a point K was reached, 9" from the starting line (12 - 14 hours). The paper was removed and after thorough air-drying was cut along lines a and b. The end B was next inserted in the second apparatus containing water-saturated phenol and the paper irrigated continuously for 14 hours. Control of the height of the water-saturated phenol in the trough is necessary as the irrigation occurs more rapidly when

the trough is full than when nearly empty. Small serrations along the base of the paper were useful for obtaining an even dripping action. Before removal the excess water-phenol mixture was removed by touching the base of the sheet with absorbent filter-paper. The chromatogram was suspended by stainless steel clips in an inverted position (to allow the solvent to drip off the edge B freshly removed from the trough), for 48 hours until all smell of phenol had disappeared from the paper. It was now treated with spray-reagents.

(iii) Spraying Reagents

Ammoniacal silver nitrate first used diagnostically by Bate-Smith (186), Erdtman's (205) diazotised benzidine (both used by Kirby et Al.) and Mitchell's (206) ferrous tartrate in the presence of 10% ammonium acetate were found the most effective spraying reagents. Ammoniacal silver nitrate in the cold was preferred due to its sensitivity and also as it gave diagnostic evidence regarding the nature of the phenols. 2% Aqueous ferric chloride was not sufficiently sensitive.

(iv) Choice of Solvent for Extraction of the Polyphenolic Tannins

Methanol is an excellent solvent for the "purified" polyphenolic fraction of the commercial extract, and is superior to ethanol which removes tannins and sugars completely from the gum during Soxhlet extraction. This appears to be in conflict with the findings of Kirby, Knowles and White (168) who showed that 19%

of the dried extract, still containing one-quarter of its weight in tannin, was insoluble both in cold acetone and in methanol. Their residue probably consists predominantly of gums and sugars, which occlude a low proportion of the less soluble tannins normally soluble in methanol, and which are themselves partly adsorbed on to hide-powder. These facts re-emphasise the necessity for the removal of the gums before attempting paper chromatography of the polyphenolic portion of the extract.

(v) Appearance of the Black Wattle Tannin Chromatogram

A marked difference was evident in the appearance of the two-way chromatograms of methanol-extracted mature fresh bark (Fig. XII) and methanol-extracted commercial black wattle tannin. (Fig. XIII).

The difference between these extracts is that the commercial tannin is subjected to atmospheric and enzymatic oxidation during the drying of the bark after stripping, (see section on colour-control, Chapter XI) and also to a limited extent during the commercial extraction and concentration processes.

In order to determine the effect of limited atmospheric oxidation, water was added to the fresh bark methanol-extract at 80°C., and kept at 60 - 80°C. for 8 hours during which it darkened considerably. A chromatogram of this oxidised tannin was run as before. (Fig. XIV).

For all Figs. : Direction 1 - Water-saturated sec-butanol.
Direction 2 - " " phenol.
Spraying Reagent : Ammoniacal silver nitrate (185)

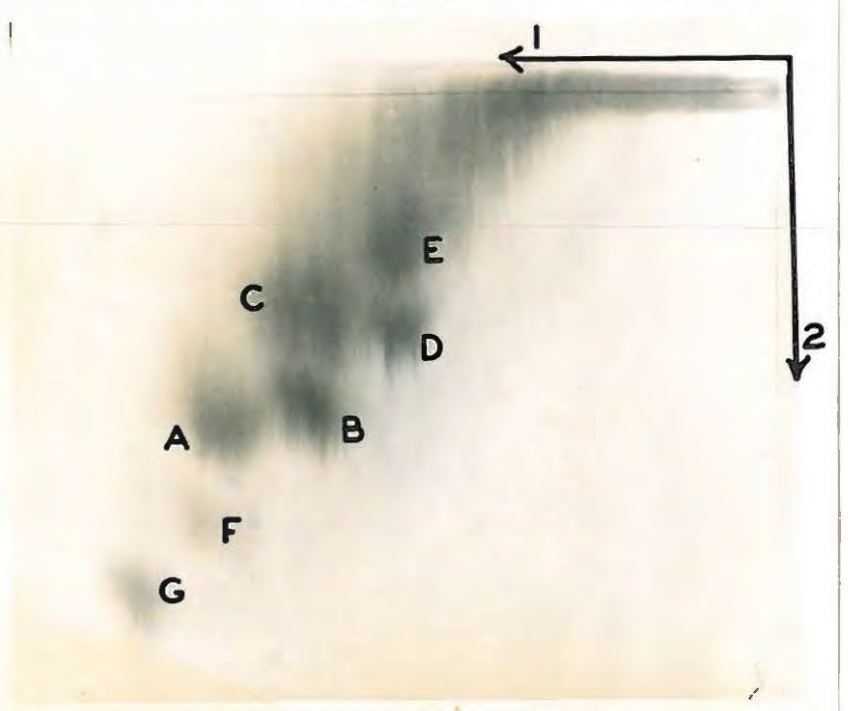



Fig. XII. Two-Dimensional Development of Methanol-Extracted Mature Fresh Bark.

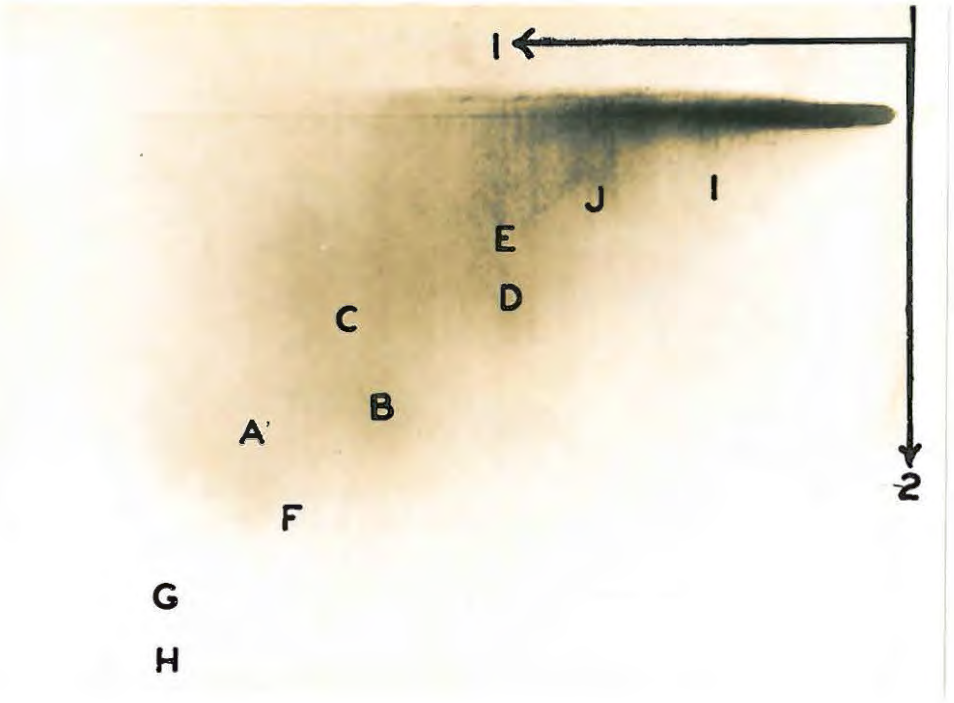



Fig. XIII. Two-Dimensional Development of Methanol-Extracted Commercial Mimosa Extract.

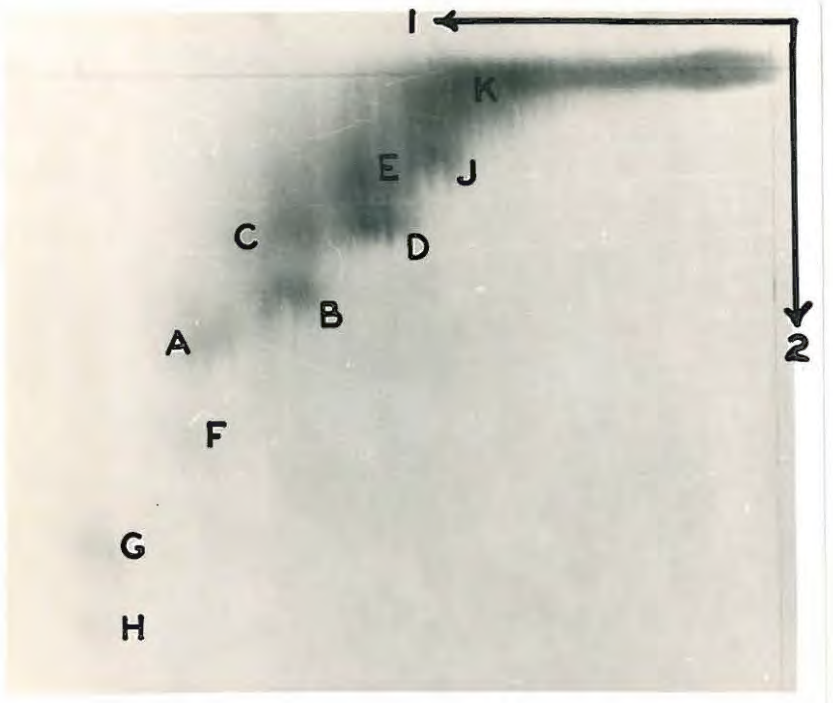



Fig. XIV.

Two-Dimensional Chromatogram of Oxidised Methanol-Extracted Mature Fresh Bark.

Oxidation, whether atmospheric or enzymatic, in the presence of sunlight or heat thus tends to increase the proportion of material of low RF value and prevents the clean separation of spots as in Fig. XII.

The areas of high concentration A, B, C, D and E form a pattern which is characteristic of black wattle tannin. When the concentration of the tannins spotted on is successively reduced the spot F first disappears followed by G while the concentration of the trail gradually diminishes. The five areas of high concentration still remain evident, however, under these conditions. By this method it is possible to assess the relative

concentrations of the various constituents. (The spot C in Fig. XII could possibly represent two compounds).

These spots may also be identified as areas of higher concentration on the chromatogram of the commercial extract, although the picture is by no means as clear. Other phenolic compounds are obviously also present here e.g. H, I, J, in Fig. XIII.

From the above it appears that the seven phenolic bodies of the fresh bark extract, represented by discrete spots on the chromatogram are capable of further condensation by atmospheric oxidation during the drying and transportation of the bark and possibly also during the extended heating period of commercial extraction. As peroxidases are known to be present in the bark (See Chapter XI), enzymes are probably responsible for much of this oxidation. A paper chromatogram of any extract furnishes a rough indication of the degree of oxidation which has occurred when compared with that of the fresh-bark extract. The trail of oxidised material is normally easily visible in ordinary light as a brown streak. This is far more pronounced with the commercial than with the fresh-bark extract. By contrast the areas occupied by the units A - G are not discernible before spraying with various reagents. Confirmation that this more highly condensed or oxidised material of low RF value is also the most astringent is obtained from Fig. 10 of Kirby, Knowles and White (168) representing a chromatogram of the polyphenolic extract leached from hide-powder with an acetone - water mixture. Here

the tannins of low RF value are present in very low concentration. Further confirmation of these facts is provided by an examination of the behaviour of the polyphenolic catechin bodies during oxidation.

(vi) The Oxidation of Catechin and Casuarin

In order to obtain a less confused picture of the behaviour of polyphenolic bodies, pure catechin was examined one- and two-dimensionally before and after atmospheric oxidation. Catechin from cube gambier consisting predominantly of d-catechin together with a minor admixture of r-catechin was isolated and purified by Perkin's (207) method. The product moved as a single spot on one- and two-dimensional chromatograms (Fig. XV).

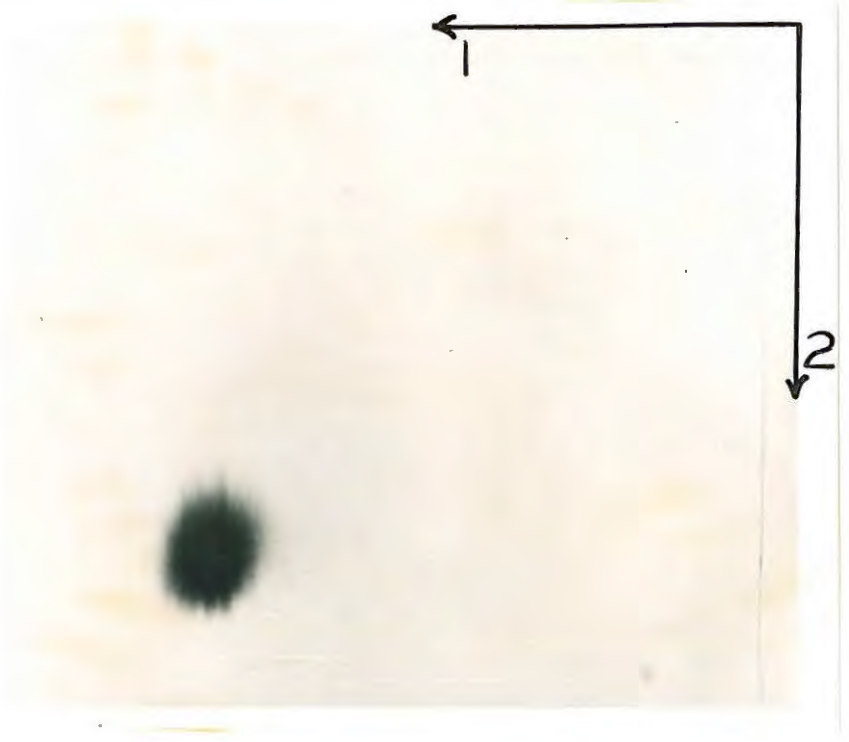



Fig. XV. A Two-Dimensional Chromatogram of Catechin from Cube Gambier

Fig. XVI. A One-Dimensional Chromatogram of Catechin from Cube Gambier. Oxidised 5 hours at 60-80°C.

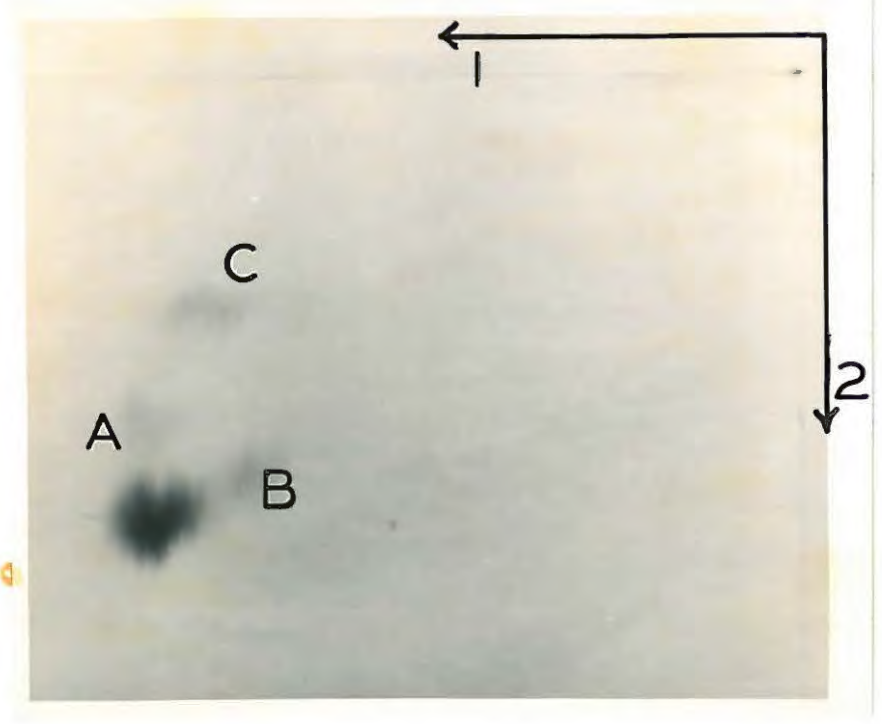



Fig. XVII. A Two-Dimensional Chromatogram of Catechin from Cube Gambier. Oxidised 5 hours at 60-80°C.

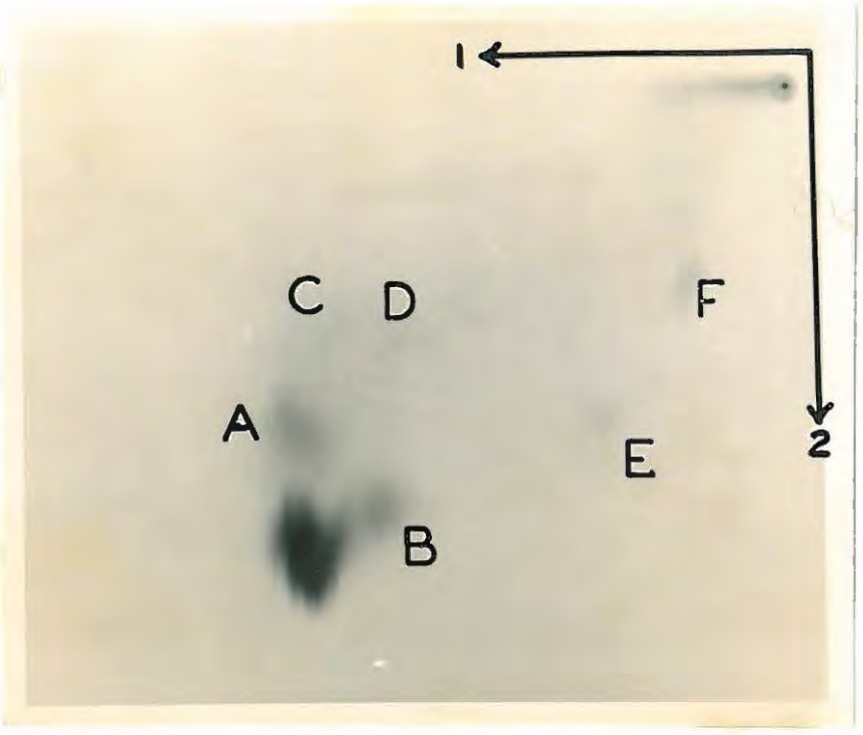



Fig. XVIII. A Two-Dimensional Chromatogram of Catechin from Cube Gambier. Oxidised 10 hours at 60 - 80°C.

After oxidation in aqueous medium at elevated temperatures (60 - 80°C) a one-dimensional examination showed the presence of a trail of both lower and higher RF value in addition to the catechin spot (Fig. XVI). Two-dimensionally the presence of a number of compounds in addition to a broad diffuse trail was evident (Figs. XVII and XVIII). It is interesting to note that two compounds (A and B) with RF values close to that of catechin are formed by oxidation. This observation shows the necessity for extreme caution in the interpretation of the many spots observed on the two-dimensional chromatogram of black wattle tannin, especially when it is remembered that enzymes are present in bark and also as Williams ( 7 ) has shown that the colour of a 0.5% infusion of

tannin increases progressively with the age of the bark.

Casuarin (Fig. XIX), a mixture of catechin and gallocatechin condenses easily on atmospheric oxidation to a single spot located at the origin. (Fig. XX).

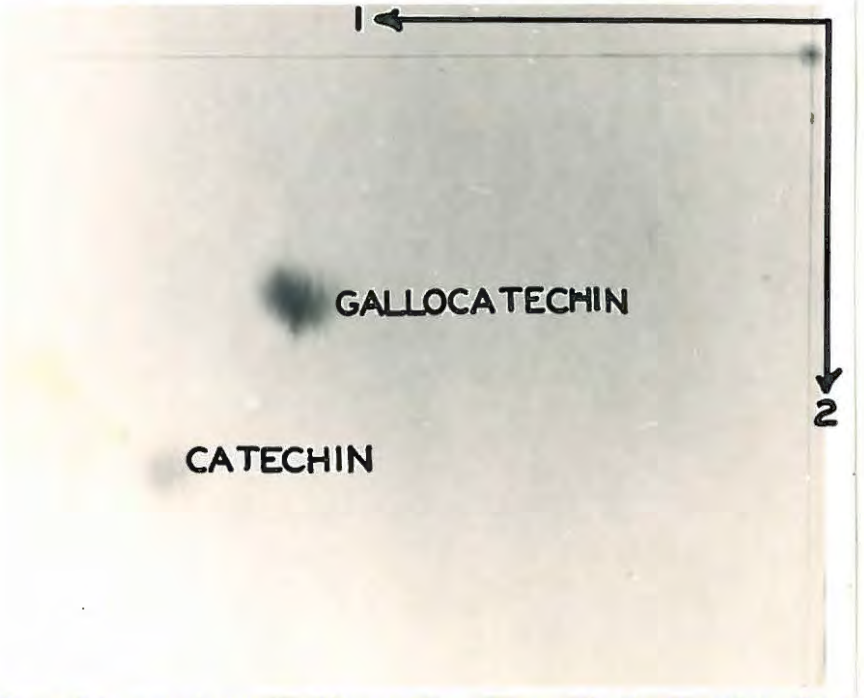



Fig. XIX. Two-Dimensional Chromatogram of Casuarin from the Bark of Casuarina equisetifolia.

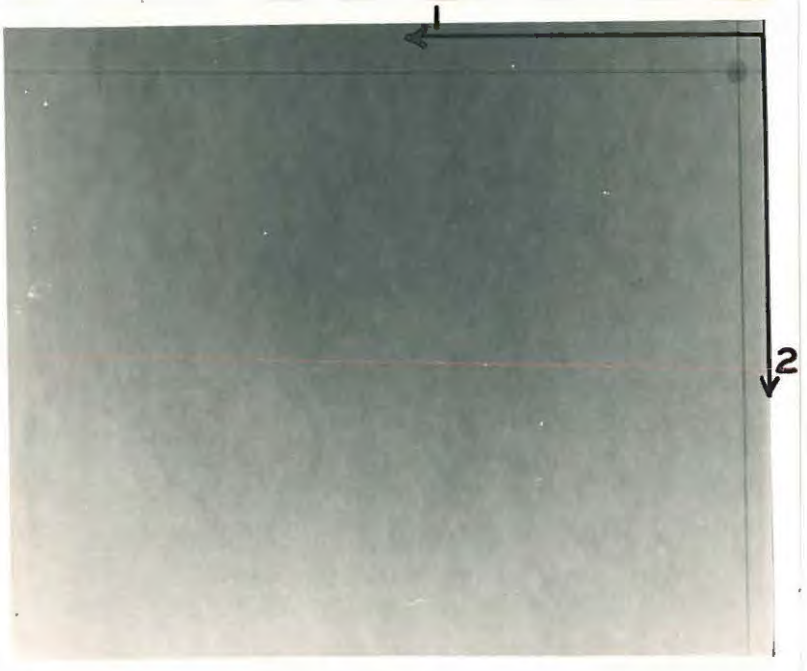



Fig. XX. Two-Dimensional Chromatogram of Oxidised Casuarin.

(vii) Fractional Precipitation of the Commercial Tannins with Lead Acetate

The separation of constituents of low RF values from the phenolic fraction of the commercial extract is easily achieved by fractional precipitation with lead acetate. The first-formed precipitates are brownish in colour but the major portion of the phenolic fraction subsequently precipitates as a colourless lead salt.

15 grms. commercial extract (66% tannin content) was repeatedly extracted with warm methanol to remove the tannins from the gum. The tannins, recovered after the removal of the methanol, were dissolved in 500 ml. water and 10% neutral lead acetate was added in small quantities with vigorous stirring. After various additions the precipitates were centrifuged (15 mins. at 3500 r.p.m) off as follows :

| | Vol. of 1% Lead Acetate Solution Added | Proportion of Total Volume of Precipitate. | Colour |
|---|---|---|---|
| Fraction I | 5 ml. | 1/7th | dark brown |
| Fraction II | 5 ml. | just < 1/7th | brown |
| Fraction III | 10 ml. | " > 1/7th | brown-white |
| Fraction VI | excess | " > 4/7ths | white |

The tannins were liberated as in the lead-salt method of separation and concentrated under reduced pressure in the presence

of nitrogen. Chromatography of each fraction (Figs. XXI - XIV) shows that the coloured material of low RF is precipitated first, and the discrete areas of high RF value last.

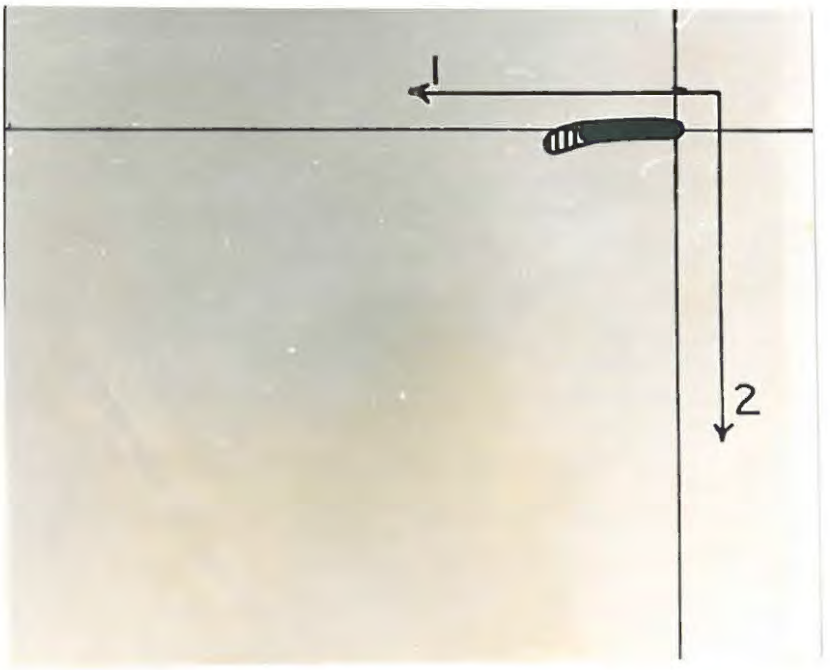



Fig. XXI. Fractional Precipitation of the Commercial Tannins with Lead Acetate. Fraction I.

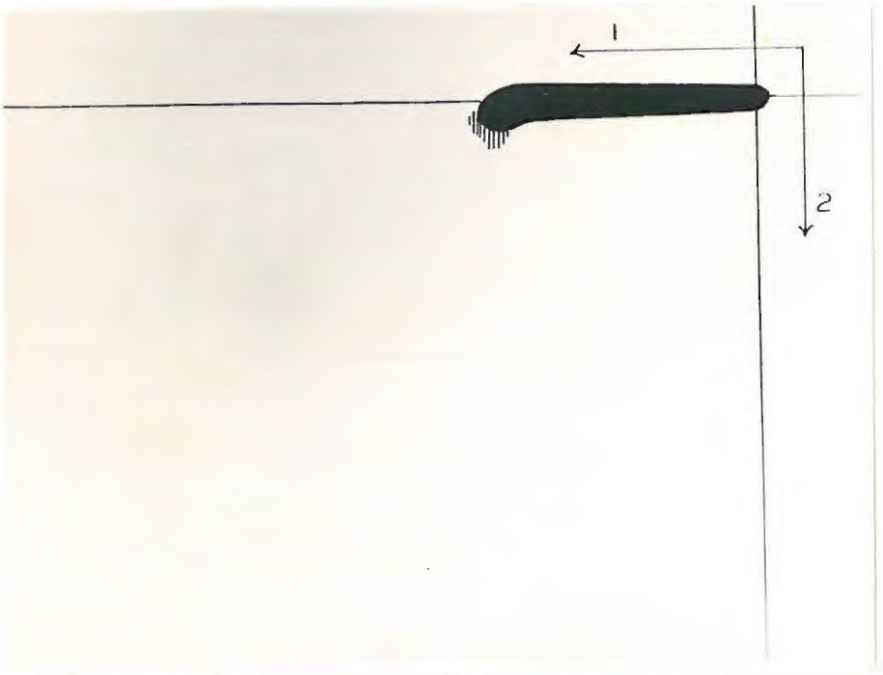



Fig. XXII. Fractional Precipitation of the Commercial Tannins with Lead Acetate. Fraction II.

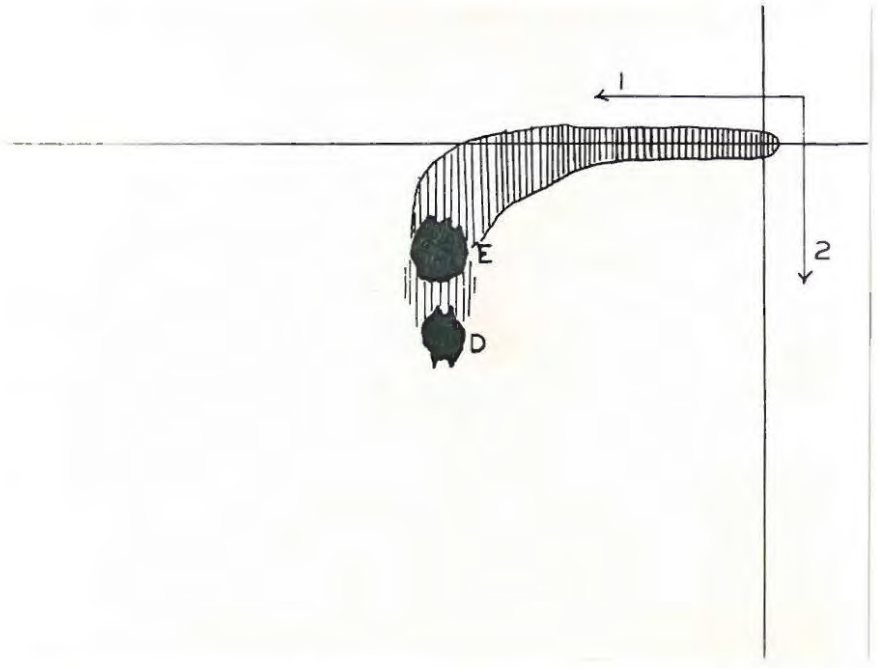



Fig. XXIII. Fractional Precipitation of the Commercial Tannins with Lead Acetate, Fraction III.

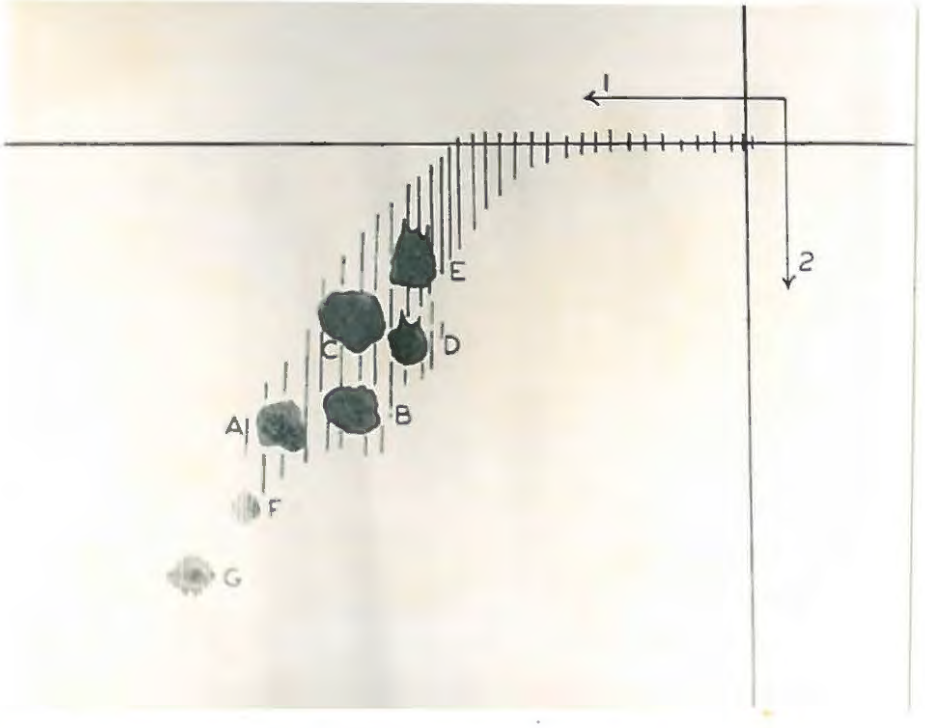



Fig. XXIV. Fractional Precipitation of the Commercial Tannins with Lead Acetate. Fraction IV.

The solvent separations used by Kirby, Knowles and White appear promising and should be applied to the phenols alone. The salting-out procedure still requires examination.

(viii) Comparison of Results from Two-Dimensional Chromatography

As previously mentioned, Kirby et Al. (168) recently demonstrated the complexity of the commercial extract by chromatography, using water saturated with tert-amyl alcohol and containing 0.2% acetic acid for the first direction and water-saturated sec-butanol for the second.

The present one-dimensional study of various solvents has shown that the second mixture gives excellent separation of tannins and catechin bodies. The first-mentioned, however, contains a predominance of water, and although it has the advantage of removing the yellow fluorescent bodies from the rest of the extract (evident also from their Fig. 1), the findings in paragraph (b) page 151 suggest that it might be an inefficient separating mixture.

The efficiency of water saturated with tert-amyl alcohol and 0.2% acetic acid mixture was accordingly tested by developing a casuarin spot one-dimensionally, and spraying the resultant chromatograms with ammoniacal silver nitrate and with ferric chloride (Fig. XXV). With the former reagent no separation was shown and with the latter a poor tendency towards separation was found.

Sprayed with Amm. $AgNO_3$

Sprayed with $FeCl_3$

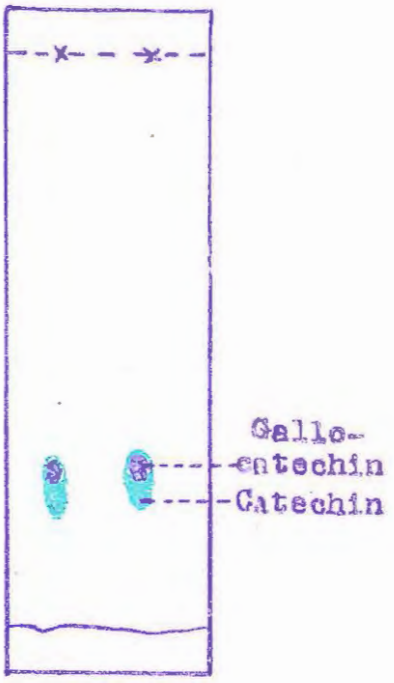



Fig. XXV. One-Dimensional Chromatogram of Casuarin Developed with Water Satd. with tert-Amyl Alcohol and containing 0.2% Acetic Acid.

Their combination of solvent mixtures was next tried two-dimensionally on casuarin (Fig. XXVI). By comparing with

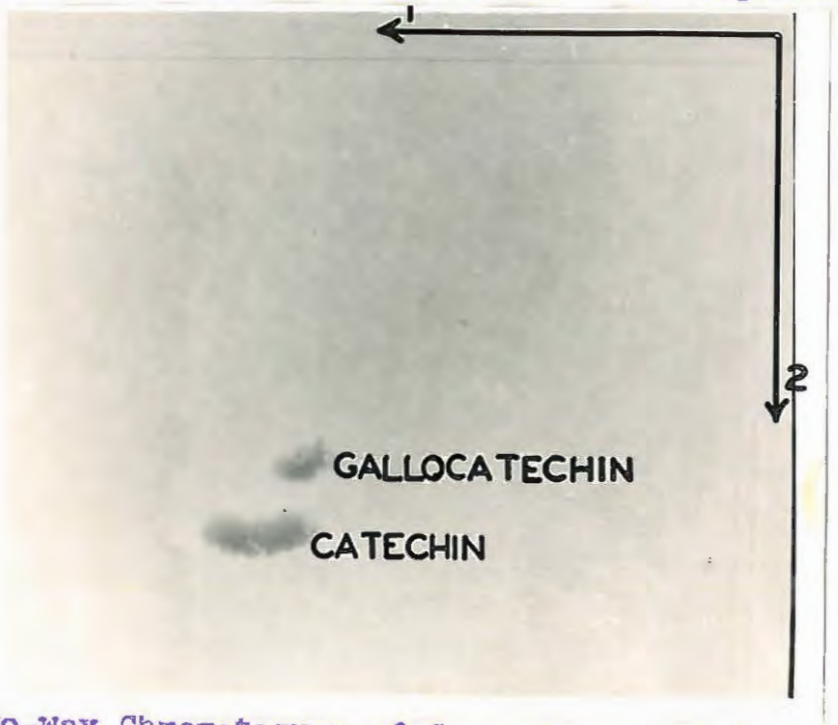



Fig. XXVI. Two-way Chromatogram of Casuarin. Kirby's et Al. Mixtures.

Fig. XIX where water-saturated sec-butanol and water-saturated phenol were used for the two-dimensional irrigation the following conclusions may be drawn :

(a) Kirby, Knowles and White's solvent mixtures give less effective separations two-dimensionally.

(b) The spots formed are also less discrete. Gambier catechin particularly appears as a longitudinal area, and it is most unlikely that this represents a tendency towards separation into stereoisomers as the longitudinal nature of the spot is due to the first solvent mixture which is incapable of separating catechin and gallocatechin (RFs 0.76 and 0.59 in butanol - acetic acid - water at 20°C.) one-dimensionally.

(ix) Two-Dimensional Chromatogram of the Yellow Fluorescent Constituents.

The two-dimensional chromatogram of the fluorescent constituents isolated by Perkin and Gunnel's method (120) and freed from gallic acid, resorcinol and the blue fluorescent constituent (RF = 0.90)(See p. 155) is represented by Fig. XXVII.

All the areas reproduced represent yellow fluorescent trails and spots, when viewed in ultra-violet light. Only three constituents A, B and C are present. B (fisetin) constitutes by far the major proportion of the mixture. A (unidentified : RF = 0.50 - 0.53 on one-dimensional chromatogram in butanol : acetic acid : water) occurs in minor proportion, and C (unidentified) as a trace only. The fluorescent areas and trails surrounding the main spots A and B are considered to represent the same compounds

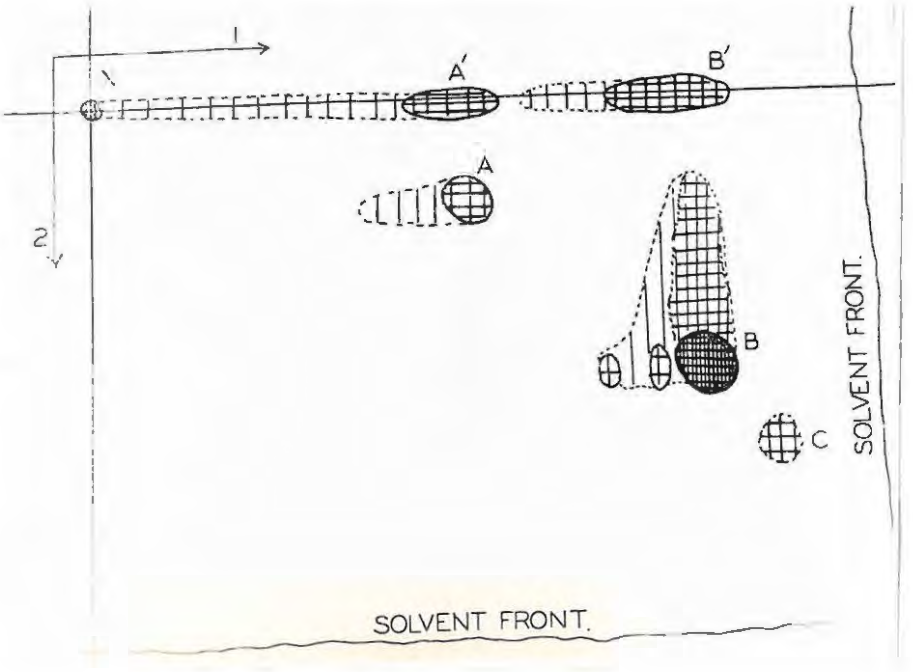



Fig. XXVII. A Two-Dimensional Chromatogram of the Yellow Fluorescent Constituents of Black Wattle Extract.

as contained in the spots themselves. The trails are caused by the high affinity of these compounds for cellulose.

The areas X, $A^1$, and $B^1$ are also clearly visible on the 2-way chromatogram developed with this solvent combination. These areas represent positions where the chromatogram was air-dried after spotting on (at X) and after one-dimensional development (at $A^1$ and $B^1$). This behaviour again results from the high affinity of these compounds for the cellulose, and low proportions of A and B appears to be irreversibly fixed at $A^1$ and $B^1$ respectively, and also at X.

(x) Summary and Conclusions

(a) The polyphenolic (tannin) portion of black wattle extract was separated into a number of constituents by paper chromatography after a detailed study of various solvent systems.

(b) One-dimensional chromatograms show that the tannins contain a low admixture of three bodies which fluoresce brightly under ultra-violet light. One of these was isolated and identified as fisetin for the first time. Gallic acid and resorcinol were also obtained by the action of hot sulphuric acid on the commercial extract during the isolation of the fisetin.

(c) Two-dimensional chromatography has confirmed the previously-surmised complexity of the extract, also recently demonstrated by Kirby, Knowles and White (loc. cit.). The solvent combination used in the present study has produced a number of very discrete spots for the first time from black wattle tannins, and is superior to that used by Kirby et Al.

(d) The chromatograms of the mature fresh bark tannins show the predominance of five or possibly six constituents. Others are present in lower concentration.

(e) These compounds are represented on the chromatograms by discrete spots which are close in RF value and could possibly represent different condensation-states of one or two fundamental tannin units polymerised or condensed in different ways and to different degrees.

(f) The commercial extracts show a far higher degree of condensation and oxidation than cold fresh-bark extracts. Confirmation of this was obtained from an oxidation-study of phenolic units, e.g. catechin and casuarin.

(g) The commercial extract must therefore contain a large number of artifacts produced by oxidation during the drying, transport and commercial extraction of the bark. For this reason future research should be concentrated on the separation of the constituents of the unheated, unoxidised fresh bark extract. Once isolated, the behaviour of each constituent on oxidation could be studied by chromatography and a better composite picture thus obtained.

(h) Welch, McGlynn and Coombs (365) found that the older cells near the outer cortex of the bark contain the greatest amounts of tannins, while the younger tissues nearest the inner cambium contains mainly soluble non-tannins. Their results indicate that the new tannin-bearing cells contain non-tannins which gradually change to tannins as the age of the bark increases. Enzymes were found present in black wattle bark (see Chapter XI). Osima and Hayashi (364) have shown that d-catechin and gallocatechin are oxidised by enzymes to reddish-brown products. These interconversions require study, and enzymatic oxidation might be responsible for the gradation of tannin particles present even in the cold fresh-bark extracts.

(i) During the above study casuarin from the bark of Casuarina equisetifolia which was identified by Osima (200) as a gallocatechin was found to contain also a major admixture of catechin. Casuarin was used for examining the efficiency with which the various solvent systems give separation of complex polyphenols.

## CHAPTER VI.

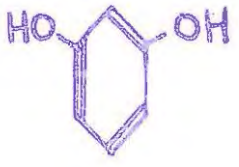
## THE DEGRADATION OF BLACK WATTLE TANNINS

From a review of previous investigations in Chapter III it is obvious that hitherto all degradations of the polyphenolic (tannin) fraction were either carried out on partly purified material, or else on derivatives originating from such material. As it is known from the present work that gums and carbohydrates form the remainder of the extract, the phenolic fission products previously isolated are thus true derivatives of the tannins themselves, and could not have originated from the non-tannins.

Similarly degradation products derived from "mimosa" extracts may be compared with those later obtained from authentic samples of black wattle bark, as this tree has been almost exclusively used for extract manufacture. Minor admixtures of green wattle barks (Acacia decurrens Willd.) were used only for a limited period during the last war. (See Chapter I).

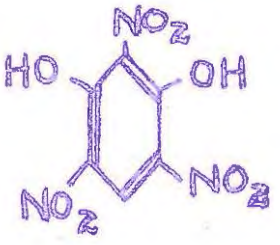
The isolation of most of the following compounds have been confirmed by successive investigators, while the identity of others, particularly the explosive nitro-derivatives, still await confirmation.

(a) Resorcinol was isolated by Stephen (149) and Williams (157)



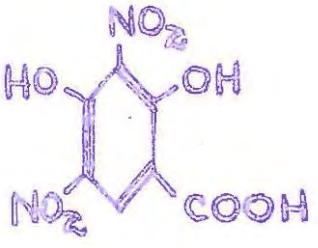
in very low yield (1 - 2%) from alkaline fusions of the acetone-extracted tannins. In spite of an intensive search neither could detect the presence of phloroglucinol, which was the only meta-hydroxy phenolic nucleus found by Russel (136) in the degradation products from similar fusions.

(b) Styphnic Acid or 2,4,6-trinitroresorcinol was first obtained



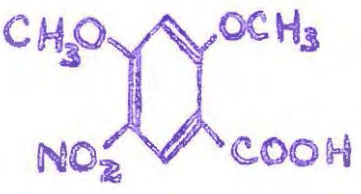

by Jablonski and Einbeck (121) by the nitrative oxidation of "mimosa" extract. The yield was equivalent to 2% resorcinol in the extract, and the same product was also simultaneously derived from quebracho extract but in higher (4.5%) yield. Styphnic acid was subsequently also isolated by Stephen (loc. cit.) from the direct nitration of the tannins and by Kirby (163) from the nitration of the methylated tannins.

(c) 3,5-Dinitro-β-resorcylic acid was obtained by Einbeck and



Jablonski (122) from the mother-liquor of the above styphnic acid. Von Hemmelmayr (144) found that when 3,5-dinitro-β-resorcylic acid was heated with nitric acid, it was quantitatively converted into styphnic acid. Einbeck and Jablonski thus postulated that 3,5-dinitro-β-resorcylic acid was the primary, and styphnic acid the secondary product of the reaction of concentrated nitric acid on "mimosa" extract. The identical product was also obtained from quebracho extract.

(d) O-dimethyl-5-nitro-β-resorcylic acid was isolated by

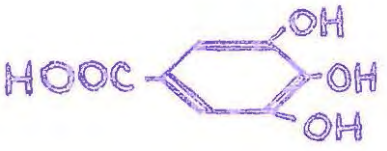



Kirby (163) in low yield (2%) from the reaction-products of concentrated nitric acid and methylated tannins. The identification of this compound indicates that after complete methylation (36% methoxyl), a proportion of the resorcinol nuclei is attached to the tannin residue by a carbon chain only.

(e) Resorcinol monomethylether was obtained by Heugh (161) in

low (1.8%) yield from the alkaline fusion of methylated tannins. This volatile oil was condensed away from the reaction-mixture after liberation.

(f) Gallic Acid. Stephen (149) obtained this acid in very low




yield ($\leqslant$1%) and partially pure condition from the alkaline fusion of acetone-salt purified tannins.

(g) O-trimethylgallic acid and veratric acid are easily obtained



by the alkaline permanganate oxidation of methylated tannins and have been isolated by almost every investigator. Corbett (146), Stephen (149), and Kirby (164) all established, and particularly Heugh conclusively identified the presence of both acids in the oxidation products. Corbett first separated the acids by making use of the different solubilities of their silver salts in water. The yield of the mixture of acids is low, usually of the order of 4%, but 8 ~ 10% yields have been claimed.

(h) Oxalic acid was identified by Einbeck and Jablonski (122),

Stephen (149) and Kirby (164) from the mixture of oxidation products which results from the uncontrolled nitration of the tannins and their methylated derivatives.

(i) Other degradation products have been obtained in a partially pure condition and were not successfully purified. Heugh (161) reduced the methylated tannins with sodium in amyl alcohol. The gum obtained after remethylation of the product yielded a strong acid on oxidation. This failed to crystallise and gave the analysis : Eq. Wt. = 222.4, % $-OCH_3$ = 27.14, %C = 60.35, % H = 6.2. It furthermore yielded veratric acid on more drastic oxidation, and represents a dimethoxyphenyl proprionic acid.

Kirby (163) obtained a neutral compound $C_{12}H_{11}N_3O_4$ (mpt. 134 - 6) from the nitration of the methylated tannins.

It will be noted that all the above degradation products were obtained in exceptionally low yields compared with those normally produced from crystalline catechin units. Nierenstein (210) obtained 17 - 27% veratric acid and 7 - 23% O-dimethylphloroglucinol from the permanganate oxidation of methylated acacatechin. Freudenberg and Maitland (123) again, isolated 9.4% resorcinol from the alkaline fusion of synthetic quebracho-catechin, and 25% veratric acid by the alkaline permanganate oxidation of the methylated derivative.

These low yields were hitherto considered the most unsatisfactory feature of the chemistry of black wattle tannins, and gave rise to much speculation. Degradations were therefore

repeated under carefully controlled conditions, and paper chromatography was used for a detailed scrutiny of the degradation products.

(1) The Controlled Alkaline Fusion of Black Wattle Tannin

The commercial tannins were freed of fisetin, the phloroglucinol derivative (RF = 0.90) and most of the second yellow fluorescent compound (RF = 0.50), by repeated ether extraction of the aqueous solution. Gums and sugars had previously been removed by the addition of ethanol and by the lead-salt technique as described in Scheme I (Chapter IV).

10 grms. of the dried (vacuum 100°C) product was rapidly ground with 50 grms. of a eutectic 6 : 1. mixture of KOH/NaOH, and placed in a metal bomb with a screw-down lid. This was heated at 200 ± 1°C. for one hour in an autoclave. The fused mass was dissolved in a minimum of water after cooling; was acidified; ether extracted, and the ethereal solution on concentration subdivided into phenolic and acidic fractions by the bicarbonate technique.

The acidic fraction (23% yield) after removal of the ether in a desiccator under vacuum, was examined by paper chromatography using butanol - acetic acid - water (4 : 1 : 5) at 22°C. A 12 inch development gave separation into 3 acidic spots which were detected by spraying the paper, after airing for 24 hours to remove all traces of acetic acid, with bromocresol-green. They appeared as yellow spots against a green background. With 2% ferric chloride spray discrete spots RF = 0.68 (blue), RF = 0.84 (green) and RF =

0.91 (violet) were strongly evident. A weak dark trail, consisting of oxidised phenolic-acid material, extended from the origin to RF = 0.90.

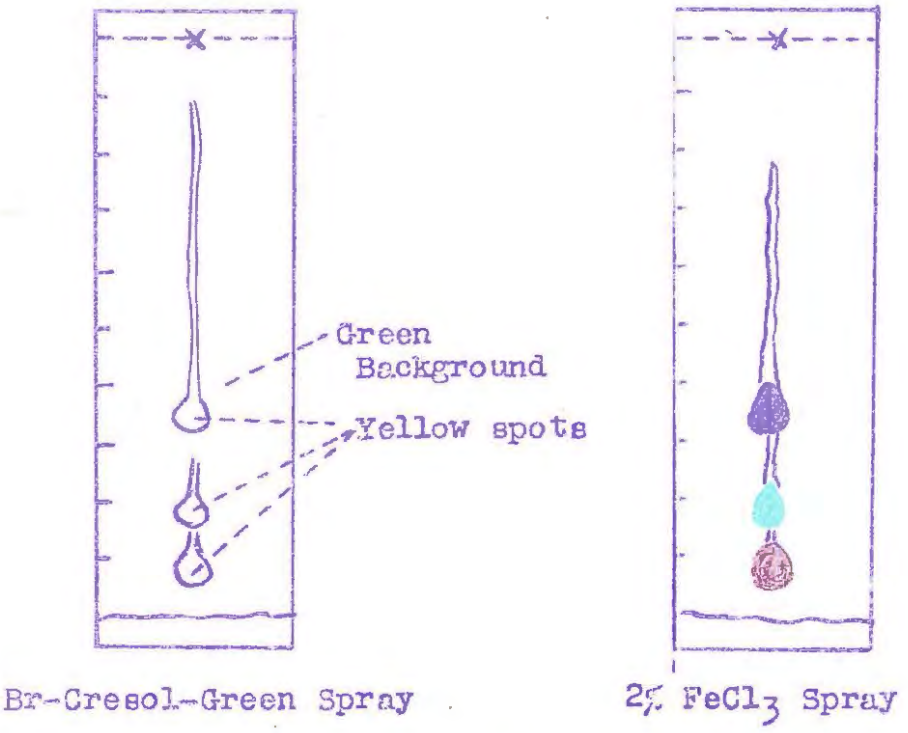



Fig. XXVIII. One-Dimensional Chromatogram of the Phenolic-Acidic Fraction from the Alkaline Fusion of Black Wattle Tannins.

The green spot was characteristic in colour and RF value of PROTOCATECHUIC ACID (See Bate-Smith (186)). A chromatogram run with a mixture of authentic gallic, protocatechuic and β-resorcylic acids confirmed their identity. (Fig. XXVIII).

β-RESORCYLIC ACID was separated from the gallic and protocatechuic acids by precipitating the ortho-hydroxy acids with a slight excess of dilute 10% solution of neutral lead acetate. The lead salts were separated and washed by centrifugation. The

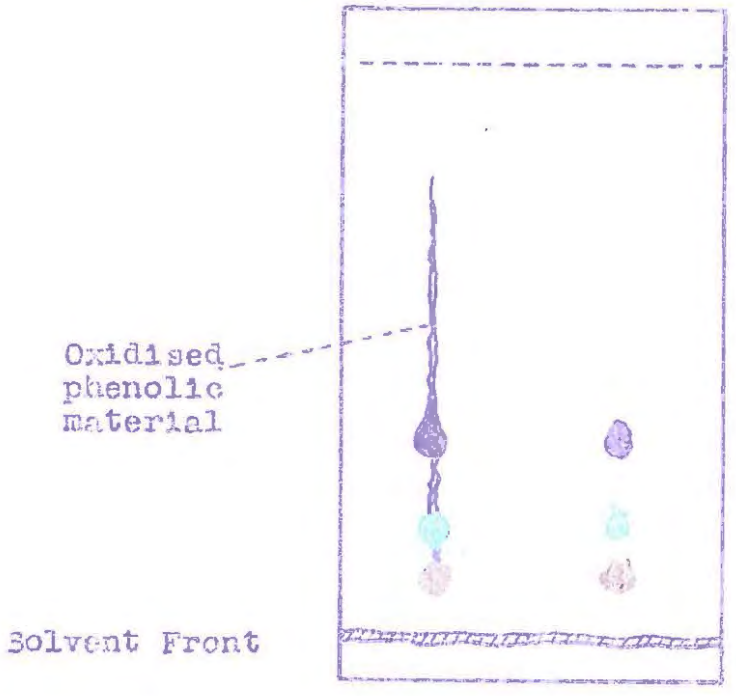



Fig. XXIX. Chromatographic Comparison of Acid Mixture with Authentic Acids. 2% $FeCl_3$ spray used.

mother liquor and washings gave 0.42 grm. of β-resorcylic acid on ether extraction. The acid was recrystallised by solution in hot water just off the boil to prevent decarboxylation. Mpt. and mixed mpt. with authentic specimen = 203°C. (decomp.) Purple colour with $FeCl_3$ in aqueous solution. Found : Equiv. wt. (anhydrous) = 154.9. Calculated for $C_7H_6O_4$ = 154. Yield = 4.2%.

The ortho-hydroxy acids liberated from their washed lead salts with dilute 2 N sulphuric acid, were ether-extracted. After drying the ethereal extract, the bulk of the ether was removed on a water-bath and the remainder under vacuum over $P_2O_5$. Yield=1.6 grms of dark acidic gum. The silver-salt method of Corbett (146) for the separation of veratric and O-trimethyl gallic

acids was attempted for the identification of these acids. The gum was methylated with excess (benzoic acid test) methanolic diazomethane and hydrolised with potassium hydroxide in methanol. Water was added and the methanol removed by ether extraction. The aqueous solution of the potassium salt was acidified and ether-extracted. This yielded 1.1 grm oil which on sublimation and re-crystallisation from water gave pure O-trimethylgallic acid. Mpt. and mixed mpt. 166°C. Found : Equiv. Wt. = 211. Calculated for $C_{10}H_{12}O_5$ : Equiv. wt. = 212. Protocatechuic acid was presumably present only in low proportion and was probably eliminated during the above methylation, hydrolysis and purification processes. The evidence from the chromatogram was considered as sufficient proof of its presence. GALLIC ACID in major proportion and PROTOCATECHUIC ACID in minor proportion mainly constitute the O-hydroxy-phenolic acidic fraction.

The phenolic fraction (20% yield), obtained as a black gum, was dissolved in water and separated into ortho- and meta-plus mono-hydroxy fractions by the lead acetate technique as in the case of the phenolic acids.

The bulk of the meta-hydroxy fraction (13%) sublimed at 110 - 120°C. in vacuum (3 mm.) This proved to be RESORCINOL 10.5%. Violet colour with $FeCl_3$. Mpt. (recrystallised from benzol) and mixed mpt. 108°C. The small amount of non-volatile residue was examined by chromatography using butanol - acetic acid - water as before. With 2% $FeCl_3$ spray two weak violet areas were visible. Other chromatograms were also sprayed with diazotised

sulphanilic acid (211), phosphomolybdic acid (212) and the author's sucrose - HCl reagent (see below). In addition to resorcinol RF = 0.90 a predominant spot RF = 0.76 was present which fluoresced blue under ultra-violet light in the presence of ammonia. From the colour reactions and RF value this was obviously PHLOROGLUCINOL. The trace of phloroglucinol is considered to be formed from gallic acid (see Barth and Schreder (213)) during alkaline fusion.

Paper chromatography of the ortho-hydroxy fraction (3%) showed the presence of three phenols (RFs = 0.76, 0.81, 0.91) of which that of RF = 0.76 predominated. The latter proved to be PYROGALLOL and gave a yellow colour with 2% $FeCl_3$ spray. Its identity was further confirmed by the different colours produced by the above spraying reagents. A small quantity could be sublimed under vacuum, but due to the additional presence of an oily material, high purity could not be achieved. More drastic fusions gave higher yields of pyrogallol and also a trace of catechol (paper chromatogram). This material after sublimation under reduced pressure (3 mm.) and recrystallisation from a benzol/ethanol (100 : 2) mixture yielded crystals of mpt. and mixed mpt. with pyrogallol 129 - 130°C. The remaining orthohydroxy phenols, which constitute a minor proportion of the fraction could not be identified, due to difficulties of separation, and the low yields obtained.

More drastic fusions (2½ hours at 240 - 260°C.) gave a decreased yield of the acidic mixture containing only (paper chromatogram) gallic and protocatechuic acids, and increased yields of resorcinol (12%) and pyrogallol.

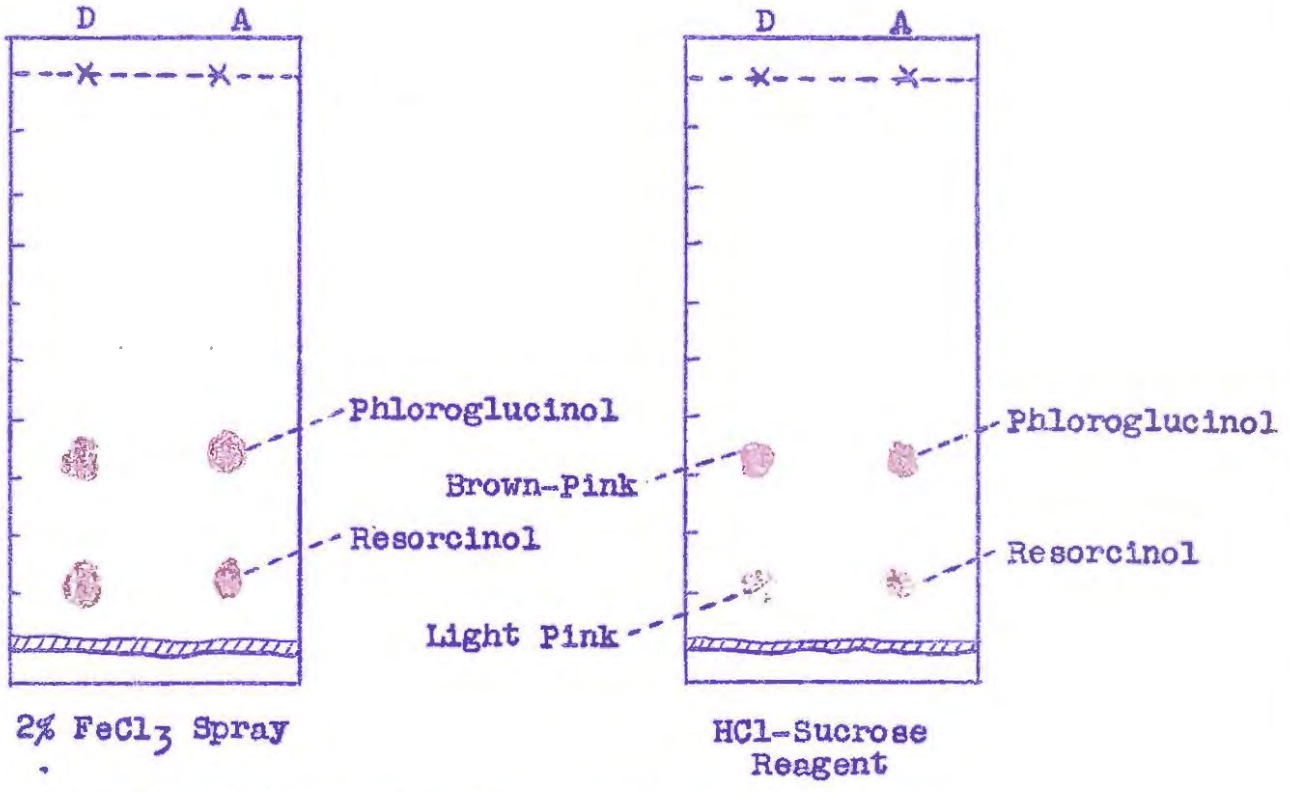



Fig. XXX Chromatogram of the Meta-Hydroxy Phenolic Fraction from Alkaline Fusion. (Butanol - Acetic acid - Water).

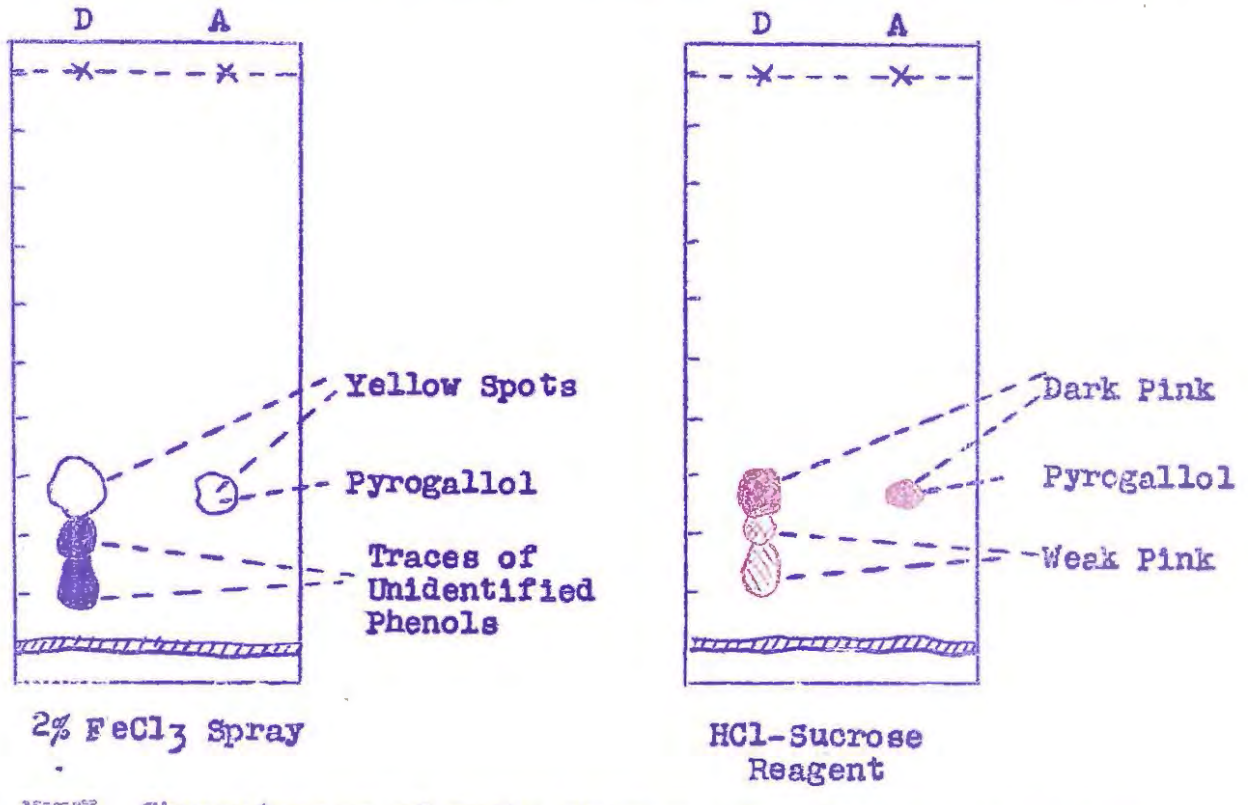



Fig. XXXI. Chromatogram of Ortho-Hydroxy Phenolic Fraction from Alkaline Fusion. (Butanol - Acetic acid - Water)

Freudenberg and Maitland (123) obtained resorcinol in 9.4% yield from the fusion of synthetic 7 : 3' : 4'-trihydroxy-flavanol. Compared to the above Stephen previously obtained resorcinol (1 - 2%) and gallic acid ( ± 1%) by the same reaction.

During the above work on the phenolic fraction a new colour-reagent was devised.

## Colour Reagents for the Paper Chromatography of Di- and Tri-hydroxy Phenols

Various colour-producing spraying reagents have been developed for the detection of di- and tri-hydroxy phenols on paper chromatograms. Excellent results are obtainable with the diazotized sulphanilic acid of Evans, Parr and Evans (211) and the phosphomolybdic acid reagent of Riley (212). Bate-Smith (186, 189) applied Partridge's (379) ammoniacal silver nitrate reagent for furnishing diagnostic evidence regarding the position and number of hydroxy groups and also used ultra-violet light, both in the presence and absence of ammonia vapour, to obtain useful information. The majority of the above class of $C_6$ compounds give characteristic coloured spots with a 2% aqueous ferric chloride spray (186, 189), although meta- and para-hydroxy phenols (for example, orcinol, phloroglucinol, resorcinol and hydroquinone) show ill-defined greyish spots.

Numerous phenols have been used for the identification (229, 230) and estimation (231) of sugars; but the reverse, that is, the use of sugars for the identification of phenols, appears to

have found little application, except in the case of a fructose - hydrochloric acid reagent for the identification of resorcinol.

Sucrose (2.0 grm.) was therefore shaken with a mixture of concentrated hydrochloric acid (10 ml.) and absolute ethanol (90 ml.). Complete solution was not achieved, but the resulting mixture, when lightly sprayed on to paper chromatograms of di- and tri-hydroxy phenols and heated for 40 - 60 sec. at 85 - 95°C., gave the colorations shown in the accompanying table in ordinary and ultra-violet light.

| Phenol | Visible light | Ultra-violet light |
|---|---|---|
| Catechol | Violet | Blue-violet |
| Pyrogallol | Dark-pink | Violet |
| Resorcinol | Light-pink | Weak yellow fluorescence |
| Phloroglucinol | Brown-pink | Maroon |
| Orcinol | Light pink fading to yellow in 1-2 hour. | Light Blue fluorescence |
| Hydroquinone | Grey | Violet |

Variations in concentration produce slightly different shades of colour. Of the above, catechol and hydroquinone require slightly longer heating periods (about 60 sec. or longer) before their characteristic colours appear. The hydrochloric acid - sucrose - ethanol reagent affects the paper only to a slight degree, as very light spraying and short heating times are necessary. Phosphoric acid, while not affecting the paper when used to replace

the hydrochloric acid, gives similar but weaker colourations. Orcinol spots when sprayed with sucrose - phosphoric acid - ethanol show more intense light-blue fluorescence under ultra-violet light.

Fructose may be used in place of sucrose in these reagents, since both give the same colorations when sprayed with resorcinol or naphthoresorcinol in the presence of hydrochloric acid (229) or phosphoric acid (230). Other sugars e.g. rhamnose, laevulose, d-galactose, xylose, d-glucose, arabinose and maltose are also effective and give similar but varying shades of colour. A cheap and easily made reagent capable of giving clearly defined characteristic colours with various polyhydroxy phenols is thus available.

(ii) Oxidation of Methylated Tannins. An Improved Technique.

On account of the consistently low yields of acids from the methylated tannins, it was previously thought likely that if a catechin-type nucleus formed the basic unit of the condensed material, the catechol and pyrogallol nuclei might play an important part in condensation and thus be bound by stable ether linkages whic prevent the isolation of easily recognisable degradation products. (149)(164)(214).

To ascertain whether this is actually the case, an improved technique was devised, and gambier catechin tetramethyl ether was oxidised under the same conditions to compare the yields obtained.

5 grms. of methylated tannins of maximum methoxyl content (36.5%) in 600 ml. of an acetone - water (1 : 1) mixture, were refluxed with the slow addition of solid potassium permanganate. A total of 65 grms. was added in 5 grm. quantities, each time the permanganate colour was discharged. The manganese dioxide was sucked off and washed with fresh acetone - water mixture. The solution and washings were successively concentrated on a water-bath, acidified, and ether extracted. After drying and removal of the ether the residue was vacuum-sublimed (3 min.) at 140 - 180°C. A red residue remained. Some unoxidised ether-insoluble methylated tannins were also recovered after the removal of the acetone. The following is a comparison of yields obtained after the application of the same technique to catechin tetramethylether of MPt. 145°C.

| | From Methylated (36.5% methoxyl) Tannins . | Gambier Catechin Tetramethylether |
|---|---|---|
| Yield of Acidic Product | 38% | 40.8% |
| Yield of Acidic Sublimate | 22% | 36% |
| Red Acidic Residue | 16% | 4.8% |
| Unoxidised Material Recovered | 14.2% | 8.8% |

The 16% of non-volatile (at 180°C. and 3 mm.) red acidic residue obtained from the tannins was re-dissolved in dry ether. A residue (5.8%) of ether-insoluble methylated tannins remained. The 10% extracted was semi-crystalline and yielded a further 3% of mixed acids when extracted with boiling water, thus increasing the total yield to 25%. It appears that the excess of impurities present prevents the easy quantitative sublimation of the mixed acids.

The white sublimates were easily hot-water soluble and recrystallised in white needles of Mpt 142°C. The equivalent weight of the recrystallised product varied from 201 to 206. The acids were separated by Corbett's silver salt method, to give only pure veratric and O-trimethylgallic acids. No evidence of any other methylated phenolic acid was obtained even from the increased yields of this method.

The equivalent weight of the mixture of the two acids varied from one oxidation to another. The values 204.6, 205.7, 200.6 were obtained from different oxidations. Stephen recorded the values 201 and 203 and Corbett 200, 201 and 198, for oxidations under different conditions which resulted in lower recoveries of acids. As the equivalent weight of veratric is 182 and that of O-trimethylgallic is 212, the latter is present in greater proportion (roughly 60 - 80%) in the mixed acids. As O-trimethylgallic acid is known to be less stable to permanganate oxidation than veratric acid (215), the pyrogallol nucleus is probably present in greater proportion than the above figures indicate. This appears

to be confirmed by evidence from the alkaline fusion.

In view of the greater instability of the predominant O-trimethylgallic acid under the conditions of degradation, the yield of acidic nuclei obtained from the methylated tannins is considered to compare favourably with that of veratric acid from catechintetramethylether under identical conditions. The predominant pyrogallol and the minor proportion of catechol nuclei in black wattle tannins thus appear to be attached by carbon chain only after methylation.

The improved yield of mixed acids obtained from methylated black wattle tannins is considered to be due mainly to two factors :

(a) Both the permanganate and methylated tannins are soluble in the solvent mixture, compared with the majority of previous oxidations carried out in aqueous medium in which the methylated tannins are insoluble. Oxidation in a single phase should give improved results.

(b) A fairly rapid oxidation at slightly elevated temperatures appears to improve the yield of degradation products. The boiling-point of acetone is probably the optimum temperature in this instance.

The sublimation technique also appears superior to the usual method of boiling of the products with water to separate the acids.

(iii) Oxidation of the Methylated Tannins after Fractionation

Kirby (164) claimed that acetone-soluble commercial tannins, after methylation with dimethyl sulphate and alkali could be subdivided into three fractions according to their solubilities in

ethanol (20%), benzene (60%), and chloroform (20%). Each fraction was passed through an alumina column mainly to remove unmethylated material. The ethanol- and benzene-soluble portions although differing only slightly analytically, (Found for ethanol-soluble fraction : C = 66.45%, H = 6.54%, $-OCH_3$ = 34.8%. Found for benzene-soluble fraction : C = 66.03%, H = 6.08%, $-OCH_3$ = 35.9%) gave veratric acid and O-trimethylgallic acid respectively on permanganate oxidation. Kirby, therefore, claimed to have effected a true chemical separation.

From the data available in this publication confirmation of Kirby's work was sought, using the above improved oxidation technique. About 20% of the methylated tannins of 32.5% methoxyl content were ethanol-soluble at room temperature, but oxidation after chromatography on an alumina column gave a typical mixture of acids and showed that no separation had been achieved. This was also the case with the ethanol-insoluble but benzene-soluble portion.

Later it was evident from Kirby's thesis (163) that the separation as indicated in his previous publication was incorrect. The fractionation depends primarily on a separation obtained after the methylation of the tannin (100 grms.) in methanol (50 ml.) with dimethyl sulphate (195 ml.) and 50% aqueous KOH (270 ml.) in three stages. Towards the end of this methylation process a brown resin separated with the potassium salts from the methanol-water mixture. The aqueous-methanol solution was decanted and the residue washed with more methanol.

The combined methanol and methanol-water soluble portions

gave 19 grms. of methylated tannin when poured into excess water. 16.5 grms. of this was ethanol-soluble and after chromatography on an alumina column gave a product which, according to Kirby, yielded only veratric acid from permanganate oxidation.

The portion insoluble in methanol and methanol-water was distributed between water (500 ml.) and chloroform (300 ml.) and the aqueous layer extracted with more chloroform. The chloroform-soluble material (80 grm.) was powdered and allowed to stand with benzene. 57 grms. were soluble and 23 insoluble. The former after chromatography on an alumina column was claimed by Kirby to give only O-trimethylgallic acid on oxidation. The latter was identical to the major fraction but less completely methylated.

The latter procedure was repeated by the writer as closely as possible, using half the quantities described. After methylation a gum precipitated as described, but the yield of methanol and methanol-water soluble methylated tannins was only 5 grms. (dry weight) compared to 9.5 grms. anticipated from Kirby's work.

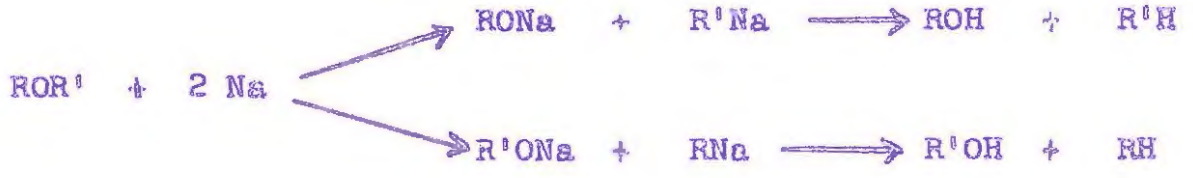
It was felt that due to the empirical nature of the method, greater detail (e.g. temperatures at which fractionations and washings occur) is essential for its successful reproduction, and further attempts were abandoned. It is interesting to note that a one-dimensional chromatogram (water saturated-sec-butanol) of the fresh bark extracted with methanol showed a small green area RF = 0.86 when sprayed with ferric chloride reagent. The

catechol nucleus thus appears to be attached to a separate chemical entity and the above separation thus appears possible.

(iv) Attempted Fissions with Sodium in Liquid Ammonia

From analytical figures alone it is evident that a high proportion of oxygen present in the amorphous tannins does not exist in the form of hydroxy groups. Carbonyl groups are also absent (See Chapter VII), and such oxygen is, therefore, presumably present as ether links which may play an important part in the formation of condensed tannins. Such ether links may either be di-alkyl, alkyl-aryl or di-aryl.

Alkyl-aryl ethers are cleaved with pyridine-hydrochloride (216), pyridylalkali (217) and aluminium chloride (218). Diaryl ethers on the other hand are cleaved by fusion with KOH at 220 - 240°C. (219), and quantitatively by sodium (220)(221) or potassium (222) in liquid ammonia. Aromatic acetals, ketals and certain phenol glucosides are also cleaved by the latter, and reaction occurs according to the scheme :




The mechanism of the fission with sodium or potassium in liquid ammonia has been discussed by Shorygin and Semechkima (223). Alkyl glucosides as well as dialkyl ethers, acetals of the aliphatic

series and dibutyl phenol (224)(225) are not cleaved under such conditions. Shorygin and Semechkina (226) also applied this cleavage method to lignin and obtained an 86% yield of dihydro-eugenol. The cleavage of methylated tannins was thus attempted with the use of sodium in liquid ammonia.

5 grm. of fully methylated tannins (36.5% methoxyl) were introduced into a large thermos flask containing 20 grms of sodium dissolved in 300 ml. liquid ammonia. The mixture was agitated continuously with a small stirrer inserted into the flask through a loosely-fitting stopper. After 6 hours reaction the ammonia was removed and the product allowed to hydrolise slowly by exposure to the damp atmosphere. Water was finally added and the alkaline solution acidified. A flocculent precipitate resulted which was sucked off. The acidic solution was ether-extracted but no small phenolic break-down products could be detected in the ethereal extract. The precipitate was partly ether-soluble and resembled the methylated or partly-methylated tannins.

The ether-soluble portion was methylated once with dimethylsulphate in the usual way. Found : % $-OCH_3$ = 32.13. The ether-insoluble portion was also analysed. Found : %-$OCH_3$ = 22.39.

It appears that over a 6-hour period no cleavage other than dimethylation takes place. No easy cleavage as with diaryl ethers was evident, and no small degradation products were indicated. Very prolonged action (cooled in dry ice) on the partly

methylated tannin (to simulate lignin) might give the desired effect, but was not attempted as the method did not appear promising.

(v) Bromination and Oxidation of the Brominated Product

Stephen (149) attempted the complete bromination of the tannins, as well as their methylated and acetylated derivatives in various solvents. Various degrees of bromination were reported, and a proportion of the bromine was always labile and easily removed with silver nitrate.

In an effort to stabilise the resorcinol nuclei in the tannins, so that they might survive subsequent permanganate oxidation which normally causes their destruction, controlled bromination was carried out as follows :

34.6 grms. of methylated tannins, dissolved in 100 ml. glacial acetic acid, were cooled in an ice-salt mixture. 8 grms. of bromine (1 equivalent per $C_{15}$ methylated unit) was added in small quantities with thorough shaking, and the reaction-mixture allowed to stand for 2 hours. The acetic acid solution was poured into iced water and the flocculent brominated product sucked off and washed with water.

After drying; the product (6 grms.) was oxidised with permanganate (62 grms.) in the same way as the methylated tannins. The acidic product isolated set to an oily yellowish semi-crystalline gum, insoluble in cold water but easily soluble in acetone, ethanol and ether. The acid was difficult to recrystallise and charcoal treatment did not remove the light yellow colour. After

recrystallising poorly from acetone-water and subsequently from ethanol it melted at 128°C., with much softening beforehand. When redissolved in bicarbonate solution some decarboxylation appeared to have occurred even during gentle heating on a water-bath.

Found : Br = 33.63%, C = 41.50%, H = 3.90%
$-OCH_3$ = 24.47%, Equiv. wt. = 296.3

Calc. for $(CH_3O)_2$ Br.$C_6H_2$.COOH :
Br. = 32.5% C = 41.38%, H = 3.45%
$-OCH_3$ = 23.80%, Equiv. wt. = 261

Bromine estimations were carried out by the semi-micro method of Peel, Clark and Wagner (227). The compound was fused with potassium nitrate, sucrose, and sodium peroxide in a Parr bomb for two minutes. After reduction of any bromate formed, bromide was estimated by the addition of silver nitrate in the usual way. The method was standardised against dibromodimethoxy-resorcinol. Found : Br. = 54.8%. Calc. for $C_8H_8O_2Br_2$ : Br = 54.1%.

The impure acidic mixture thus approximated to a mono-bromodimethoxybenzoic acid containing some impurity. Separation of the mixture by chromatography on a silica gel (228) has yet to be attempted.

(vi) Atmospheric Oxidation of Black Wattle Tannins.

The permanganate oxidation of the tannin was found by

Corbett (146) to result in the complete disruption of all phenolic nuclei. Shuttleworth (232) more recently studied the effect of atmospheric oxidation on alkaline solutions of the tannin. From various titration-curves it was found that a relatively large amount of acids of varying strengths was formed, and Shuttleworth's conditions were thus repeated on an increased scale in order to identify these acids by paper chromatogram.

10 grms. of lead-salt purified tannins were dissolved in 250 ml. water and 60 ml. N NaOH added. Air from a pressure system was bubbled through the solution at a steady rapid rate (2 bubbles per second) for about 3½ hours. The oxidised solution was acidified, ether extracted and the acid fraction isolated by the bicarbonate technique. A small quantity of reddish gum resulted which smelt strongly of formic acid. The gum was examined on a paper chromatogram using butanol - acetic acid - water (4 : 1 : 5) as partitioning mixture. A mixture of gallic, protocatechuic and $\beta$-resorcylic acids was spotted on simultaneously for comparison (Fig. XXXII).

The developed chromatogram of the oxidation mixture showed a brown streak from the origin to about RF = 0.90. This probably consisted of unseparated oxidation-products. Under ultraviolet light two prominent blue areas were discernible in this streak at RFs = 0.68 and 0.88. Sprayed with 2% ferric chloride a weak blue spot at RF = 0.68 and a still weaker violet spot at RF = 0.90, corresponding to gallic and $\beta$-resorcylic acids respectively, could be discerned. Formic and possibly other aliphatic acids together with traces of the aforementioned two

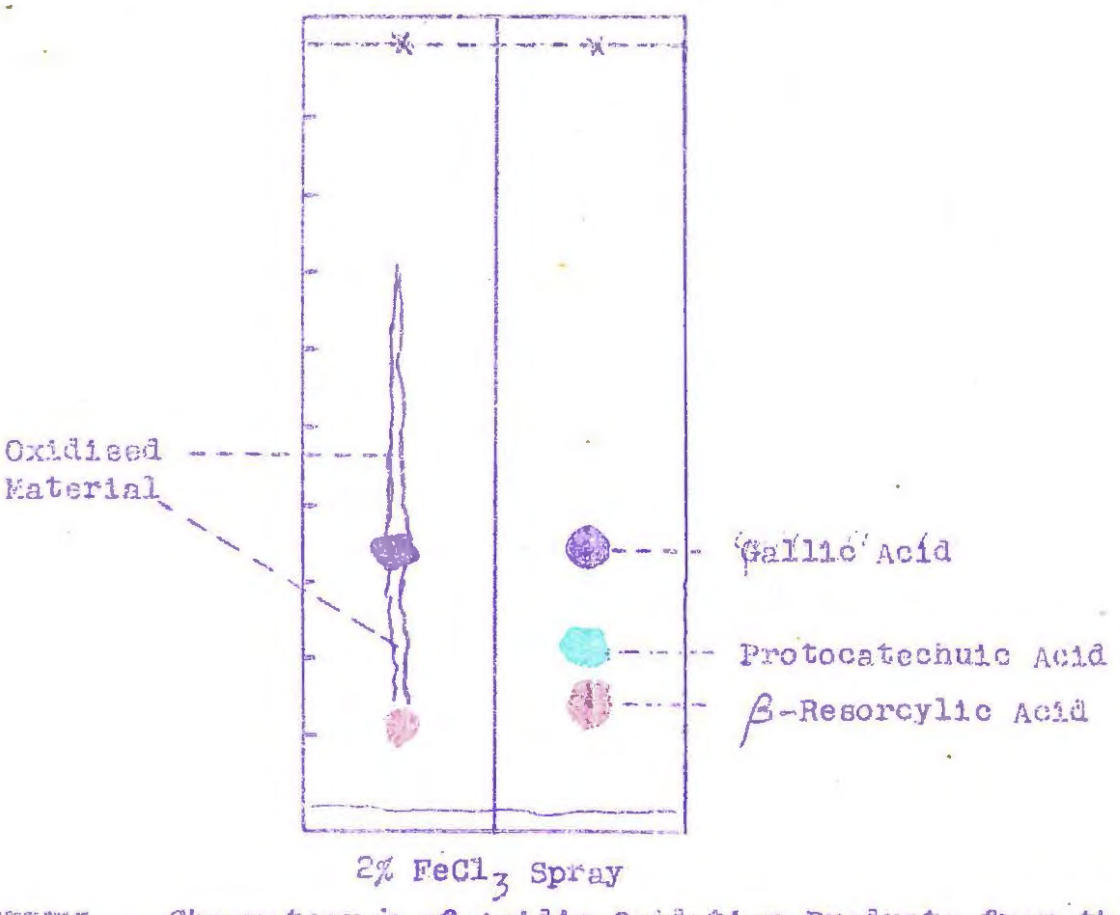



Fig. XXXII Chromatogram of Acidic Oxidation Products from the Alkaline Atmospheric Oxidation of Black Wattle Tannins

phenolic acids thus constitute the endproducts of alkaline atmospheric oxidation.

The predominant presence of formic and possibly other aliphatic acids is not surprising as biologically important substances containing phenolic nuclei are easily degraded in a stepwise manner, ending eventually in the simple aliphatic acids, as a result of oxidative bacterial metabolism (233).

(vi) Ester Linkages in Black Wattle Tannins

Strong acids are known to cause the condensation of black wattle tannins rather than their hydrolyses, while strongly

alkaline solutions have been shown by Shuttleworth (232) to cause degradation either in the presence or absence of atmospheric oxygen.

Hydrolysis of the extract with hot concentrated sulphuric acid during the isolation of fisetin, however, was found to produce low proportions of gallic acid and resorcinol, and it would be of interest to determine whether the purified tannins could be hydrolised once they were stabilised by methylation.

Tannin methylated with dimethyl sulphate was considered unsuitable as it had already been subjected to drastic alkaline treatment in hot methanol, (which could have already caused hydrolysis) during the methylation process. Diazomethane - methylated tannin (34.0% methoxyl) was thus subjected to hydrolysis with sulphuric acid and saponification with alkali under the usual conditions. No acidic hydrolysis-products could be isolated, and this shows the absence of ester linkages in the tannins.

(vii) Summary and Discussion of Fission Products

From the present investigation on the tannin the following facts have been established :

(a) Improved degradative techniques coupled with superior separation methods and paper chromatography have shown that high yields of phenolic nuclei are obtainable from black wattle tannins, compared with the exceptionally low yields previously obtained. From controlled alkaline fusion a 43% mixture of ether-soluble degradation products was obtained consisting of : $\beta$ -resorcylic

acid (4.2%), gallic acid (about 11%), and protocatechuic acid (low proportion), resorcinol (10.5%), pyrogallol (about 1%) and phloroglucinol (trace). The phloroglucinol is considered to originate from the gallic acid under the conditions of fusion. The above figures represent yields isolated and, therefore, do not account for manipulative losses. A very low proportion of ether-soluble oxidised material also accompanied the phenolic bodies.

(b) β-Resorcylic acid was isolated from the tannin for the first time without the prior stabilisation of the nucleus by nitration. β-resorcylic and protocatechuic acids, pyrogallol and traces of phloroglucinol were identified amongst the degradation products of alkaline fusion for the first time. Resorcinol and gallic acid were obtained in greatly improved yields.

(c) From a study of the fusion reaction it was evident that the phenols were formed by the decarboxylation of the corresponding phenolic acids at elevated temperatures in the presence of alkali. Only potential β-resorcylic, gallic and protocatechuic acid nuclei thus constitute the tannins.

(d) Ester and diaryl-ether linkages appear absent.

(e) Increased yields of methylated acids have been obtained from the methylated tannins after alkaline permanganate oxidation. In this reaction the resorcinol nucleus was destroyed and no evidence of its presence could be detected in spite of the improved yields.

The behaviour of the tannins is in this respect similar to that of the catechins and related $C_{15}$ compounds. The isolation of fully methylated catechol and pytogallol carboxylic nuclei, shows that after methylation these are attached to the tannin residues by carbon chain only.

(f) Atmospheric oxidation of the tannins in alkaline solution yielded very low quantities of gallic and $\beta$-resorcylic acids which are the predominant nuclei. They are accompanied by larger amounts of aliphatic acids, possibly mainly formic acid.

(g) Bromination of the tannin followed by permanganate oxidation yielded what appears to be a monobromo-dimethoxybenzoic acid, accompanied by impurities from which it could not easily be freed.

(h) A new sucrose - HCl spraying-reagent has been found to assist identification of $C_6$ phenols obtained on alkaline fusion.

(i) From Kirby's earlier work it appears that at least a low proportion of the resorcinol nucleus is not joined in a pyrane ring after methylation. Kirby also claimed the separation of the methylated tannins into two chemically-dissimilar fractions. Although other evidence shows that this is possible, no separation could be achieved in this work due to inadequate description of an empirical method.

(j) The improved alkaline degradation technique is most suitable for investigating the constitutional nature of the various constituents of the black wattle tannin fraction.