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STUDIES ON NATURALLY OCCURRING PLANT PHENOLS

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ABSTRACT

Various monocotyledenous and dicotyledenous plants have been examined and found to possess an enzyme capable of deaminating 3,4-dihydroxyphenyl (β-) alanine (DOPA) with the formation of trans-caffeic acid. The activity of this enzyme in barley, dandelion and wheat is relatively high, whereas in broad-bean leaves it is low and in potato tubers tissues, negligible. It is possible that in the broad-bean, this low activity may be, at least in part, responsible for the high concentration of DOPA in the tissues.

Broad-bean leaves also contain a tyrosine deaminase.

It has been shown that "foreign" phenols can move rapidly up and down the stems of broad-bean plants and that glucosylation of many of these compounds readily occurs. Phenols fed into the cut ends of the main veins of leaves quickly appear in the sieve tubes of the stem. This has been proved by the use of the aphid, Macrosiphium pisi. Aphid techniques have also strongly suggested that the sieve tubes of broad-bean and willow normally contain phenolic compounds.

The reaction of boron trichloride and methanol on cinnamic acid derivatives has been investigated in detail and a procedure has been devised which aids the identification of these acids in plant tissues. Using this method all glycosidic, esterified and methoxylated derivatives are converted to the corresponding mono-, di-, or tri-hydroxy-methyl cinnamates which can be identified on paper chromatograms or electrophoretograms. Thus the cinnamic acids present can be classified into one or more of these families.

The paper electrophoretic examination of high molecular weight phenols as azo dye derivatives has also been investigated. These phenols have been coupled with diazotised p-aminobenzoic or diazotised sulphanilic acids. The resulting dyes possess strongly dissociated acidic groupings and therefore exhibit relatively high electrophoretic mobilities in comparison with the unsubstituted phenols.

ACKNOWLEDGMENTS

CONTENTS

INTRODUCTION AND DISCUSSION
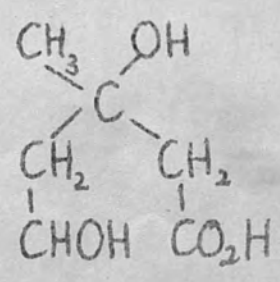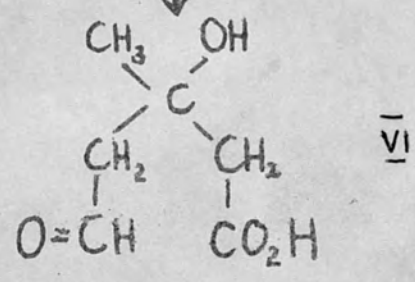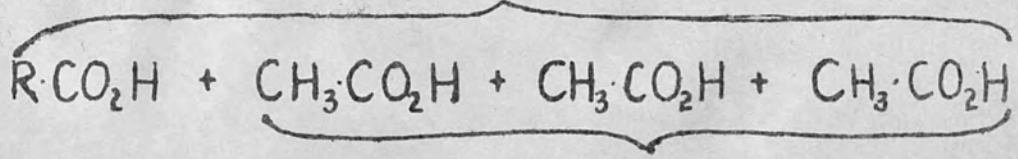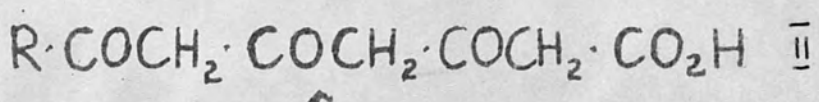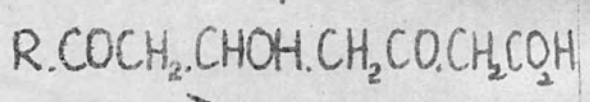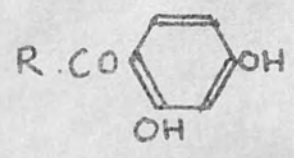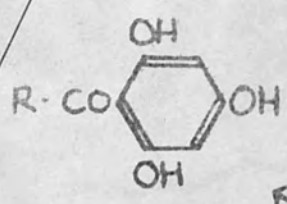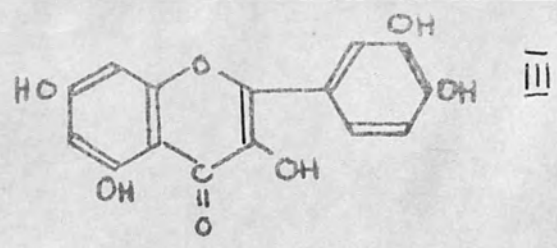
FIG I

DOPA DEAMINASE IN PLANTS

Biosynthesis of Phenolics

There exists, at present, two accepted pathways which lead to the build-up of naturally occurring phenols in plants (see Fig. I).

The first is the "acetate unit" pathway,[1,2,3,4] whereby the production of many aromatic compounds with the resorcinol and phloroglucinol hydroxyl patterns, e.g. the flavonoids, is postulated. The compounds arise by head to tail linkages of acetate units. Using isotopically labelled acetate it has been demonstrated that the biosynthesis of fatty acids in plant tissues from acetic acid, or acetic acid together with higher fatty acids,[5,6] proceeds without loss of carbon. For example, the synthesis of butyric acid (I) in Clostridium kluyveri proceeds as follows:

$$CH_3.CO_2H + CH_3CO_2H \longrightarrow CH_3.CO.CH_2.CO_2H$$

$$\downarrow + CH_3.CO_2H \qquad Fig. (II)$$

$$CH_3.CH_2.CH_2.CO_2H \quad (I)$$
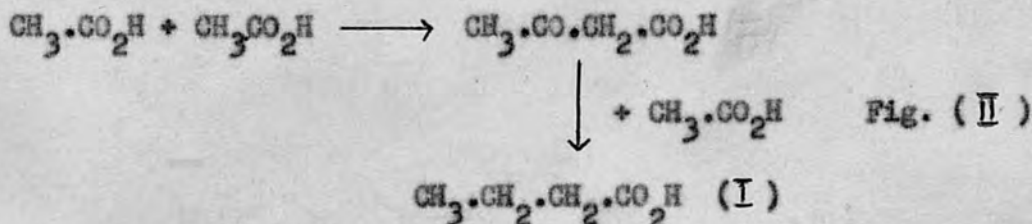
No enzyme studies have been made of the mechanism of acetate assimilation by plants or bacteria, leading to the formation of the lower fatty acids, nor to the role played by coenzyme A[7] (3', 5'-diphosphoadenosine-4'- phosphopantetheine) in their synthesis.

However, the synthesis of higher fatty acids (e.g. citric, succinic and malic acids) from acetate units proceeds by way of the intermediates of the tricarboxylic acid cycle.[20] Coenzyme A is necessary not only for the activation of acetate, but also for the conversion of all the carboxylic acids into an active form. No plausible explanation has been found for the chemical mechanism of acetate activation because of the coenzyme A molecule has a number of functional groups, which make several different reaction mechanisms possible. Lynen has, however, established that the thiol group is the active centre of the coenzyme A molecule.

The β-keto acid intermediate ($\bar{\text{I}}$) in the build-up of acetate units is, at present, purely hypothetical, and may exist only as an enzyme bound complex, probably of the coenzyme A ester.

Birch[8] noticed that in many naturally occurring phenols, the hydroxyl groups in the aromatic ring were on alternate carbon atoms, e.g. ring A of flavonoids such as quercetin ($\bar{\text{III}}$).

$$(\bar{\text{III}})$$
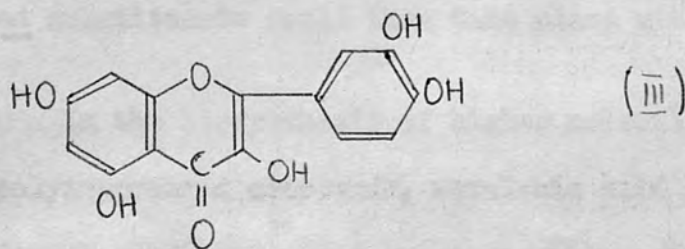
The first biochemical evidence to support the theory that naturally occurring aromatic compounds arise by head-to-tail condensations of acetic acid molecules was provided by Birch, Massy-Westropp and Moye [9] when they found that <u>Penicillium griseofulvin</u> formed 6-methyl salicylic acid ($\overline{IV}$) from ^{14}C-carboxyl-labelled acetate (Fig.$\overline{III}$).

<u>Fig. $\overline{III}$</u>

$$4.CH_3^*.CO_2H \longrightarrow$$

$(\overline{IV})$
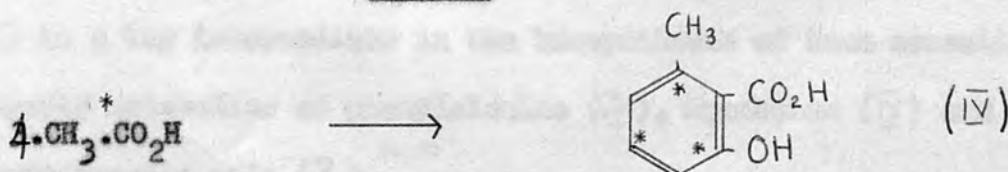
The pathway for the formation of a phloroglucinol ring is shown in Fig. $\overline{I}$, where $R.CO_2H$ can be any naturally occurring acid such as a fatty acid, a terpenoid acid, a branched chain acid or a cinnamic acid. The formation of resorcinol derivatives can be explained by reduction of a carbonyl group, not involved in cyclisation, in a non-aromatic intermediate. This is more likely than the reductive removal of a hydroxyl group from the benzene ring. Introduction of more oxygen atoms <u>ortho-</u> or <u>para-</u> to existing oxygenated substituents could then take place without difficulty.

In the biosynthesis of higher molecular weight compounds such as polyisoprenoid compounds, mevalonic acid ($\overline{V}$) has been found to be a direct precursor [10] and, more recently, attention has been focused towards establishing the intermediates between acetate and

mevalonate.[11]   One such intermediate is thought to be 3-
hydroxy-3-methyl-glutaraldehydic acid ($\overline{VI}$).

A second pathway for the formation of phenols arose
from the observations of Davies[12] who, in 1954, published the
results of work on intermediary metabolism in microorganisms.
He established, by the method of mutants that shikimic acid[13]
($\overline{VII}$) is a key intermediate in the biosynthesis of such essential
aromatic metabolies as phenylalanine ($\overline{VIII}$), tryptophan ($\overline{IX}$) and
p-aminobenzoic acid ($\underline{\overline{X}}$).[14,15]

($\overline{IX}$)   ($\underline{\overline{X}}$)
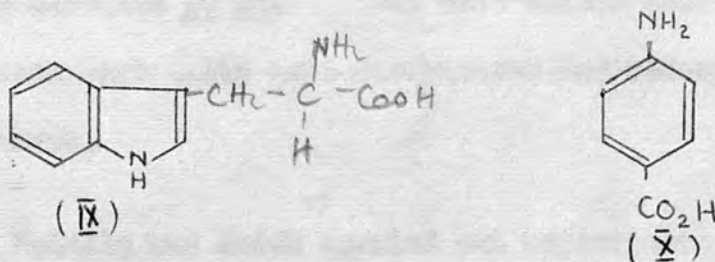
Shikimic acid ($\overline{VII}$) is first formed from the condensation
of a tetrose ($\underline{D}$-erythrose-4-phosphate [$\overline{XI}$]) and a triose (phospho-
enol pyruvate [$\overline{XII}$]) by way of a 2-keto-3-deoxy-$\underline{D}$-araboheptonic acid-
7-phosphate[16,17] ($\overline{XIII}$). The latter compound cyclises to give 5-
dehydroquinic acid ($\overline{XIV}$) and this is then converted to 5-dehydroshikimic
acid ($\overline{XV}$) by loss of one molecule of water which is catalysed by 5-
dehydroquinase. This enzyme, which apparently requires no co-
enzyme, has been extracted from Escherichia coli.[17] and Vicia faba.
5-Dehydroshikimic acid ($\overline{XV}$) is reduced by an NADP-dependent de-
hydrogenase to shikimic acid ($\overline{VII}$). A C_3 side chain, arising from
pyruvic acid is then added to an activated phosphate derivative of

shikimic acid ($\overline{\text{VII}}$) with the formation of prephenic acid ($\overline{\text{XVI}}$)
via the intermediary Z_1. The chemical structure of Z_1 has not been
positively identified although it is thought to be an ~~pre-arranged~~ [isomeric]
form of prephenic acid. ($\overline{\text{V}}$) Prephenic acid is the branching point
of the system. It is unstable and readily decomposes (non-enzymically)
to phenylpyruvic acid ($\overline{\text{XVIII}}$) or to p-hydroxyphenyl-pyruvic acid ($\overline{\text{XIX}}$).
There is no reason to believe that there is a pathway from phenyl-
pyruvic acid ($\overline{\text{XVIII}}$) to the corresponding p-hydroxy derivative ($\overline{\text{XIX}}$).
The presence of these ~~various~~ [cyclohexane carboxylic acid] intermediates shown in Fig. 1 was
confirmed [in higher plants.] by Hasegawa et al.[18,19] ~~who also showed that shikimic acid~~
~~and cyclohexane carboxylic acid derivatives had widespread distribution~~
~~in plant tissues.~~

McCalla and Neish[37] carried out experiments feeding shikimic
acid to Salvia splendens. Subsequent examination of the plants
showed that both phenylalanine ($\overline{\text{VIII}}$) and tyrosine ($\overline{\text{XX}}$) had been
synthesised. This, and other reported studies[22,23] showed that
phenylpyruvic acid ($\overline{\text{XVIII}}$) and p-hydroxyphenylpyruvic acid ($\overline{\text{XIX}}$) can
both undergo transamination reactions with the formation of phenyl-
alanine and tyrosine, respectively. Tyrosine ($\overline{\text{XX}}$) has an alternative
pathway via enzymic hydroxylation of phenylalanine ($\overline{\text{VIII}}$)[22,24]
and 3,4-dihydroxyphenylalanine (DOPA-) can be formed by the
hydroxylation of tyrosine.[25] A pathway to cinnamic acid [$\overline{\text{XXI}}$] from
phenylalanine ($\overline{\text{VIII}}$) via phenyllactic acid ($\overline{\text{XXII}}$) - Fig. $\overline{\text{IV}}$ was put
forward by Neish.[27]

Fig. IV

CH_2-CH CO_2H NH_2

(VIII)

CH_2-CH CO_2H OH

(XXII)

$CH=CH.CO_2H$

(XXI)
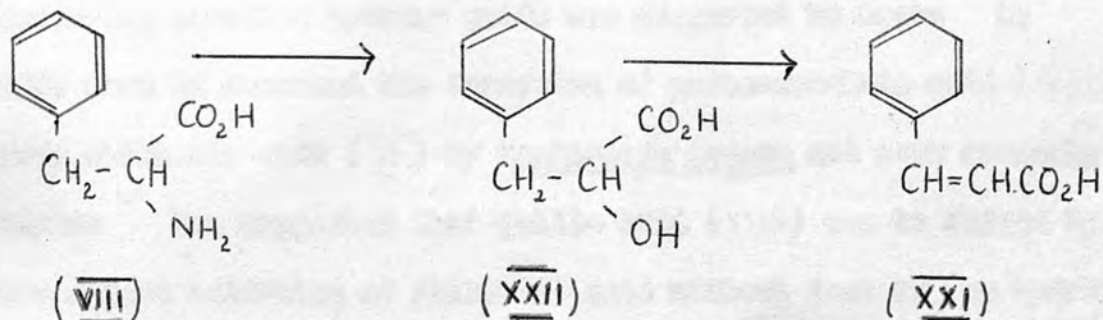
In 1961, Koukol and Conn published results
showing that phenylalanine could also be converted to cinnamic
acid (XXI) by direct deamination using enzymes from Gramineae
and Leguminosae.[26] Neish[28] later discovered an enzyme, tyrase,
which effected the conversion of tyrosine to trans-p-coumaric acid
(XXIII) in Gramineae, also by direct deamination. This enzyme was
not found in any of the legumes that he examined. Further evidence
for the presence of deaminases in plants is provided by our own
studies which are discussed later (see page 18).

An alternative pathway for the formation of the
phenolic cinnamic acid derivatives involving hydroxylation and
methoxylation, was put forward earlier by McCalla and Neish in
1959.[29, 30] They found that p-coumaric acid could be formed very easily
from cinnamic acid in Salvia splendens that that p-coumaric acid
(XXIII) could in turn, by converted to caffeic acid (XXIV), which
then gave rise to the two commonly occurring methoxylated cinnamic
acids, ferulic acid (XXV) and sinapic acid (XXVI).

A route (shown in Fig. $\bar{1}$) to the commonly
occurring aromatic hydroxy acids was suggested by Cross[21] in
1958 when he reported the formation of protocatechuic acid ($\overline{XXVII}$)
from shikimic acid ($\overline{VII}$) by Neurospora crassa and more recently
Haslam[31] has suggested that gallic acid ($\overline{XXVIII}$) can be formed by
the direct oxidation of shikimic acid without loss of the hydroxyl
functions (Fig. $\bar{1}$).

Both the acetate and shikimic acid pathways are
apparently required for the formation of phenolics with a C_{15}
skeleton. These compounds are very widely distributed in the plant
kingdom.[33,34,35,36] Several groups of workers have carried out
experiments using isotopically labelled compounds related to
shikimic acid, phenylalanine and acetic acid. In this way, it has
been shown that both the flavonol, quercetin ($\overline{III}$)[23] and the
anthocyanidin, cyanidin ($\overline{XXIX}$)[37] are formed by a combination of the
two biochemical pathways. ^{14}C-Labelled shikimic acid ($\overline{VII}$), p-
coumaric acid ($\overline{XXIII}$), phenylalanine ($\overline{VIII}$) and cinnamic acid ($\overline{XXI}$)
can all act as precursors of the $C_6^{(B)}$-C_3 portion of quercetin in
buckwheat.

$\overline{XXIX}$
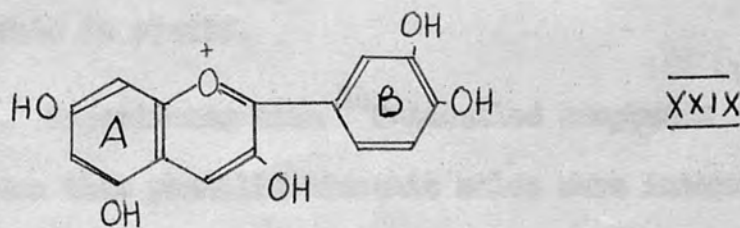
There is no evidence, at present, as to whether the hydroxylation occurs before, or after the formation of the C_{15} nucleus. Ring A arises from acetic acid as was shown by feeding ^{14}C methyl- and carboxyl-labelled acetic acids.

The different classes of C_6-C_3-C_6 compounds once formed in the plant are capable of interconversion by oxidation or reduction of the C_3 moieties. The details of these interconversions are still speculative as intertransformation between the main groups of flavonoids in the plants has not so far been shown.

Once the pathways to the simpler naturally occurring phenolic and other aromatic compounds had been satisfactorily examined, a multitude of questions concerning their transformation to more complex products, such as lignin, were raised. Hibbert[38,39,40] [41] suggested that simple precursors of lignin were interconverted by reactions similar to those in the respiration of carbohydrates. Neish[27] considered it more probable that the phenolic cinnamic acids were intermediates, since they were so widely distributed in trace amounts in plants.[42]

Experiments with ^{14}C-labelled compounds[43,44] substantiated the idea that phenolic cinnamic acids were intermediates in lignin formation. Conversion of ^{14}C-labelled tyrosine to

lignin occurred readily in certain plant species, such as
members of the Gramineae. Neish[28] suggested that a tyrase
(tyrosine deaminase) was necessary for one stage in the
conversion of tyrosine to lignin.

Acerbo[45] found that ^{14}C-labelled p-hydroxyphenyl-
pyruvic acid (XIX) was converted to lignin in sugar cane.
As sugar cane also contains tyrase the following sequence of
reactions has been postulated by Neish (Fig. $\overline{V}$).

p-hydroxyphenylpyruvic acid

$\downarrow$

L-tyrosine   (Fig. $\overline{V}$)

$\downarrow$

trans-p-coumaric acid

$\downarrow$

LIGNIN
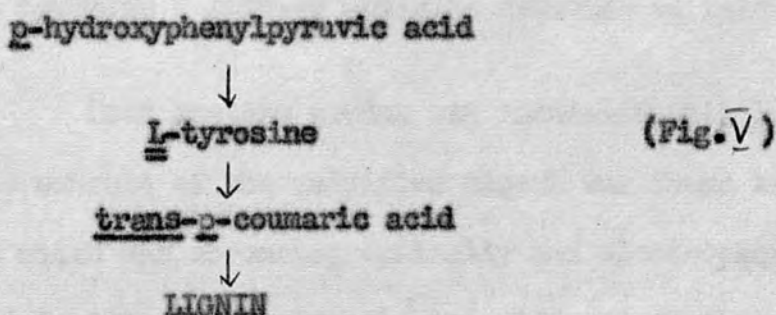
It seems, likely, therefore, that the C_6-C_3
compounds are the most likely precursors of secondary phenolics
substances in plants.

FURTHER STUDIES WITH PLANT DEAMINASES

In view of the relatively high concentrations of
tyrosine ($\overline{\overline{XX}}$) and 3,4-dihydroxyphenylalanine ($\overline{\overline{XXX}}$) in <u>Vicia faba</u>
tissues, it was of interest to examine this plant for the
corresponding deaminases. Neish[28] had shown that tyrase was absent
in the legumes, pea (<u>Pisum sativum</u>), lupin (<u>Lupinus albus</u>) and
clover (<u>Melilotus alba</u>), but had not examined the broad bean
(<u>Vicia faba</u>) and so preliminary experiments were carried out to
see if a tyrosine deaminase could be detected in this plant.

Bean acetone powder was incubated with <u>L</u>-tyrosine and
the ether extract of the acidified digest was found to contain a
compound which was chromatographically and electrophoretically
identical to <u>cis-p</u>-coumaric acid and differed on chromatograms
from <u>trans-p</u>-coumaric acid (Table $\overline{I}$).

The $\lambda_{max.}$ of the enzyme-synthesised compound
(measured in dilute alkali) was 290 mμ (<u>i.e.</u> the same as for
<u>cis-p</u>-coumaric acid), as compared with 333 mμ for the <u>trans</u>-isomer
(Fig.$\overline{VI}$). The reason why the <u>cis</u>-isomer is formed with the broad-
bean tyrosine deaminase, but not with the other plant deaminases
examined by Neish, is not at present understood. Our experimental
procedures were similar to those of Neish, except that a Tris-HCl
buffer, instead of a borate buffer, or a mixture of borate and

Fig $\overline{VI}$

$\overline{I}$ trans-p-coumaric acid
$\overline{II}$ cis-p-coumaric acid
$\overline{III}$ enzymic product.
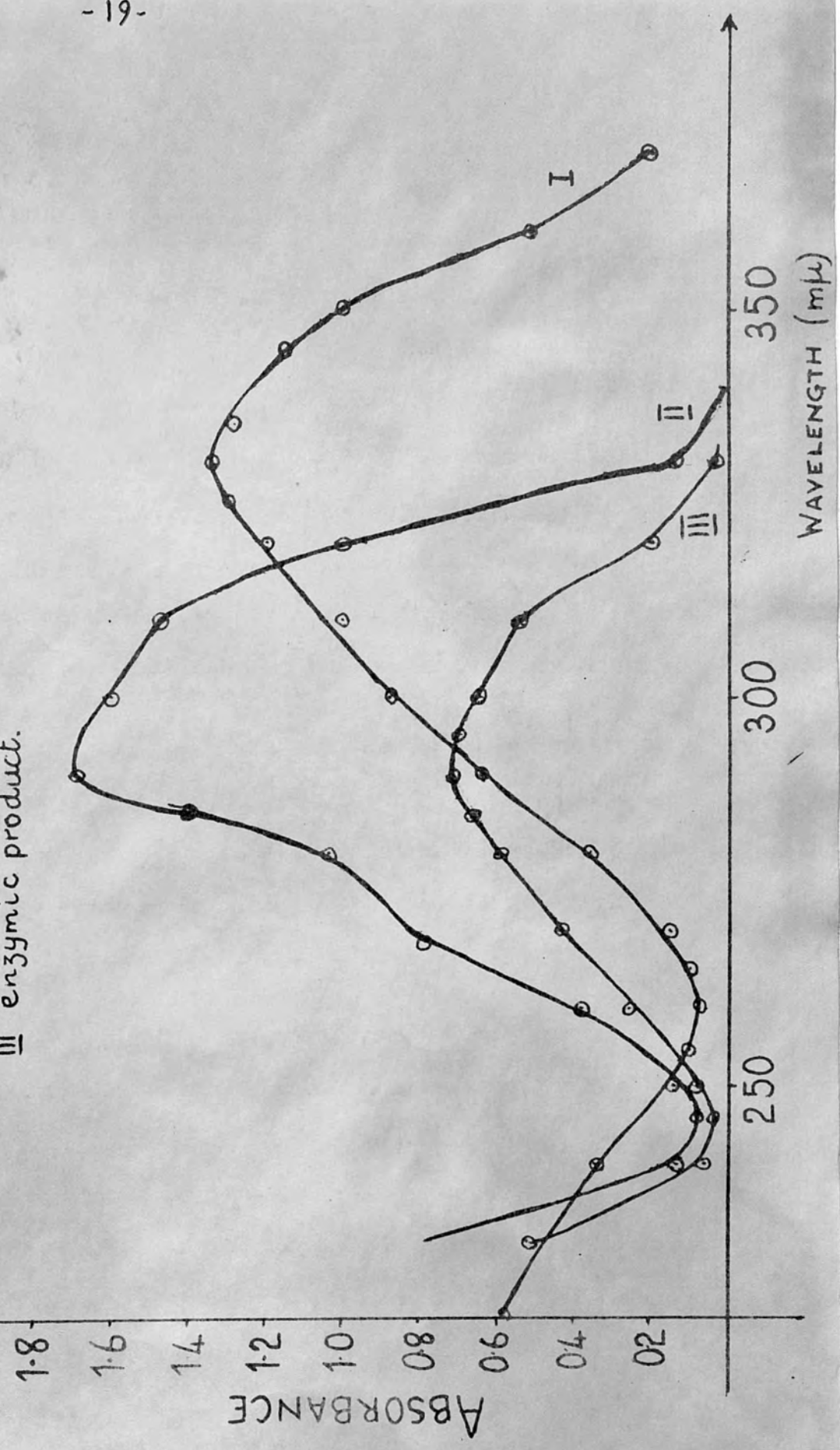
Chromatographic and Electrophoretic Examination of Tyrosine deaminase product in V.faba

Table I (and Caption)

	Tyrosine	trans-p-coumaric acid	cis-p-coumaric acid	Reaction product
R_f values in:				
Butanol;ethanol;water (A)	0.14	0.29	0.60	0.60
Ethyl acetate; acetic acid;water (B)	0.23	0.90	0.89	0.89
2% Aqueous Acetic acid (J)	0.20	0.36	0.66	0.65
M_{SA} values in:				
Sodium borate buffer (A)	0.64	1.00	1.01	1.00
Ammonium molybdate buffer (B)	0.20	0.55	0.55	0.55
Sodium acetate buffer (C)	0.18	0.44	0.44	0.44
Colour with Spray reagent A	Pink	Orange→Blue	Orange→Blue	Orange → Blue

Tris-HCl buffers, was used in the enzyme digests. It is, of course, possible that the broad-bean deaminase and Neish's tyrase function by slightly different mechanisms. It should also be emphasised again that Neish was unable to find a deaminating enzyme in the three leguminous species that he examined.

The presence of DOPA has not been noted in many species of plants, but it has been shown to occur in some Vicia species and, by chromatography, we have shown that Isatio tinctoria, Ajirga reptaus and Toireya myristica also contain traces of this.

Outside the plant kingdom DOPA is of more common occurrence, and in both the plant and animal kingdoms is an intermediate in the biosynthesis of melanin.[46] It is a particularly interesting compound because it is both an amino acid and an ortho-dihydric phenol.

In the broad-bean, DOPA occurs in unusually high con-centrations and is certainly responsible for the blackening of the tissues when the plant is injured, and at the onset of senility. It is also interesting to note that dopamine (XXXI) is responsible for the blackening of bananas (Musa sapientium).[49]

OH

OH

$CH_2-CH_2.NH_2$
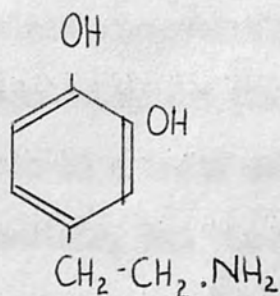
(XXXI)

The reason why such large quantities of DOPA are
present in V. faba may be due to either an abnormally high
rate of synthesis, or to the inability of the plant to metabolise
the amino acid once it has been formed. It was therefore decided
to examine suitable plants in an attempt to discover a DOPA
deaminase and, in particular, to see if this enzyme was lacking
in the broad bean. The deamination of DOPA is, of course, a
possible biosynthetic pathway for the formation of caffeic acid. (XXIV)

A preliminary examination of leaf acetone powders from
several plant species (this consisted of incubating the powders
with DOPA and examining the products in ether extracts of the
digests) suggested that a DOPA deaminase existed, and that there
was a particularly high activity in the dandelion (Taraxacum sp.)

The product obtained by incubating a dandelion leaf acetone

powder with DOPA was examined exhaustively, on paper
chromatograms and electrophoretograms (Table $\overline{\text{II}}$). In the presence
of molybdate ions, it produced a brown coloured, charged complex,
a typical reaction of ene-diols, and the presence of this
grouping in the molecule was substantiated by the observed high
electrophoretic-mobility in borate buffer (A).

The presence of a carboxyl group in the product was
suggested by the mobility in acetate buffer, (pH 5.2).
In all systems the product was indistinguishable from authentic
trans-caffeic acid. The ultra-violet spectrum of the compound
was also identical to that of authentic trans-caffeic acid and it
also showed the characteristic hypsochromic and bathochromic
shifts with alkali[48] and borate ions, respectively (Fig.$\overline{\text{VII}}$).
The latter change in spectrum is, of course, also strong evidence
for the existence of an ene-diol grouping. Catalytic hydrogenation
of the enzymic reaction product resulted in the formation of a
compound, which was indistinguishable from hydrocaffeic acid (β-3,4-
dihydroxyphenylproprionic acid) on paper chromatograms (R_f values
in solvents A and B, 0.79 and 0.93, respectively), and electrophoretograms
(M_{SA} value with buffer C, 0.81), and gave the same blue-coloured azo
dye with the diazotised p-nitroaniline/sodium hydroxide spray reagent.

Chromatographic and Electrophoretic Examination of DOPA deaminase product

Table II (and Caption)

R_f value in:	Reaction product
Butanol:ethanol:water (A)- plain paper	0.71
molybdate paper	0.08[*]
Ethyl acetate:acetic acid:water (B)	0.89
Toluene:acetic acid:water (F)	0.00
10% Aqueous acetic acid (G)	0.41
Phenol, water (H)	0.34
2% Aqueous acetic acid (J)	0.80
M_{SA} value in:	
Sodium borate buffer pH 10.0 (A)	1.16
Ammonium molybdate buffer pH 5.2 (B)	0.98[*]
Sodium acetate pH 5.2 (C)	0.48
Ammonium phosphate pH 7.2 (E)	0.61

[*] Brown molybdate complex formed

FIG VII

I ORIGINAL EXTRACT FROM CHROMATOGRAM
II $\frac{3}{250}$ M Na.OEt ADDED TO I
III $\frac{3}{250}$ M BORIC ACID ADDED TO II
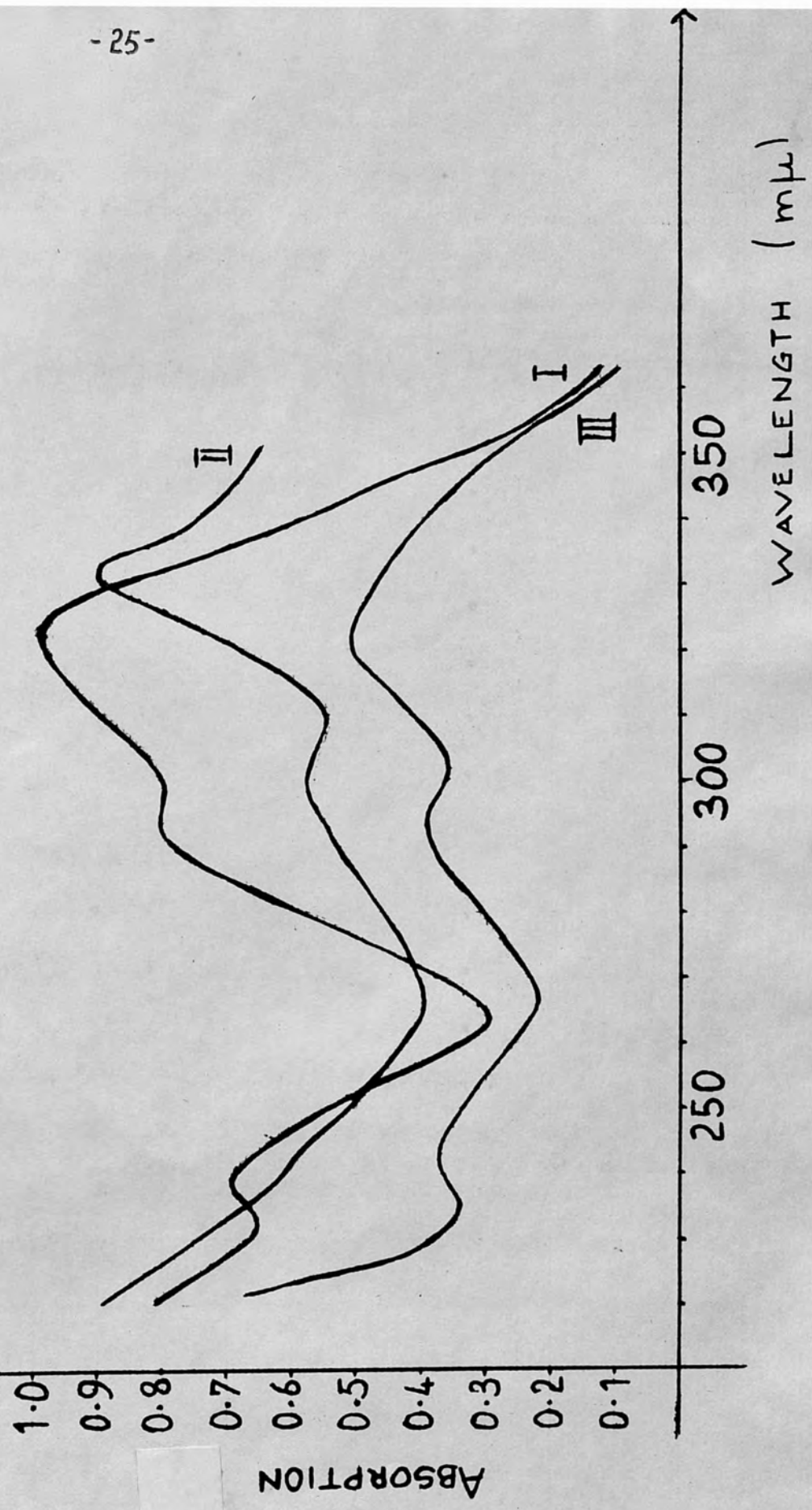
During the course of the enzyme reaction, it was
established with Nessler's reagent[50] that ammonia was also
produced. The enzyme producing caffeic acid from DOPA in the
dandelion is therefore a true deaminase. (Fig. $\overline{\text{VII}}$)

Fig $\overline{\text{VII}}$

$(\overline{\text{XXX}})$ $(\overline{\text{XXIV}})$
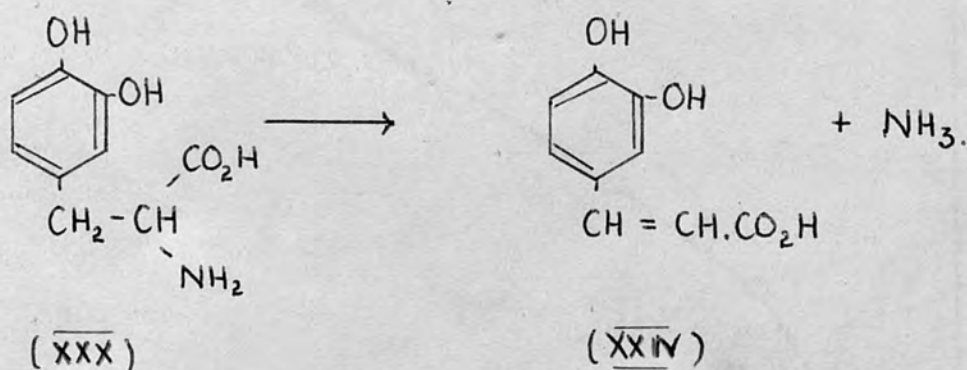
Quantitative estimation

The DOPA deaminase was estimated by measuring the
rate of formation of caffeic acid. The acetone powder, and DOPA
in buffered solution, was incubated for a suitable period of time
at 25°. The solution was then acidified, extracted with ether and
the extract resolved on a paper chromatogram with butanol:ethanol:
water solvent (solvent A). (The latter procedure was included to
remove trace impurities, which were apparently derived from the
acetone powder during the incubation. The caffeic acid spot was
then located with U.V. light and measured spectrophotometrically at $320 m\mu$
after extraction from the paper with water (Fig. $\overline{\text{IX}}$). One unit

FIG. XIII
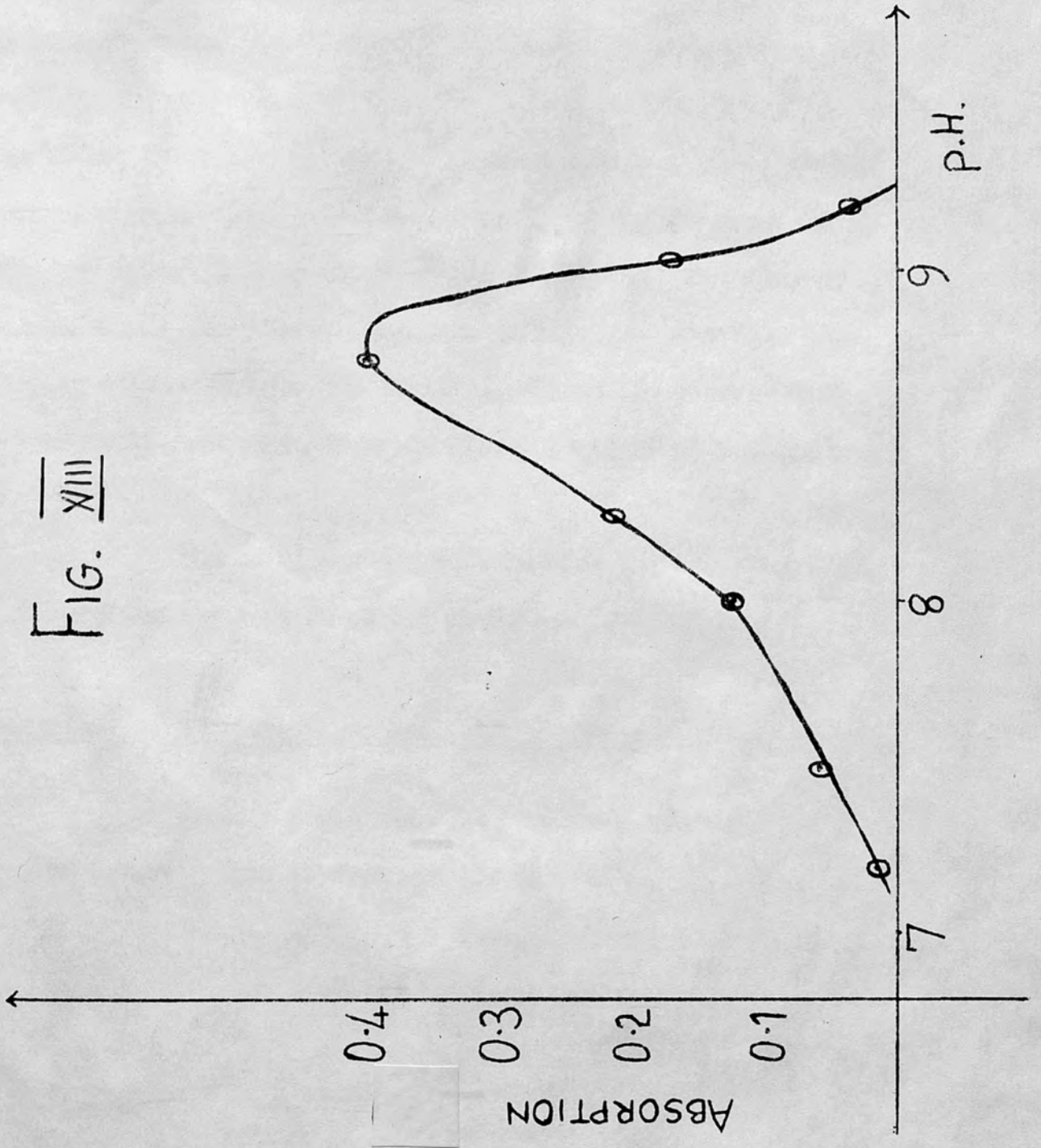
of DOPA deaminase was defined as the amount of enzyme which would catalyse the formation of 1mM caffeic acid in 1 hr. under the conditions described in the experimental section. The rate of formation under these conditions was linear for the first 20-30 mins. of incubation and then rapidly decreased. Browning of the solutions, even in the presence of nitrogen, may have been largely responsible for the "tailing off" of the rates. Unit measurements were taken from the linear portions of the graphs.

The pH of maximum activity for the dandelion DOPA deaminase was shown to be pH 8.8 in Tris-HCl buffer (Fig. $\overline{\text{VIII}}$).

Comparison of DOPA deaminase activity in different plants

Table $\overline{\overline{\text{III}}}$ lists the DOPA deaminase activities of acetone powders prepared from various species of plants.

Table III

Source	Age (days)	Yield of acetone powder (% fresh wt.)	Units of DOPA deaminase per g. acetone powder
Dandelion leaves	?	10.6	1.17
Wheat shoots	7	11.4	0.43
Barley shoots	6	6.2	2.03
Bean stem and leaves	40	10.9	0.12
Potato tuber (unpeeled)	?	14.2	0.00

Dandelion and barley (Hordeum sp.) possessed relatively high deaminase activities, whereas broad-bean gave a low activity and no activity could be detected in potato tuber tissue.

Broad-bean cotyledons, germinated and ungerminated also contained no detectable DOPA deaminase. In the case of the broad-bean leaf and stem there did not appear to be much variation in enzyme activity with the age of the plants (Table IV).

Table IV

Source	Age (days)	Units of DOPA deaminase per g. acetone powder
Bean stem and leaves	7	0.10
	14	0.10
	21	0.11
	28	0.12
	35	0.10
	42	0.12
	49	0.13

Effect of Inhibitors and Activators on DOPA deaminase

Table V summarises the effect of various potential enzyme activators and inhibitors on the formation of caffeic acid by DOPA deaminase.

Table $\overline{V}$

Reagent 1ml. 0.01M added (final concentration-0.01M) to 9ml enzyme solution	Relative rate (% of control)
Sodium molybdate	43
Sodium borate	68
p-chlore mercuriacetate *phenyl*	47
potassium cyanide	86
L-cysteine	120

Neish found that with crude barley tyrase preparations, cysteine produced inactivation, but the thiol activated purified preparations. p-Chloromercuribenzoate inactivated all preparations as did cyanide.

With dandelion acetone powder DOPA deaminase, molybdate had a marked inhibitory effect probably due to complex formation with the enzyme substrate. Borate also produced a decrease in activity, presumably for the same reason. Borate buffers used by Neish for determining tyrase activity could therefore not be used with DOPA deaminase. The deaminase would appear to require -SH groups for its activity as it was inhibited by p-chloremercuriacetate *phenyl* and activated by cysteine. Slight inhibition also occurred with cyanide.

In conclusion, it may be said that an enzyme capable of deaminating DOPA with the formation of caffeic acid exists in some monocotyledenous and dicotyledenous plants. At the present time, it is not known whether this enzyme plays an important part in the synthesis of caffeic acid in the plant. The apparent substrate for the enzyme, DOPA, has only been reported in the literature to occur in a few species, and it is difficult to ascertain whether it is a rare compound in the plant kingdom, or w hether it is commonly occurring in small quantities which, in the past, have escaped detection. The presence of little, or no, detectable DOPA in a species could mean that it was not formed, or once formed, was very rapidly metabolised, perhaps via DOPA deaminase. The low deaminase activity in the broad-bean may well account for the abundance of DOPA in this plant, although an abnormally high rate of synthesis of this compound could also be an important factor. Tyrosine also appears to be at a high level in the bean and this could also result from the inability of the plant to metabolise DOPA, as tyrosine is presumably a direct precursor of DOPA. In addition, it is interesting to note that bean tissues contain only small amounts of cinnamic acid derivatives.

Future studies in connection with DOPA deaminase should include a survey of the occurrence of DOPA in plants, and a quantitative examination of the relationship between this phenolic amino acid, the caffeic acid derivatives (e.g. ferulic

acid) and the DOPA deaminase activity of tissues. Another

interesting line for investigation, in view of inhibitions by

molybdate and borate, would be the effect, _in vivo_, of

deficiencies in these ions (and possibly those of other trace

elements) on the DOPA and the related cinnamic acid derivatives

in the tissues.

Although it is apparent from the preceding discussion

that a great deal is now known about the biosynthetic pathways

leading to the formation of phenolics, virtually no information is

available in the literature regarding the translocation of these

compounds and their direct precursors. The translocation of

phenols has now been examined and is discussed in the second section

of this thesis (page 34).

TRANSLOCATION OF PHENOLS IN THE BROAD-BEAN (Vicia faba)

It is no longer possible to picture translocation
in plants as consisting simply of an upward movement of
absorbed mineral elements in the xylem and a downward
movement of elaborated nutrients in the phloem. It is
now known that organic compounds move in the xylem as well
as in the phloem, and some inorganic ions move in the
phloem.[52,53] It is true to say, however, that the phloem
content of a plant is mainly organic matter, 90% being
carbohydrates. Zimmermann[54] in his review of transport in
the phloem reports that movement in opposite directions at
any one time and in any one channel has never been found,
and that most papers describing movement in opposite
directions can be explained in terms of upward movement
in the xylem and re-translocation from the leaves through
the phloem.

Little work has been reported on the trans-

location of phenolics in plants. It has not been es-
tablished whether natural plant phenols can move down the
phloem from the leaves where they are biosynthesised,
or whether the complex, high molecular weight phenols found
in the stems and branches of plants, such as the tannins
or lignin, arise by translocation of smaller, non-phenolic
molecules, e.g. acetate, which are then converted to
phenolic compounds in these organs. Hathway[55] has stated
that ~~pyrogallol~~ gallocatechins can be translocated intact in
Quercus sp.,
but little conclusive evidence has been put forward for
the translocation of higher molecular weight phenolic
compounds.[56,57] U.S.

The broad-bean (Vicia faba) has now been used
to investigate this problem further. This plant was chosen
as it is easily cultivated and has a relatively simple
phenolic content.

An examination of the bean leaf showed that the
main phenolics present were rutin (quercetin-3-rutinoside,
kaempferol glycosides, quercetin and quercetrin).

phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine

and small amounts of unidentified derivatives of p-

coumaric, caffeic, ferulic and sinapic acids.[35]

OH

HO ... OH ─ _Rutin_

OH ... O glucose-rhamnose

Kaempferol
glycoside ─ HO ... OH

OH ... O-glycoside
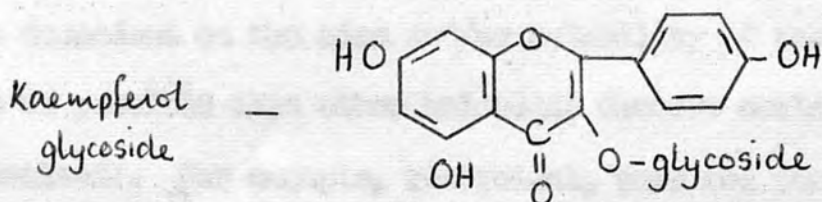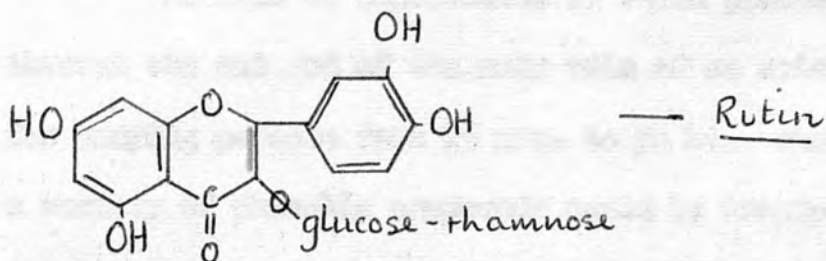
    The stem epidermis and inner stem tissues also

exhibited a similar phenolic pattern.

    To ascertain whether any phenols could be trans-

located around the plant, experiments were carried out

using "foreign" phenols, i.e. those not found naturally

in the broad-bean, and which could be detected easily in

the plant tissues by chromatographic methods. The movement

of these compounds was measured in cm./10 min. down

the main stem, and also relative to the movement of

resorcinol (Table VI).

Results of experiments in which plants were fed,
through the cut end of the main vein of an apical leaf,
for varying periods from 15 min. to 24 hr., showed that
a variety of phenolic compounds could be transported down
the stem of the plant. The rate of movement appeared to
be dependent on the size and/or solubility of the molecule
(it is possible that other metabolic factors could also be
involved). For example, resorcinol, when fed through an
apical leaf reached all parts, including the root, of a
30 cm. high plant within 30 min., whereas quercetin took
10 hr. to move a similar distance. Tables VI and VII show that   _(p 92, 93)
phenolic glycosides move more rapidly than their cor-
responding phenolic aglycones. In this connection, Roberts [59]
has suggested that because flavones and flavonols, but not
the corresponding glycosides, are adsorbed strongly by
cellulose   that translocation of these aglycones can
be hindered unless glycosylation can occur in the 3-
position. Whether glycosylation of the flavonoids which

were fed through the vein of the leaf, occurred at some stage, is unknown, but both quercetin and kaempferol were found throughout the plants as aglycones after 24 hr. feeding.

In most cases, the low molecular weight foreign phenols were partially converted to the corresponding glucosides, and the latter compounds were found together with the aglycones throughout the plants. In the natural state, it is possible that only glycosidic or other hydrophilic derivatives are normally translocated.[60-64] Most phenolic aglycones have a toxic effect on the plant.[51] These phytotoxic compounds are usually lipophilic in character, but possess hydrophilic groups and are therefore surface active. Such compounds could interfere with the function of cell vacuoles, by interaction with the tonoplast membrane. Addition of further hydrophilic groups, such as glycosyl, would nullify the toxicity. In the experiments described, the phenols were fed in excess and therefore the plant would be incapable of completely converting the phenols to glucosides. When small amounts of resorcinol were fed, only resorcinol-β-glucoside appeared to be translocated.

Translocation down from the leaves occurred at
a much slower rate than the upward passage of phenols
which had been fed in through the cut ends of the main
stems of bean plants. Compounds fed by the latter
method were probably taken upward in the transpiration
steam. The comparative rates of movement, relative to
resorcinol, for upward feeding are given in Table $\overline{\text{VII}}$. (p 93)
Feeding time had to be limited to 1 min. in this case
because of the rapid movement.

Two foreign sugars, L-rhamnose and D-ribose were
also fed in through the cut stems, and it was surprising
to find that they moved more slowly than many of the phenols
that were tested. Ribose had a rate of 0.42 relative to
resorcinol and rhamnose a relative rate of 0.25. The
difference in rate of the two sugars was possibly due to
the greater solubility of D-ribose in aqueous solutions.

During the feeding experiments with D-ribose a
reducing sugar (X) with a low mobility on paper chromatograms
was formed in small amounts. It had R_G values similar to
those of pentose disaccharides and also gave a colour reaction

typical of a sugar with a pentose reducing group (pink)
with Spray B. With increased time of feeding there was
also an increase in the formation of (X) and the gradual
appearance of a second compound (Y) (R_G 0.59 in solvent
B) which also gave a pink colour with Spray B.

It seemed probable at first that (X) was a di-
saccharide formed from the D-ribose. Experiments with
the broad-bean plants of various ages showed that (X) was
only produced by plants from 3 to 12 weeks old.

Electrophoretic examination showed that compound
(X) was non-acidic (zero mobility in acetate buffer, pH
5.2 (C)), and that it had a higher mobility in borate
buffer, pH 10.0 (A) than xylobiose (4-O-β-D-xylopyranosyl-
D-xylose) and therefore probably possessed more adjacent
hydroxyl groups, capable of complexing with the borate,
than xylobiose. (X) also gave a positive reaction with
triphenyltetrazolium chloride (spray reagent H), and
therefore was not a 1→2 linked disaccharide, and it gave a
blue colour with aniline-diphenylamine (spray reagent E),
which is characteristic of a 1→4 linked disaccharide.

β-Glucosidase had no effect on the unknown sugar. In-
sufficient material was available for further studies
but these preliminary results suggested that (X) was a
1→4 linked pentose disaccharide, the linkage possibly
having an α-configuration, and from the electrophoretic
rate of movement at least one monosaccharide residue could
have been D-ribose.

A Heptose?

The direct conversion of a monosaccharide to an
oligosaccharide, if such is the case, is unusual in intact
plant tissues, and warrants further investigation.

Another technique for investigating the movement of
phenols in plants has also been used. The foreign phenol
was dissolved in Tween 40 (Exp. XIII) p 94 and a droplet of this
solution placed on the leaf epidermis and allowed to diffuse
into the plant. Here again the phenol, and the correspond-
ing glucoside (in the case of resorcinol, catechol, quinol
and saligenin) were detected in the stems of plants which
had this treatment. Movement of the compound down the plant
appeared to be much slower using this method. This was
presumably due to the slow diffusion of the phenol through
the epidermis to the vascular tissue. One advantage of this
method is, however, that it causes no mechanical damage to

the plant and the phenols may enter the vascular system by
a process which is somewhat nearer to the one which occurs
naturally.

65-69

In 1957 Mittler published results he obtained
utilising a completely new technique for examining the
phloem content of plants. It has been long accepted that
an aphid, during feeding, inserts its stylet specifically
into the phloem sieve tubes of the host plant. Mittler
devised a method for cutting the stylet away from the
feeding aphid, leaving it embedded in the sieve tube.
The sieve-tube sap of a stem is under considerable turgor
pressure, which forces the sap up the isolated stylet and
it can be made to exude for many hours from the cut end.
The sap can be collected in a capillary tube, the exudation
(translocation) rate measured and the contents of the sieve
tubes examined qualitatively and quantitatively. The
advantage of this technique is that the exudate comes only
from the phloem and not from the phloem plus surrounding cells.
With other methods for examinind phloem contents, such as
making incisions in the vascular tissue, [70,71] contamination from
surrounding tissue is usually inevitable.

Mittler worked with Tuberolachnus salignes, a large aphid from which it is relatively easy to cut the stylet. In the present study, experiments were carried out using Tuberolachnus salignes feeding on Salix daphnoides, in an attempt to discover phenolic compounds in the phloem of this plant. These experiments had to be curtailed as cell sap could not be induced to exude from the cut stylets. Further investigation into the method, showed that Mittler's results were a culmination of many month's work, and the number of times that the stylets had success- fully exuded sap were relatively few, due possibly to the varying turgor pressures in the willow stems at different periods of the year.

A second approach to this project was an exam- ination of the honeydew of Tuberolachus salignes feeding on Salix daphnoides (Exp.XIV) on paper chromatograms, the idea being that any phenols appearing in the honeydew would probably have been derived from the willow phloem. Phenolic compounds were detected in the honeydew using Spray A and ultraviolet light, but different collections of honeydew from the same stems, appeared to contain different phenolic contents.

The honeydew was next compared with the leaf and bark
contents of Salix daphnoides (Exp.$\overline{XV}$) and although there
was some similarity in the phenols present in the bark,
leaf and honeydew, results were not very satisfactory as
the phenols common to all three extracts were not easily
identifiable but tyrosine and possibly cinnamic acid derivatives did
appear to be present (Table $\overline{XVI}$).

As many of the phenolics in the broad-bean had been
previously identified, aphids feeding on this plant were
next examined for phenolics. Colonies of Microsiphum pisi
were cultivated on a number of broad-bean plants. This
species of aphid was too small to apply the cut stylet tech-
nique but large quantities of honeydew were collected and
examined on paper chromatograms for phenolic content. When
feeding on the bean leaves the insects excreted honeydew on
to the lower leaves and this could be easily collected and exam-
ined (Exp.$\overline{XVII}$) chromatographically.

In this way, it was shown that the honeydew did possibly
contain phenols that were present in the host plant (e.g.
DOPA and flavonoids were identified) and, presumably these
compounds originated from the leaf phloem and were therefore
translocatable materials.

Extracts of whole feeding aphids were also examined on paper chromatograms (Exp. XVI) and compared with the honeydew which had been "milked" from insects which had been feeding on the same item and methanolic extracts of the stem. These experiments showed that the honeydew and stem and insect body extracts all contained similar substances including DOPA and flavonoids (Table XVII).

Colonies of Microsiphum pisi were cultivated on the stems of the beans, and the plants were then fed with foreign phenols through the main veins of apical leaves (Exp. XVIII). The results in Table show that the smaller molecular weight, phenols such as quinol, catechol, resorcinol and ferulic acid, were found in the aphid extracts and therefore were presumably present in the phloem. It was noted that only the free phenols were found in these experiments and not the accompanying phenolic glycosides as might be expected. This may mean that the foreign phenols are not glucosylated in the sieve tubes but in other stem tissues. However, another possibility is that phenolics found in the honeydew or in the aphid are modifications of the phloem constituents. Hence with whole-aphid experiments the possible effect of the aphid itself on these compounds should always be considered.

The enzyme activity associated with the aphid
body was therefore examined (Exp.XIX9XX) Results showed that
buffered aqueous extracts of the insects contained α- and
β-glucosidases, α-galactosidase and an esterase, capable
of hydrolysing chlorogenic acid. Phenolase (catecholase)
was also present which oxidised catechol and catechin
(Exp.XIX) and which would presumably oxidase other ortho-
dihydric phenols.   It is not known where in the aphid
body these enzymes were located but it is reasonable to
assume that they are present in the gut, and therefore
could and probably do modify phenolics originating from
the phloem.   Another interesting possibility is that these
enzymes may be partially derived from the plant itself.
In any case, these enzymes constitute a definite hazard in
examining whole aphids or honeydew for phloem contents.
This disadvantage does not arise with the aphid-head technique.

These series of experiments have shown that phenolic
compounds were present in the phloem of willow and broad-bean
and, therefore, presumably were being translocated. They have
also indicated that foreign phenols fed into the leaves rapidly
enter the sieve-tubes of the stem. The actual mechanism of
translocation of all organic substances is at present a

controversial subject. It seems most probable that naturally
occurring phenolics are translocated as glycoside derivatives or as
derivatives of other hydrophilic substances. Low mol. wt. phenols
are rarely found in the free state in plant tissues. No phenolic
glycosides were found in the aphids which had fed on plants
that had been treated with foreign phenols. This does not
necessarily mean, however, that the glycosides were not present
in the phloem, as these could easily have been hydrolysed by
aphid enzymes. (There is some evidence to suggest that the aphid
can hydrolyse arbutin whilst feeding (Table $\overline{XXI}$)). The stylet
technique would obviously be the best method for checking this if
a suitable aphid for the broad-bean could be found. Alternatively,
foreign phenols could be fed to Salix sp. and the stylets of Tuberolachnus
salignus used.

The Identification of Naturally Occurring Cinnamic Acid Derivatives

During the past ten years boron trichloride has proved to be one of the most reactive substances known. It is a strong Lewis acid, and therefore the majority of organic functional groups can react with this reagent because they mostly contain an element with a lone pair of electrons. Gerrard and Lappert[75] have reviewed the reaction of boron trichloride with all the main classes of organic compounds.

Cinnamic acid derivatives are widely distributed throughout the plant kingdom.[42] It has been shown that they are capable of inhibiting the growth of parasitic microorganisms[74] in certain species and van Sumere showed[75] that they have an inhibitory effect on spore germination of wheat stem rust.

Cinnamic acids have been found to behave as plant growth factors.[76-78] Although some plants contain free hydroxycinnamic acids,[28] the majority of these compounds have their carboxyl groups esterified with glucose or quinic acid.[79-82] In addition, the phenolic hydroxyl groups may be methylated, as in ferulic ($\overline{XXV}$) and

sinapic acid (XXV) derivatives. In rare instances, a phenolic hydroxyl group may be glucosylated. Examples of such compounds are given below: (Fig $\bar{X}$)

Fig $\bar{X}$

CH_2OH

O ‖ $O \longrightarrow C \longrightarrow CH = CH$

$\underline{OH}$

HO

OH

- $\underline{\rho}$ - coumaryl glucoside

OH
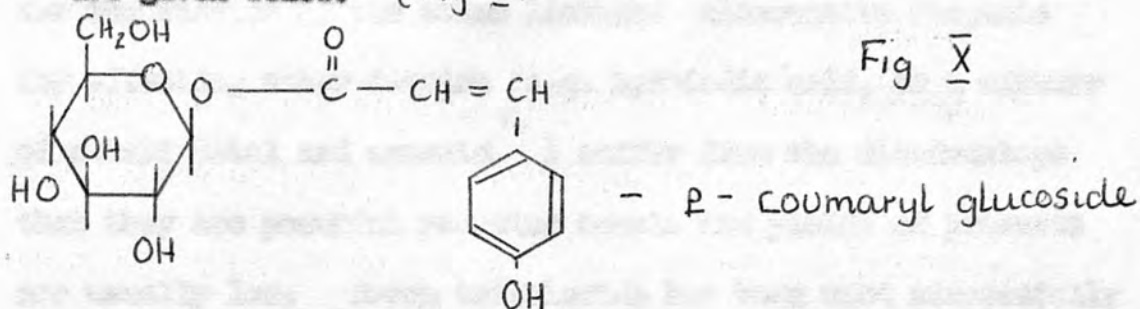
ALSO $\underline{o}$- and $\underline{m}$- coumaryl glucosides
caffeoyl and ferroyl glucosides
sinapyl glucoside ; 3,4,5-trimethoxycinnamic glucoside

$\underline{o}$-coumaryl gentiobioside,
caffeoyl gentiobioside,
$\underline{\rho}$-coumaryl rutinose.

CH_2OH

O $O \longrightarrow \langle phenyl \rangle \longrightarrow CH = CH. CO_2H$

$\underline{OH}$

HO

OH

OH

- ALSO caffeic acid, 3-β-glucoside

ALSO   caffeic acid, 4-β-glucoside

OH

OH

chlorogenic acid

$CH = CH - C$ ‖ O

HO

OH

$HO \longrightarrow$

CO_2H

HO
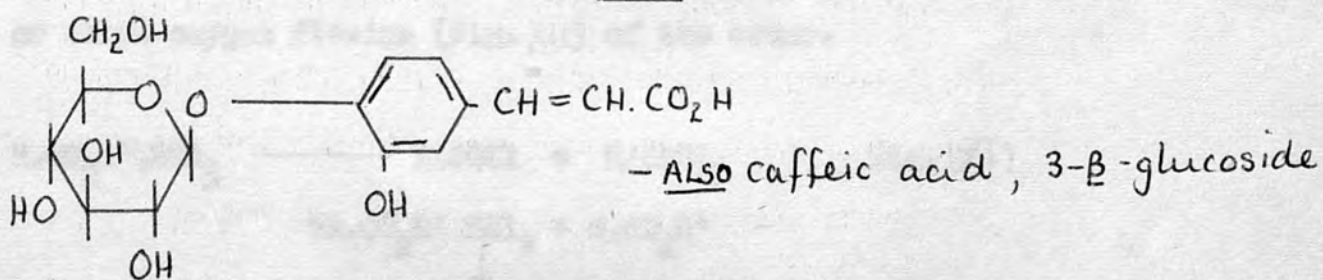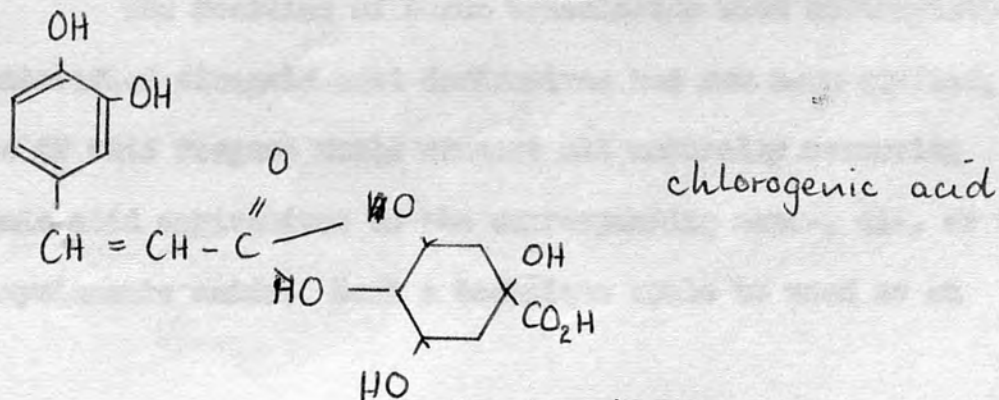
ALSO   cinnomyl quinic acid

The reaction of boron trichloride with ethers
is of particular interest because it provides a simple method
for the fission of the ether linkage. Alternative reagents
for effecting ether fission (e.g. hydriodic acid, or a mixture
of alkali metal and ammonia [84]) suffer from the disadvantage
that they are powerful reducing agents and yields of products
are usually low. Boron trichloride has been used successfully
by Allen, Bonner, Bourne and Saville for the dealkylation of
sugar derivatives. The reaction conditions using boron tri-
chloride are very mild and hence the yields are good.

With carboxylic esters [83], boron trichloride forms a
complex which then decomposes with acyl-oxygen fission (Fig.$\underline{XI}$)
or alkyl-oxygen fission (Fig. $\underline{XII}$) of the ester.

$$R.CO_2R'.BCl_3 \longrightarrow R.COCl + R'OBCl_2 \qquad \text{Fig.}(\underline{XI})$$

$$2R.CO_2R'.BCl_3 + R.CO_2R'$$
$$\downarrow$$
$$(RCO_2BCl)_2O + 3R'Cl + RCOCl \qquad \text{Fig.}(\underline{XXII})$$
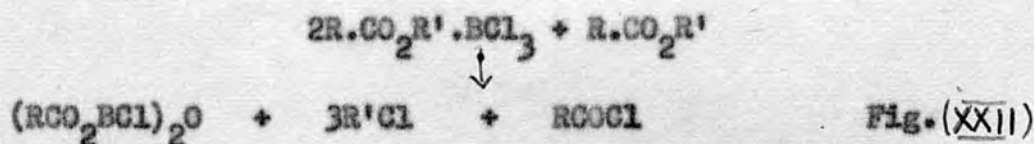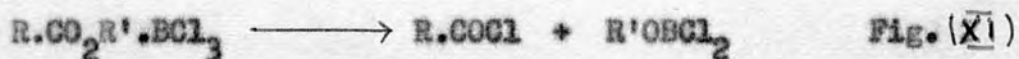
The reaction of boron trichloride with methoxylated
and esterified cinnamic acid derivatives has now been studied,
to see if this reagent would convert all naturally occurring
cinnamic acid derivatives to the corresponding mono-, di-, or tri-
hydroxycinnamic acids. Such a technique could be used as an

aid to the identification of cinnamic acids in plant tissues.

Initial experiments were carried out with authentic ferulic (XXV), sinapic (XXVI), chlorogenic (XXXV) and 3,4-dimethoxycinnamic acids. The derivatives, in dichloromethane, were treated with boron trichloride (Experiments XXII and XXVII) at -78°. The reaction mixtures were left to stand at room temperature for several hours, before excess boron trichloride was removed under reduced pressure. The reaction products were then distilled exhaustively with methanol to destroy any borate complexes, and to remove borate as methyl borate. Finally, the products were examined on paper chromatograms and electrophoretograms. The results are given in Table VIII.

Results of Initial Experiments with Boron Trichloride/Methanol

Table VIII (and Caption)

Starting Material	R_f values		M_{SA} values			Ultraviolet light		Colour with spray A
	Solvent A	Solvent B	Buffer A	Buffer B	Buffer C	Fluorescence	Fluorescence and ammonia	
Ferulic acid	0.86	0.92	0.45	0.78	0.00	Light blue	Green-blue	Brown
Sinapic acid	0.80	0.87	0.61	0.82	0.00	Blue	Blue	Yellow
Chlorogenic acid	0.86	0.92	0.45	0.76	0.00	Light blue	Green-blue	Brown
3,4-dimethoxy-cinnamic acid	0.86	0.92	0.45	0.78	0.00	Light blue	Green-blue	Brown

The products gave the same colour reactions as the expected hydroxycinnamic acids but behaved differently on paper chromatograms and electrophoretograms. In particular no strongly acidic groupings appeared to be present.

It was then realised that the distillation step with methanol was methylating the carboxyl groups of the hydroxycinnamic acids which had been initially formed.

In order to check this, large scale experiments were carried out reacting boron trichloride, followed by methanol, with cinnamic

acid itself and with p-coumaric, caffeic and sinapic acids.
Cinnamic acid (Experiment $\overline{XXII}$) yielded 66% of a finely
crystalline compound $\overline{(XXXV)}$ which analysed as methyl cinnamate
and had the correct melting point and molecular weight.
Further confirmation of structure came from comparative
infrared and ultraviolet spectroscopy (Experiments $\overline{XXIII}$; Fig. $\overline{XIII}$ α
$\overline{XIV}$) and the preparation of a dibromoderivative (Experiment $\overline{XXV}$
Fig. $\overline{XV}$).

Chromatographic and electrophoretic examination of the
ester $\overline{(XXXV)}$ showed that it was identical with authentic methyl
cinnamate (Table $\overline{IX}$) and the compound $\overline{(XXXV)}$ also gave a positive
ester reaction when treated with ferric chloride and hydroxalamine
hydrochloride (Experiment   ).

As a final confirmation of structure $\overline{(XXXV)}$ was
hydrolysed with alkali, and cinnamic acid $\overline{(XXI)}$ and methyl
alcohol, identified as hydrolysis products (Experiment $\overline{XXIV}$).

Similarly, p-coumaric acid $\overline{(XXII)}$ gave a white
crystalline solid $\overline{(XXXVI)}$ in 75% yield. Comparative examination
in the infrared region showed this product to be identical
with authentic methyl-p-coumaric, thus confirming the analytical
results. Hydrolysis of $\overline{(XXXVI)}$ with alkali gave p-coumaric acid
and methyl alcohol.

Fig XVI
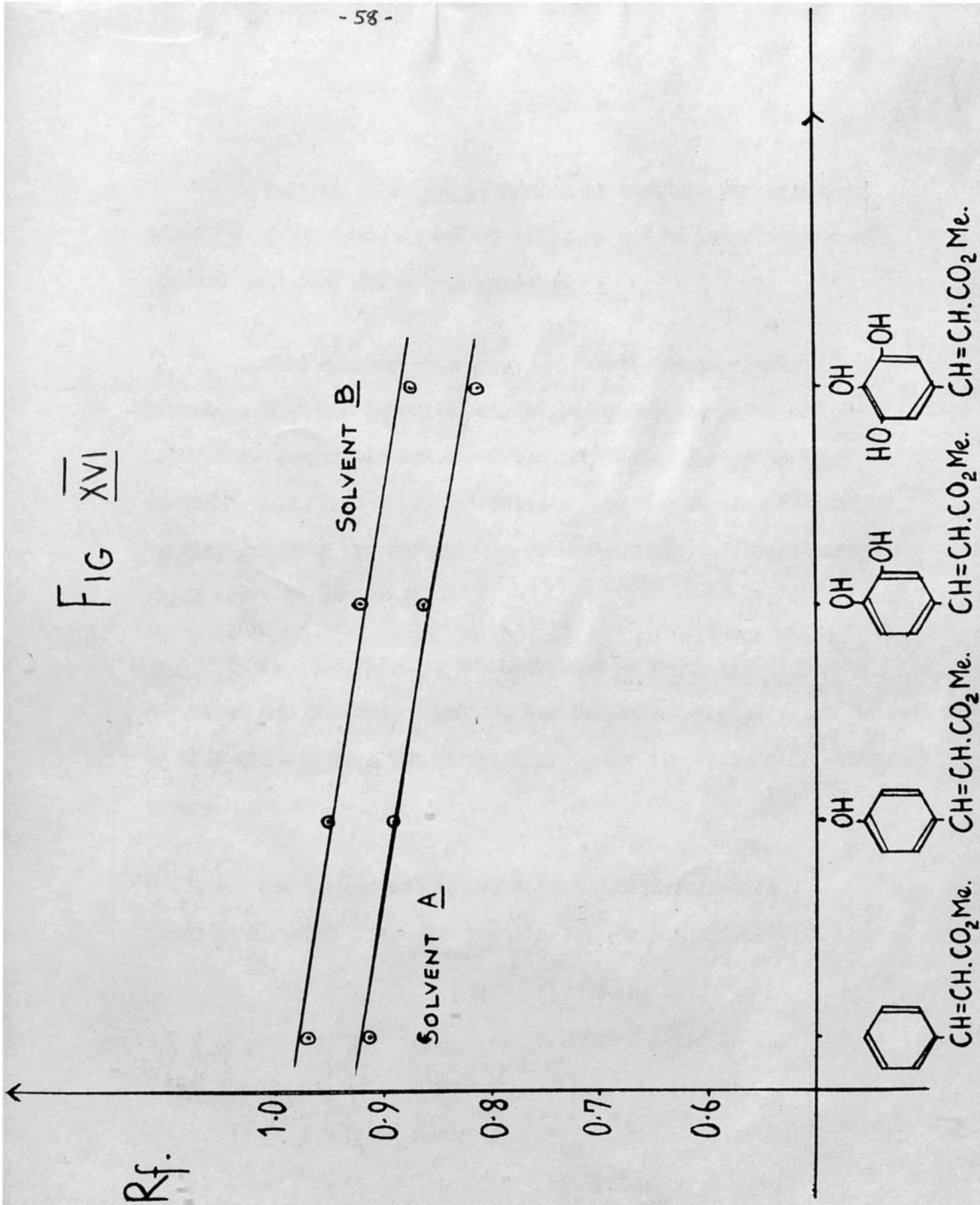
Caffeic acid (XXIV) yielded a crystalline compound (61% yield) which analysed as methyl caffeate and on hydrolysis gave caffeic acid (XXIV) and methyl alcohol.

With sinapic acid (XXVI) 3,4,5-trihydroxylmethyl cinnamate (XXXVII) was obtained, as judged by melting point and paper chromatographic and electrophoretic characteristics (Table IX and Fig. XVI). The product (XXXVII) was further identified by conversion to the more stable triacetate (XXXIX). (Experiment XXVII) which analysed correctly.

When considering the mechanism by which the reactions described can proceed, there are two functional groups which have to be examined, i.e. the methoxyl group and the carboxylic acid group.

The suggested mechanism of demethoxylation is shown in Fig. XVII.

Fig XVII

$R.B_2O_3 + HCl +$ $O-H$ $O.BCl_2$ $+ CH_3Cl.$

$CH_3 \quad Cl$

$O: \quad B - Cl$

Cl

$-78°$

CH_3

$O.BCl_2 + Cl^{\ominus}$

ROH

25°
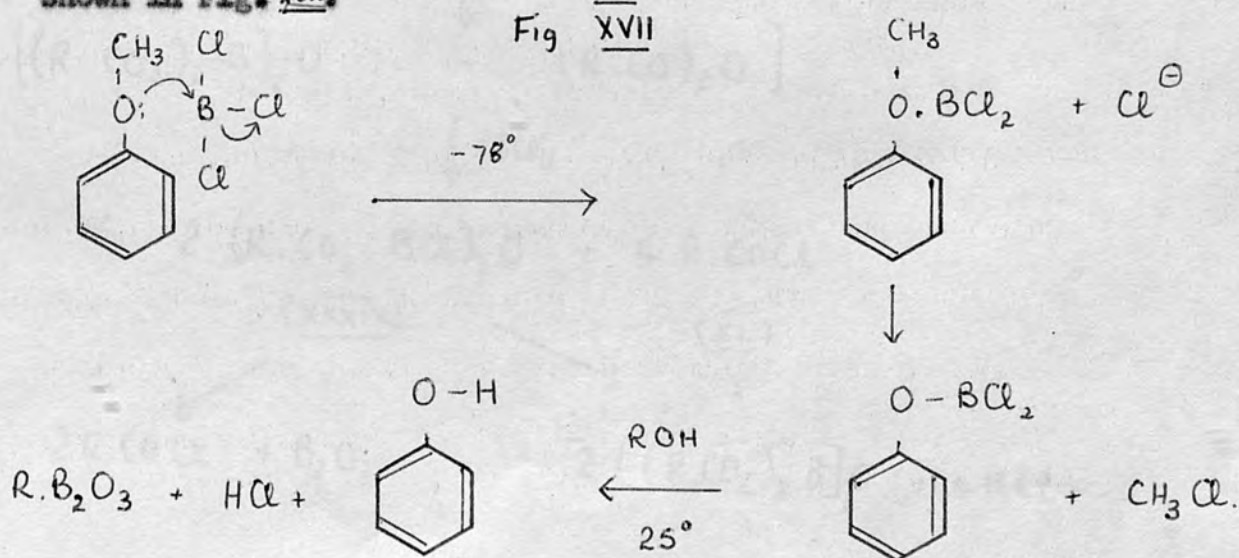
The reaction of boron trichloride with carboxylic acid was examined in 1870 by Gustavson[87] who stated that the product from acetic acid and boron trichloride was acetyl chloride, but later it was shown that this was only one of several products. For instance, if the carboxylic acid was present in excess the product was tetraacetyldiborate and acetic anhydride.[73,88] Further reaction with boron trichloride yielded dichlorodiacetyl diborate ($\overline{XXXIX}$)[89] which on gentle heating gave acetyl chloride ($\overline{XL}$). The mechanism is, therefore, complicated because a number of side reactions occur simultaneously with the main reaction. The main reaction is shown in Fig. $\overline{XVIII}$

$$\text{Fig } \overline{XVIII}.$$

$$2\ BCl_3 \quad + \quad 6\ R.C \overset{O}{\underset{OH}{\diagdown}}$$

$$\downarrow -6\,HCl$$

$$2B\ (O.COR)_3$$

$$\downarrow$$

$$[(R.CO_2)_2\ B]_2\,O \quad + \quad (R.CO)_2\,O\ ]$$

$$\downarrow 2BCl_3$$

$$2\ (R.CO_2.BCl)_2\,O \quad + \quad 4\ R.COCl$$
$$(\overline{XXXIX.}) \qquad\qquad (\overline{XL})$$

$$2R.COCl + B_2O_3 \qquad\qquad 2\ [(R.CO_2)_2\ B]_2\,O \ + \ 4\,HCl$$
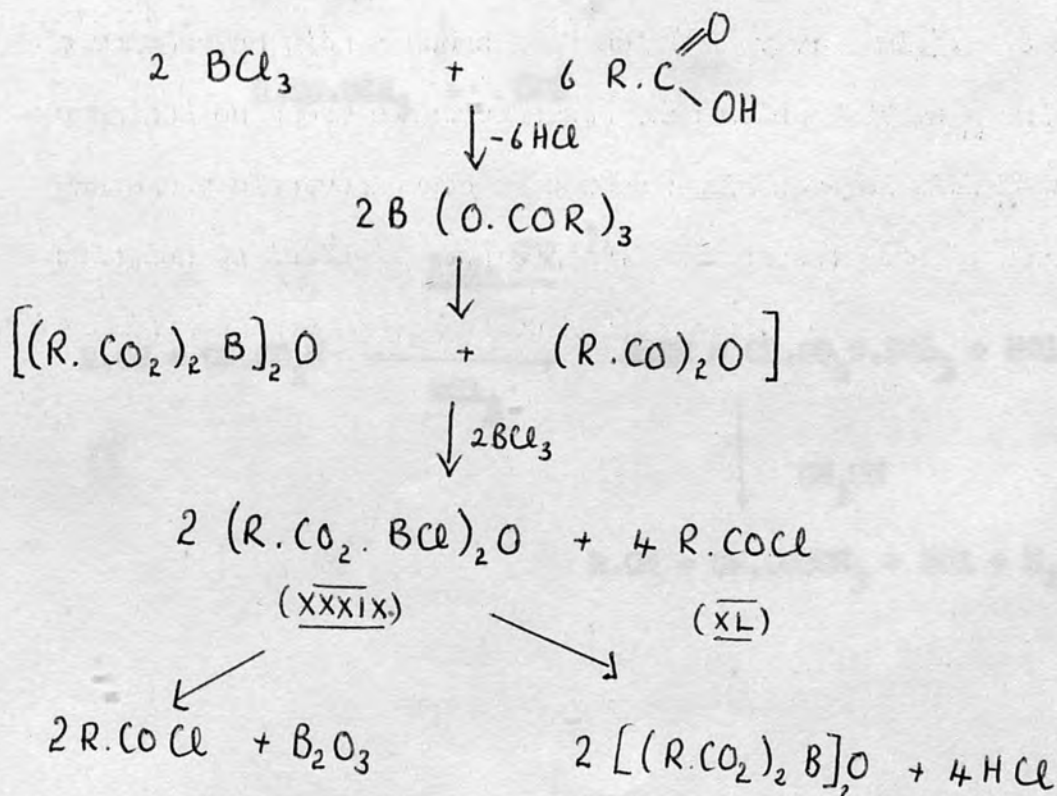
Fig. XVIII shows that during the course of the reaction, an anhydride (XLI) is produced and this, in turn, reacts with the boron trichloride to give the acyl chloride.[87]

Thus the principal product expected immediately after treatment of the cinnamic acid derivatives with boron trichloride would be the corresponding acyl chloride, and the addition of methanol to this would result in methyl ester formation by either of the two mechanisms suggested in Fig. XIX and XX.

Fig. XIX

$$R.COCl \quad + \quad CH_3OH$$

$$\downarrow$$

$$R.CO.OCH_3 \quad + \quad HCl$$

Fig. XX [?]

$$R.CH = CH.CO_2H \xrightarrow[BCl_3]{} R.CH = CH.CO.O.BCl_3 + HCl$$

$$\downarrow CH_3OH$$

$$R.CH = CH.COOCH_3 + HCl + H_2O$$
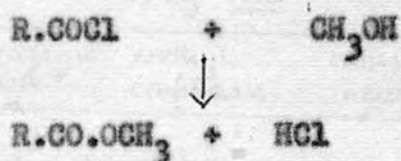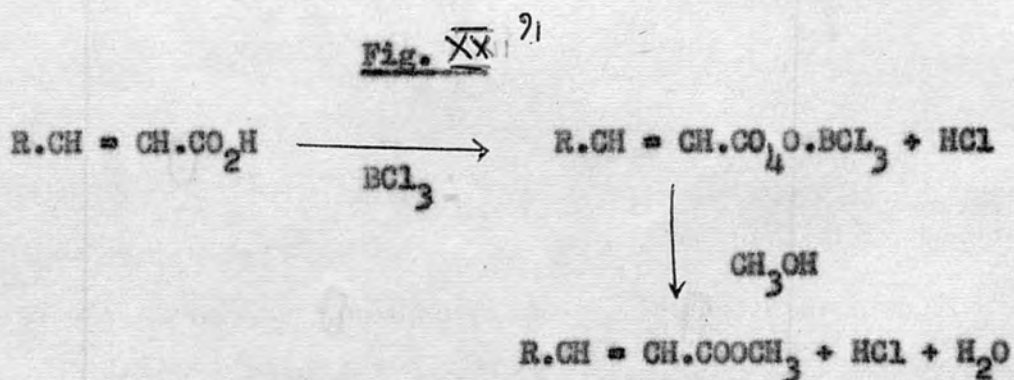
It is not known which of these esterification reactions predominates. Both almost certainly contribute to the reaction.

Attention was then turned to the application of the method to the identification of naturally occurring cinnamic acid derivatives, that is to classify these compounds into one of three hydroxycinnamic acid "families". An extract of beans of Coffea sp., which have a high chlorogenic acid content, [92] was treated with boron trichloride and methanol (Experiment XXIX) and, as expected, methyl caffeate was produced. The methyl caffeate was further identified by alkaline hydrolysis, which yielded caffeic acid (Fig. XXI).

Fig XXI

| chlorogenic acid | methyl caffeate | caffeic acid | hydrolysis product |

← Solvent front
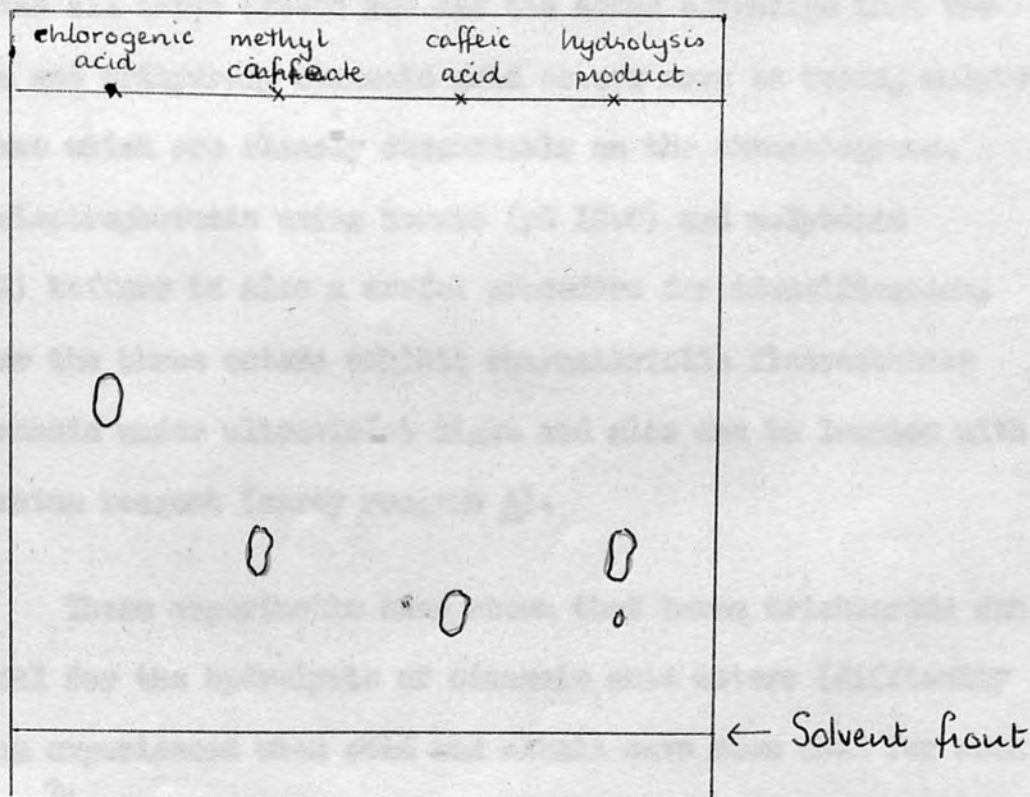
The flowers of Antirrhinum majus contain small amounts of p-coumaric (XXIII), caffeic (XXIV), and ferulic acid (XXV) esters.[93] The boron trichloride/methanol procedure showed satisfactorily that an extract of these flowers did contain members of the p-coumaric acid and caffeic acid "families". (Experiment XXX).

Table IX shows the comparative chromatographic and electrophoretic behaviour of the naturally occurring cinnamic acids and the hydroxycinnamates derived from them. The best method found for the identification of the latter compounds was paper chromatography using ethyl acetate/acetic acid/water solvent (solvent B) and molybdate impregnated paper. This system clearly separates all three esters and has the added advantage that the caffeic and trihydroxy cinnamic acid esters move as brown, molybdate complexes which are clearly discernable on the chromatograms. Paper electrophoresis using borate (pH 10.0) and molybdate (pH 5.2) buffers is also a useful procedure for identification. On paper the three esters exhibit characteristic fluorescences with ammonia under ultraviolet light and also can be located with a diazonium reagent (spray reagent A).

These experiments have shown that boron trichloride can be useful for the hydrolysis of cinnamic acid esters (difficulty has been experienced when acid and alkali have been used for such reactions[94]) and the removal of methoxyl groups, thus exposing the "parent" hydroxycinnamic acid in the form of a methyl ester.

There is, also no doubt that boron trichloride would hydrolyse naturally occurring glycosides such as caffeic acid-3-β-glucoside (XLII).

Table IX

Summary of chromatographic and electrophoretic properties and colour reactions on cinnamic acids and their methyl esters

	R_f values					M_{3A} values				Appearance in U.V. light + NH_3	Colour with diazotised p-nitro-aniline (Spray A)
	Butanol;Ethanol; water (A) with plain paper	with borate treated paper	Ethyl acetate; acetic acid; water (B) with plain paper	Toluene; acetic acid; water with plain paper	with molybdate treated paper	Forestal solvent E	Borate buffer A	Molybdate buffer B	Acetate buffer C		
p-coumaric acid	0.89	0.36	0.96	0.96	0.05	0.84	0.98	0.44	0.35	Violet	Mauve
caffeic acid	0.71	0.03	0.89	0.29	0.00	0.78	1.04	0.72	0.20	Light-blue	Yellow-Brown
Sinapic acid	0.86	0.28	0.90	0.89	0.11	0.86	0.64	0.28	0.21	Green	Red
methyl-p-coumarate	0.94	0.96	0.97	0.96	0.05	0.86	0.38	0.00	0.00	Violet	Mauve
methyl caffeate x	0.86	0.61	0.92	0.63	0.00	0.82	0.45	0.78	0.00	Light-blue	Yellow-Brown
3,4,5-trihydroxyl methyl cinnamate	0.60	0.48	0.87	0.34	0.12	0.89	0.61	0.82	0.00	Blue	Brown

x Tends to streak in most systems

Paper Electrophoresis of High Molecular Weight Phenolics

The technique of paper electrophoresis has been applied in the last ten years with great success to the separation of mixtures in various groups of compounds[95] including the phenolics.

Michl[96] in 1952 published the results of his investigations into the electrophoresis of o-dihidric phenols in borate buffer, and Coulson and Evans[98] used borate buffer for the examination of phenolic carboxylic acids and other phenolic derivatives. Sinomura and Berbalk and Schier[99] have also reported the use of paper electrophoresis for studies with phenolics.

More recently Pridham[97] has investigated the movement of phenolic compounds in various buffer solutions. He showed that, like sodium borate, sodium molybdate solution can be used as a specific reagent for the detection of o-dihydric phenolic compounds with ene-diol groupings, and that other structural features can be revealed by the choice of suitable electrolyte solutions. It was suggested that the rate of movement of phenolic compounds on electrophoretograms was dependent on three main factors:

(a) Molecular weight

(b) Degree of dissociation of functional groups

(c) Ability of the phenol to form charged complexes
with the components of the electrolyte solution.

As yet, however, paper electrophoresis has not been developed to a stage where it can be routinely applied to high molecular weight phenolic compounds, such as the flavonoids, which generally have very low mobilities because of their relatively high mass/charge ratios. Thus, most workers have tended to use chromatography rather than paper electrophoresis for the examination of these phenols. There are, however, some reports of the application of the latter technique to these molecules.

In order to exhibit electrophoretic mobility a substance must have a net charge. If a molecule contains an equal number of acidic and basic groups it can be made to acquire a net charge by adjusting the pH of the electrolyte. Thus anthocyanins in their [106,107] usual salt forms do not migrate measurable distances in neutral buffer solutions, but will migrate in acidic solutions owing to the positive charge on the ring oxygen atom (XLIII).
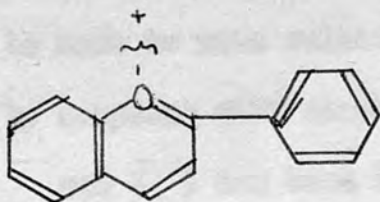
XLIII

Betanins and flavocyanins move quite rapidly at pH 4.6 and
at lower pH values.[102],[103]

Electromigration of flavonoids has also been achieved by
using borate buffers[104] with current densities of 0.1-1.6 mA/cm.
Under these conditions flavones (XLIV), flavonols (XLV) and
glycosidic derivatives of these compounds move in relation to
the number of cis-hydroxyl groups present in the sugar moiety, and
the number of ene-diol groups attached to the aromatic rings.
Both hydroxyl arrangements complex with borate, giving negatively
charged species.
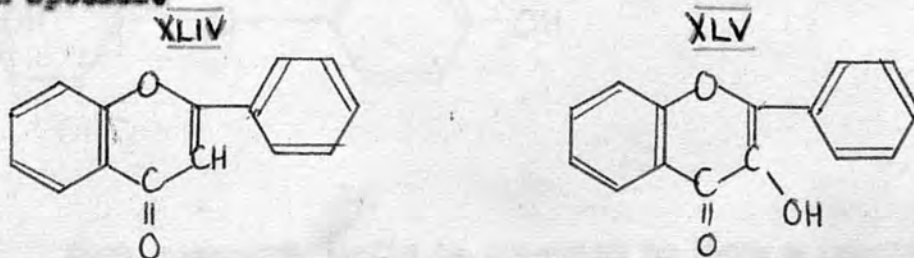
If no such grouping is present, migration is negligible and
so the method is not applicable to all compounds in these classes.

Many high molecular weight phenolic compounds still cannot
be usefully examined by high voltage electrophoresis owing to
low rates of movement. A method whereby the high molecular weight
compounds can be made to move relatively quickly on paper electro-
phoretograms, by coupling with diazotised carboxylated and sulphonated
acids (Tables X and XI) has been investigated. The technique provides
a useful additional method for the analysis of mixtures of these phenols.

Thus, phenols have been coupled with p-amino benzoic acid, giving azo dyes with strongly dissociated carboxyl groups. For example, with arbutin the dye (XLVI) is formed.
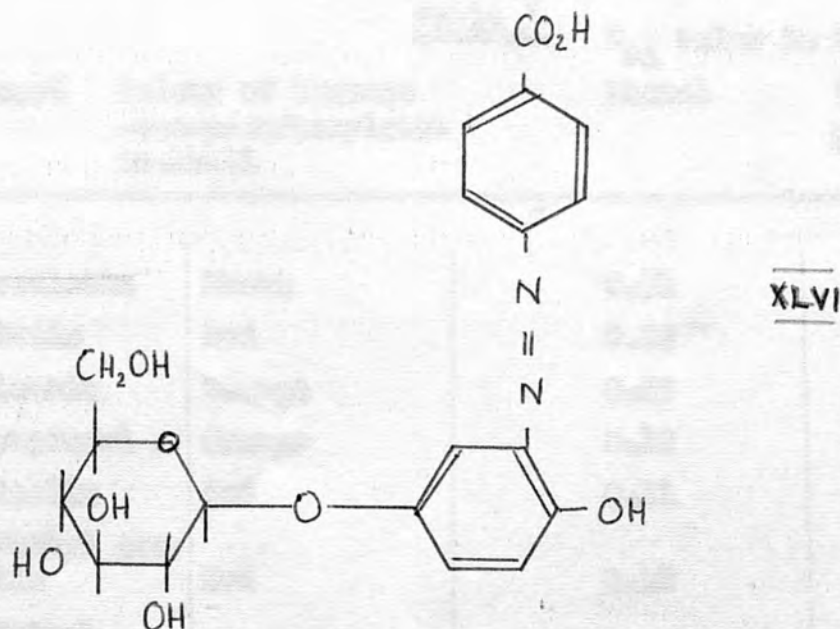
Such compounds would be expected to have a greater electrophoretic mobility than the free phenols owing to the presence of the carboxyl groups. Preliminary experiments carried out on low mol. wt. phenols showed that this was so, and that the azo dyes migrated approximately twice as far as the parent phenols in borate buffer.[105]

Subsequent examination of higher molecular weight compounds gave similar results. Thus, quercetin ($\overline{II}$) and kaempferol ($\overline{XLVII}$) which differ structurally by only one hydroxyl group, on position

3' can be separated satisfactorily as the azo dye derivatives.
The free phenols are difficult to separate clearly.

Phenol	Colour of benzene -azo-p-carboxylates in alkali	M_{2A} value in Borate Buffer Phenol	(A) Benzene-azo- p-carboxylate
Aesculetin	Mauve	0.51	0.65
Arbutin	Red	0.22	0.50
Catechin	Orange	0.65	0.86
Kaempferol	Orange	0.12	0.23
Luteolin	Red	0.11	0.35
4-Methyl aesculin	Red	0.48	0.60
4-Methyl umbelliferone	Red	0.52	0.78
Phloridzin	Orange	0.42	0.61
Quercetin	Orange	0.15	0.32
Syringin	Red	0.09	0.34
Resorcinol-β-glucoside	Red	0.16	0.30
Rutin	Orange	0.12	0.34

Table X

Table XI

Phenol	Colour of benzene -azo-p-sulphonates in alkali	M_{BA} value in Borate buffer	
		Phenol	benzeneazo- p-sulphonate
Aesculetin	Cerise	0.51	0.90
Arbutin	Red	0.22	0.81
Catechin	Orange	0.65	1.00
Kaempferol	Yellow	0.12	0.73
Quercetin	Yellow	0.15	1.20
Syringin	Red	0.09	0.47
Phloridzin	Orange	0.42	0.96

Diazotised sulphanilic acid was found to be a better reagent, in that the azo dyes formed with the phenols moved more rapidly than in the case of diazotised p-amino benzoic acid. This was presumably due to the fact that sulphonic acids are stronger acids than the corresponding carboxylic acids.

In sodium acetate buffer (pH 5.2) compounds possessing only phenolic groups are insufficiently ionised to allow electrophoretic migration, but those having an additional carboxyl group move rapidly towards the anode.

In view of this, the azo dyes formed with diazotised

p-amino benzoic acid were examined in this buffer, and, as expected,
they were mobile, whereas the parent phenols were not. (Table XII).

Table XII

Phenol	Azo dye in acetate buffer (C)	Phenol in Acetate buffer (C)
Quercetin	0.10	0.00
Kaempferol	0.14	0.00
Catechin	0.08	0.00
Arbutin	0.23	0.00
Luteolin	0.12	0.00
Phloridzin	0.31	0.00

The azo dye derivatives can be prepared from very
small specimens of the phenol by the normal diazo coupling
reaction, followed by paper chromatographic purification. The
latter effectively removes excess reagent and coloured interfering
substances. The procedure can also be used in conjunction with
paper chromatography. Thus, an unknown phenol may be examined
on a chromatogram, together with standard compounds, the paper sprayed
with diazotised p-aminobenzoic acid (or diazotised sulphanilic acid)
and a tentative identification made. The coloured spots can then be

eluted from the chromatogram and re-examined by
electrophoresis, and the identification confirmed.   As
the azo dye is strongly coloured at high pH values the progress
of the electrophoretic migration can be observed in borate
buffer (pH 10.0).

GENERAL METHODS

GENERAL METHODS

Paper Chromatography

Paper chromatography was carried out using Whatman No. 1 and No. 3 papers using the descending technique, and the following solvent systems; all proportions given are by volume, except where stated.

A Butan-1-ol, ethanol, water (40:11:19) [106]

B Ethyl acetate, acetic acid, water (9:2:2) [107]

C Ethyl acetate, pyridine, water (2:1:2 organic phase) [108]

D Butan-1-ol, acetic acid, water (2:1:1) [109]

E Acetic acid, conc. Hydrochloric acid, water (30:3:10) [34]

F Toluene, acetic acid, water (4:1:5) [35]

G 10% acetic acid [109]

H Phenol, water (bottom layer) [110]

I Pyridine, acetic acid, water (1:2:2)

J 2% acetic acid [109]

K Butanol, pyridine, water (6:4:3) [111]

$$R_G \text{ value} \quad \frac{\text{distance travelled by carbohydrate}}{\text{distance travelled by glucose}}$$

Molybdate paper was obtained by "dipping" Whatman No. 3 paper in Buffer B solution and allowing it to dry in air, before spotting.

Spray Reagents

A   Diazotised p-nitroaniline solution and N-sodium hydroxide [109]
(Phenols)

B   p-Anisidine hydrochloride (1% in 1-butanol) followed by [112]
heating at 100° for 5 min. (Reducing sugars)

C   B.D.H. Universal Indicator adjusted to pH 8.5-9.0 by
addition of dilute ammonia

D   Potassium periodatocuprate solution and rosaniline in [114]
acetic acid (Polyhydroxy compounds)

E   Diphenylamine/aniline solution ($1 \rightarrow 4$ linked carbohydrates) [115]

F   Ninhydrin (0.2% in 1-butanol) [128]

G   Diazotised p-amino benzoic acid and N-sodium hydroxide [116]
(Phenols)

H   Triphenyltetrazolium chloride (2% methanolic solution) [117]
(Reducing oligosaccharides other than $1 \rightarrow 2$ linked)

Paper Electrophoresis

Paper electrophoretic examination of compounds was carried out using Whatman No. 1 and No. 3 paper for 30 min. at 55 v/cm. The buffers used were:

A   0.2 M-sodium borate (pH 10.0)[118]

B 8.1×10^{-3} M-ammonium molybdate (pH 5.2)[119]

C   0.2M-sodium acetate (pH 5.2)[120]

D   0.05 M-Tris-HCl (pH 8.8).[120]

E   0.2 M-ammonium phosphate (pH 7.2)[120]

The mobilities of phenols were expressed as M_{SA} values.[97]

$$M_{SA} = \frac{\text{distance moved by phenol}}{\text{distance moved by salicylic acid}}$$

The mobilities of carbohydrates were expressed as M_{GLU} values.

$$M_{GLU} = \frac{\text{distance moved by carbohydrate}}{\text{distance moved by glucose}}$$

Infrared Measurements

All infrared measurements were in Nujol mulls with an
Infracord Spectrophotometer (Model 137).

Ultraviolet measurements

All measurements in the ultraviolet region were
carried out with a Unicam S.P.500 in silica cells (1 cm.).

EXPERIMENTAL

Experiment 1

Preparation of Acetone Powder

Fresh plant material was macerated with
acetone at -20° in a waring blendor. The resulting powders
were filtered off in a Büchner funnel and washed five times
with more acetone at -20°.   Last traces of acetone were
removed under vacuum and the powders were stood in desiccators
at 4°.

Experiment II

Preparation of DOPA deaminase solutions

The acetone powder (ca. 0.5 g.) was mixed with
about 100 times its weight of 0.05 M-Tris-HCl buffer (pH 8.8;
25°). The mixture was cooled in an ice bath and stirred at
occasional intervals during 30 min. It was centrifuged at 3500 g.
at 0° for 20 min. and the tissue residue discarded.

Experiment III

Incubation of Amino-acid with Acetone Powder

A sample of acetone powder solution (Experiment II)
(0.2 g.) was incubated at 25° with 0.05M-Tris-HCl buffer
(20 ml.; pH 8.8) containing 0.1% L-Tyrosine or 0.2% DL-DOPA.
After 3 hr. the mixture was diluted with water (10 ml.), filtered
and the pH of the filtrate adjusted to 6-7 with 5N-HCl.   The
solution was then extracted with ether (50 ml.) and the ether
extract evaporated to dryness on a rotary evaporator.   The residue
was finally dissolved in ether (1 ml.) for chromatographic
examination.

Experiment IV

Quantitative Estimation of Caffeic Acid Produced by DOPA Deaminase

The DOPA deaminase solution (prepared by extracting
approx. 0.5 g. acetone powder with 50 ml. 0.05 M-Tris-HCl buffer,
pH 8.8 (see Exp. II)), was incubated at 25° with 0.05 M-Tris-HCl
(50 ml.; pH 8.8) containing 0.2% DL-DOPA. At intervals of 10, 20,
60, 120 and 180 min. portions (10 ml.) were removed from the mixture,
acidified with 5N-HCl (to pH 6-7) and extracted with ether (20 ml.).
The ether extracts were concentrated to dryness and the residue
redissolved in ether (1 ml. each). The ethereal solutions were applied
quantitatively to a chromatogram which was developed with Solvent A

for 16 hr. The paper was allowed to dry at room temperature and then examined under ultraviolet light to detect the caffeic acid spots. The "strips" of paper containing the caffeic acid were cut out, eluted with aqueous methanol (80% v/v; 5 ml.) and the absorbance of each solution measured at 320 mμ.

A graph of incubation time against absorbance was plotted for each acetone powder (Fig. $\overline{IX}$) and units of activity per g. of powder calculated from the linear portion of each graph.

N.B. The unit of activity is defined as the amount of enzyme forming 1mM of caffeic acid in 1 hr. under the standard conditions described above.

Fig IX
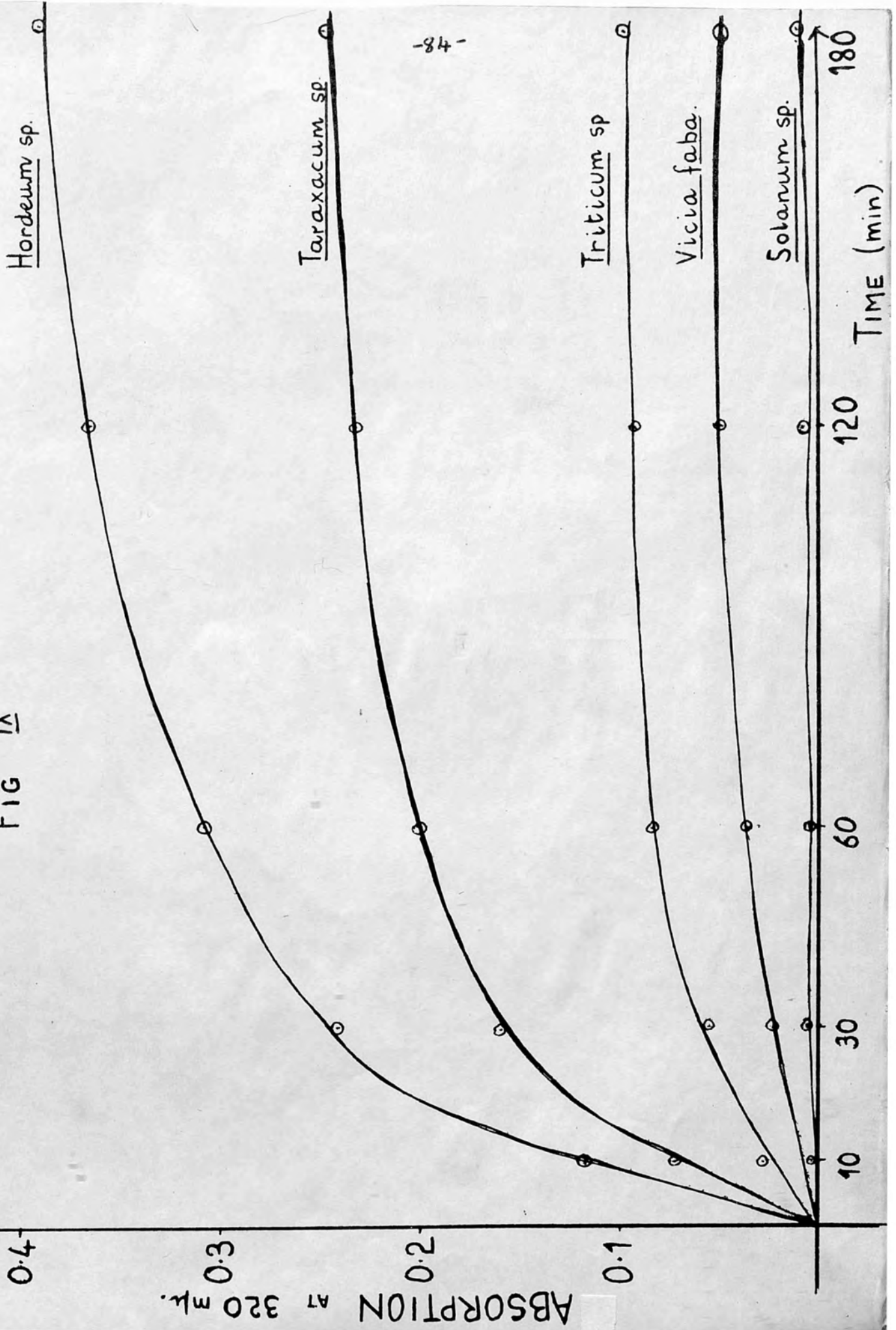
Experiment V

28

Conversion of trans-p-coumaric acid to cis-p-coumaric acid

A sample of p-coumaric acid (0.10 g.) was dissolved in a slight excess of sodium hydroxide (0.5 N). This solution was irradiated for 24 hr. by a 100 W, 366 Hg. source, shining directly on the surface of the solution from a distance of 3 in. After irradiation, an excess of N-hydrochloric acid was added and the product extracted by shaking with ether (30 ml.). The ether extract was evaporated under vacuum and the residue recrystallised from toluene (m.p. 131°). The sample was shown to be free from the trans-isomer by examination of the ultraviolet spectrum.

Experiment VI

Detection of Ammonia from DOPA Deaminase

Ammonia was detected in the DOPA deaminase digests by the use of Nessler's reagent. Control digests using boiled enzyme solutions produced no ammonia.

Experiment VII

Preparation of Hydrocaffeic acid

Authentic caffeic acid (1 g.) in ethanol
(20 ml.) was hydrogenated at room temperature using a palladium oxide
catalyst (10 mg.).   The product was purified by paper chromatography
(Solvent A) and crystallised from ethanol-water, M.pt. 140°
(lit.[124]   m.p. 139°).

Experiment VIII

Caffeic acid obtained by the action of dandelion
DOPA deaminase on DOPA was hydrogenated under similar conditions
and the product examined directly on paper chromatograms (Solvents
A and B) against the hydrocaffeic acid prepared in Experiment VII.

Experiment IX

Isolation of 3,4-dihydroxyphenylalanine (DOPA) from Broad-bean leaves

Streaks of leaf extract (in aqueous methanol 80% v/v) were made on Whatman 3MM papers and the chromatograms developed with solvent K (DOPA has an R_f value of 0.5 in this solvent and is easily separated from the faster running flavonoids and cinnamic acid derivatives in the extract). The DOPA bands eluted from the papers with water and the eluate concentrated to produce a crystalline compound (m.p. 260° with decomposition).

The ultraviolet spectrum (measured in aqueous solution) of the extracted DOPA showed absorption maxima at 230 mμ, 280 mμ, and 310 mμ, compared with 230 mμ, 282 mμ and 315 mμ for authentic DOPA. The infrared spectra of the extracted compound and an authentic specimen of DOPA were identical, and the two specimens also co-chromatographed in solvents A (R_f 0.24) and B (R_f 0.35). In addition they showed similar electrophoretic mobilities in buffers A and C.

Experiment $\overline{X}$

Other Phenolics in the Broad-bean

Fresh leaves (100 g.) were macerated with
aqueous methanol (80% v/v; 250 ml.). Chlorophyll was
removed by continually shaking the extracting with petroleum
ether (b.p. 40°-50°). The extract was then filtered and
concentrated under reduced pressure at 40°.

The extract was examined on paper chromatograms
using solvents $\underline{A}$, $\underline{B}$, $\underline{E}$ and $\underline{K}$. These chromatograms showed
the presence of quercetin, quercetrin, rutin and kaempferol
glycosides. The exact nature of the kaempferol glycosides
was not examined.

The remaining extract was hydrolysed with $\underline{N}$-H_2SO_4
(3 hr.; 100°). The resulting yellow precipitate was
filtered off, dissolved in aqueous methanol (80% v/v; 5 ml.)
and fractionated on sheets of borate impregnated Whatman 3 MM
paper using solvent $\underline{A}$.

One main fraction resulted (R_f 0.78) which was
crystallised from aqueous methanol (m.p. 270° decomp.). The
ultraviolet and infrared spectra of this material was identical
with those of authentic kaempferol.

A slower moving fraction (R_f 0.06) obtained from the paper failed to crystallise, but paper chromatographic studies in acidic solvents (B and E) indicated that it was quercetin. Comparative ultraviolet and infrared examination with authentic quercetin confirmed this.

The aqueous, acidic solution remaining after removal of the flavonols was neutralised with barium carbonate, concentrated and examined on paper chromatograms in solvent B. Glucose, arabinose and rhamnose were detected.

The extract was also examined on chromatograms for free cinnamic acids in solvents A, B and F. The results are tabulated in Table. XIII

Table XIII

R_f in solvent:			Colour with spray A	Inference
A	B	F		
0.71	0.82	0.00	Brown	Caffeic acid
0.86	0.93	0.15	Blue	Ferulic acid
0.80	0.89	0.11	Red Blue	Sinapic acid
0.88	0.94	0.05	Orange Purple	p-Coumaric acid

Experiment $\overline{XI}$

Translocation Experiments

Aqueous solutions (0.1 w/v) of various phenols and phenolic glycosides were fed into the cut ends of the main veins of apical leaves of the broad-bean for varying periods of time (10 min.-3 hr.). This was effected by partially dissecting the main veins from the laminae, dipping the veins into the phenolic solutions and then cutting them at the apices, with the veins beneath the surfaces of the solutions (Fig. $\overline{XXII}$).

The plants were then cut into sections (leaves, cotyledons, root and 1 cm. portions of stem), quickly macerated with aqueous methanol (80% w/v) and examined on paper chromatograms using solvents A, B, E and G.

Plants 30cm. high were used in all cases

Fig $\overline{XXII}$

0.1% solution of phenol
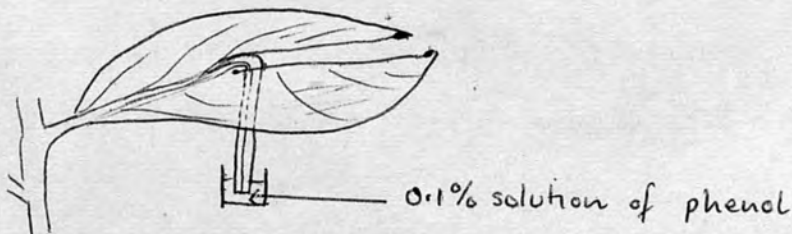
Experiment X̄I

Rate Measurements

Aqueous solutions (0.1% w/v) of various phenols and phenolic glycosides were fed into the leaves of broad-bean plants as described in Experiment X̄I . The plants were allowed to feed for 15 min., after which time the stems were cut into 1 cm. sections and the distance the phenolic derivatives had moved, determined by paper chromatographic examination of aqueous methanolic extracts of the sections using solvent A̲ and spray reagent A̲ as described in Experiment .

Table VI

Phenol	Distance moved down stem in 10 min.(cm.)		Relative mobility to Resorcinol
		cm/b	
Resorcinol	10	60	1.00
Catechol	9	54	0.90
Phloroglucinol	9	54	0.90
Quinol	8	48	0.80
Aesculetin	14		1.40
Quercetin	2	12	0.20
Kaempferol	3	18	0.30
Caffeic Acid	7	42	0.70
Ferulic Acid	5	30	0.50
Saligenin	7	42	0.70
Arbutin	15	90	1.50
Aesculin	14	84	1.40
Resorcinol β-glucoside	18	108	1.80
Salicin[x]	11	66	1.10

x   Detected with Spray Reagent D

Rate of Upward Movement of Phenolics in Vicia faba

Table VII

Phenol	Distance moved up stem in 1 min.(cm.)	Relative movement to Resorcinol
Resorcinol	15	1.00
Catechol	14	0.93
Phloroglucinol	13.5	0.90
Quinol	12	0.80
Aesculetin	15	1.00
Quercetin	2	0.13
Kaempferol	2	0.13
Caffeic acid	10	0.67
Ferulic acid	6	0.40
Saligenin	10	0.67
Arbutin	23	1.53
Aesculin	21	1.40
Resorcinol β-glucoside	30	2.00
Salicin	16	1.07
Rhamnose	3.8	0.25
Ribose	6.3	0.42

Experiment XIII

Introduction of Phenols into Broad-bean Leaves using Tween-40

An aqueous solution of Tween-40 (0.02% w/v; 5 ml.) was added to an aqueous solution of catechol (0.1%, w/v; 5 ml.). A droplet of this solution was then placed on the main vein of a broad-bean leaflet and left for approximately 10 min. The leaf was washed in distilled water, dried and cut into sections. These sections, together with the stem of the plant, were macerated in aqueous methanol (80% w/v) and examined on paper chromatograms in Solvent A. (See Table XIV).

Table $\overline{XIV}$

	Phenol fed	Phenols found in leaf and stem *
Stem	Resorcinol	Resorcinol, resorcinol β-glucoside
Petiole and lower leaf section	Catechol	Catechol, catechol β-glucoside
Middle of leaf	Saligenin	Saligenin, salicyl β-glucoside
Apex of leaf	Quinol	Quinol, arbutin

* Identified chromatographically against known standards (M.J. Saltmarsh & J.B. Pridham. Biochem J. in print.)

Experiment XIV

Experiment with Tuberolachnus salignes

Honeydew from Tuberolachnus salignes feeding on a short branch of Salix daphnoides was collected by allowing it to fall on a glass plate placed beneath the branch. The honeydew was dissolved in water and examined on chromatograms using Solvents A, B and G, the following materials being observed:

Table XV

Solvent	R_f value	Appearance under U.V. light plus NH_3	Colour with spray A
A	0.04	Blue fluorescence	-
	0.32	-	Pink
	0.48	Blue fluorescence	-
	0.55	-	Red
B	0.12	Blue fluorescence	Blue
	0.19	-	Orange
	0.25	Bright blue fluorescence	-
	0.32	-	-
	0.40	Yellow	Yellow
	0.62	Bright blue fluorescence	Red
G	0.85	-	Orange
	0.04	Blue fluorescence	-

Experiment XV

Comparison of Phenolic Components of Honeydew from Tuberolachnus salignus with those of leaf and bark from Salix.daphnoides

Honeydew collected from Tuberolachnus salignus as in Experiment   was compared chromatographically using Solvent A and B with methanolic extracts (80% v/v) of leaves and bark of willow.

Table XVI

Solvent	Bark R_f	Colour reaction	Leaf R_f	Colour reaction	Honeydew R_f	Colour reaction	Possible identity
A					0.03	Blue (U.V.)	
			0.01	Blue(U.V.)			
			0.19	Blue(U.V.)			
	0.46						
	0.62	Red with Spray A			0.62	Red with Spray A	Tyrosine
	0.77	Blue(U.V.)					
	0.84	Blue(U.V.)					
B					0.13	Blue(U.V.)	
	0.34	Blue(U.V.)	0.3	Blue(U.V.)	0.34	(Blue(U.V.)	
			0.48	Blue(U.V.)			
	0.59	Red with Spray A Blue with Spray H F			0.59	Red with Spray A Blue with Spray H F	Tyrosine
			0.60	Blue(U.V.)			
			0.72	Blue(U.V.)	0.72	Blue(U.V.)	Cinnamic acid derivative
	0.92	Blue(U.V.) Red (Spray A)			0.91	Blue(U.V.) Red (Spray A)	Cinnamic acid derivative

BEW

EHW

Experiment $\overline{XVI}$

<u>Chromatographic Comparison of Extracts of Aphids Feeding on Stems</u>

<u>of Broad-bean, with Stem Content, and Aphid Honeydew</u>

<u>Macrosiphum</u> <u>pisi</u> were induced to feed on the stem
of a broad-bean plant for 24 hr. This was effected by stripping
all but the apical leaves from the plant and then blocking the
passage of the aphids to these with cotton wool. (<u>M.pisi</u> normally
feeds on leaves of broad-bean).

Approximately half of the aphids were then removed
from the stem, anaesthetised with a jet of carbon dioxide (to
prevent excessive movement) and their heads embedded in grease
on filter paper. The flap covering the anus of each insect was
then stimulated with a needle until a large globule of honeydew
had been excreted. This was collected in a capillary tube. The
remaining insects were removed from the stem and extracted with a
small volume of distilled water at room temperature. Aqueous meth-
anolic (80% w/v) extracts of the bean stem were also prepared, and
concentrated.

The three extracts were then compared on
chromatograms using solvents $\underline{A}$, $\underline{B}$ and $\underline{G}$ and on paper electrophoretograms
with buffer solutions $\underline{A}$ and $\underline{C}$. Both were sprayed with spray reagent
$\underline{A}$. The observations are recorded in Table $\overline{XVII}$ and   , where comparable
R_f values of compounds found in the three extracts are given.

Table XVII

Solvent	R_f value of compounds found in honeydew, aphid and stem	Colour with Spray A
A	0.18	Blue/Brown (DOPA)
	0.39	Red
B	0.28	Blue/Brown (DOPA)
	0.55 (not honeydew)	Red
	0.74	Yellow (Flavonoids?)
G	0.79	Orange
	0.89	Red

Experiment XVII

Comparison of Phenols in Microsiphum pisi Honeydew and Broad-bean leaves

Broad-bean leaves, covered with honeydew from M.pisi., were cut from the plant and washed in a beaker of cold, distilled water, care being taken not to damage the leaves. The resulting solution was filtered and concentrated to a syrup under reduced pressure. An aqual number of "clean" leaves was treated in an identical manner. The two solutions were compared on paper chromatograms using solvents A and G.

Table XVIII

Solvent	R_f value of compounds found on honeydew covered leaves but not on "clean" leaves	Colour with Spray A
A	0.03 0.29	Brown/Orange Red
G	0.15 0.41	Brown/Orange Red

Experiment XVIII

Location of Foreign Phenols in Broad-bean with Macrosiphum pisi

Aphids were induced to stem-feed on broad-bean plants (as described in Exp.), for 18 hrs. The main vein of an apical leaf was then cut and dipped into an aqueous solution (0.1%, w/v) of a foreign phenol. After 1 hr. the aphids were removed, extracted with distilled water, and chromatograms of one extract developed with solvent A and sprayed with Spray reagent A.

Table XIX

Phenol fed	Phenol found in aphid
Quinol	Quinol
Catechol	Catechol
Resorcinol	Resorcinol
Arbutin	Quinol, Arbutin
Rutin	-
Quercetin	-
Ferulic Acid	Ferulic Acid
Caffeic acid	Caffeic Acid

Experiment XIX

Phenolase Activity in Macrosiphum pisi

(a) Qualitative

Two large aphids were extracted with distilled
water (2 ml.). Catechol (0.01 g.) was dissolved in 0.05 M-
sodium acetate buffer (pH 5.6; 2 ml.) and added to the aphid
extract. The reaction mixture was incubated at 30° for 30 min.
A control, using boiled aphid extract was treated similarly.
Appreciable darkening of the digest was observed. After 24 hr.
the solution was dark brown but the control digest was only
partly discoloured.

A similar experiment was carried out using catechin
(3,4,7,3',4'-pentahydroxy flavan). This was again oxidised to
dark coloured products by the aphid preparation. With tyrosine
no darkening resulted.

(b) Quantitative [126]

An aqueous solution of catechol (0.5 M, 2 ml.) was
added to a 0.01 M-sodium acetate buffer (20 ml.) containing aphid
extract (1 ml., 5 large aphids per ml. buffer solution). Two
control reaction mixtures were also prepared.

(i)   0.01 M-sodium acetate buffer (20 ml.) and 0.5 M-catechol solution (2 ml.).

(ii) 0.5 M-catechol solution (2 ml.) and boiled aphid extract (1 ml.) and 0.01 M-sodium buffer (20 ml.).

The three solutions were incubated at 35° and their absorptions measured against 0.01 M-sodium acetate buffer at 420 m at varying periods of time up to 30 min. (Fig.XIII).

Fig XXIII
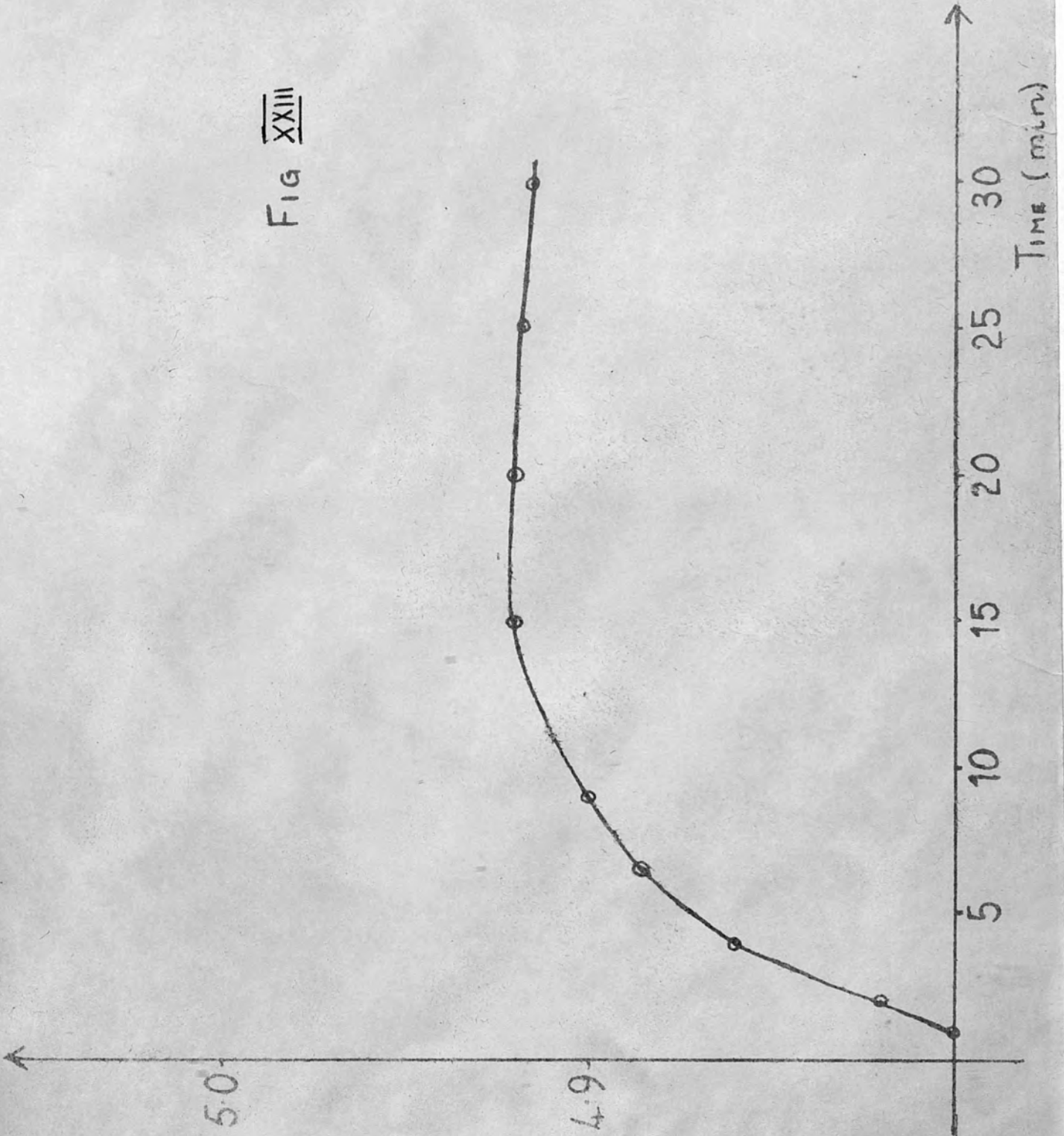
Experiment <u>XX</u>

<u>Hydrolase Activity in Macrosiphium pisi</u>

   Five large aphids (<u>M.pisi</u>) were extracted with
0.05 <u>M</u>-sodium acetate buffer (pH 5.6; 2 ml.). The extract
was incubated with the substrate (0.5% w/v) at 25°for 36
hr. Control experiments using boiled aphid extracts were also
set up. The products were examined on paper chromatograms
using solvent <u>B</u> and Spray reagents <u>B</u> and <u>A</u> (with glucose as a
standard) - see Table. <u>XX</u>

<div align="center">Table <u>XX</u></div>

Substrate (0.5% w/v)	Hydrolysis product	Inference
Maltose	Glucose (trace)	
isomaltose	Glucose	<u>a</u>-glucosidase present
Methyl α-<u>D</u>-glucoside	Glucose	
Raffinose	Galactose Sucrose (trace)	α-Galactosidase present
Cellobiose	-	-
Methyl <u>β</u>-<u>D</u>-glucoside	Glucose	β-glucosidase present
Gentiobiose	Glucose	

Experiment $\overline{XXI}$

Experiment $\overline{XX}$ was repeated using phenolic glycosides and

chlorogenic acid as substrates.

Table $\overline{XXI}$

Substrate	Hydrolysis product	Inference
Arbutin	Glucose Quinol	β-glucosidase present
Salicin	Glucose Saligenin	
Rutin	Glucose Quercetin	
Chlorogenic acid	Caffeic acid Quinic acid*	esterase present

✱ Detected with Spray reagent $\underline{\underline{D}}$

Action of Boron Trichloride on Cinnamic Acid

Cinnamic acid (4.3 g.) in methylene dichloride (80 ml.) was cooled to -78°, and boron trichloride (12 ml.) at the same temperature, was added. After 2 hr. at -78° the reaction mixture was left to stand overnight at room temperature. The excess boron trichloride was removed under reduced pressure and borate removed by repeated distillation with methanol. The reaction mixture was then concentrated to dryness under reduced pressure and distilled in a Jackson unit at 0.1 mm. Between 68° and 72° a pale yellow oil distilled over which, on cooling, yielded white crystals of methyl cinnamate, m.p. 35°. The product (xxxv) gave a crimson colour (a positive ester test) when treated with hydroxalamine hydrochloride and ferric chloride.

(Found: C, 74.51; H, 6.33; -OCH$_3$, 19.09%.

Calculated for C$_{10}$H$_{10}$O$_2$: C, 74.05; H, 6.22; -OCH3, 19.13%)

lit.[121]   m.p. 34.7°.

Molecular weight (measured cryoscopically in benzene) was 166 (methyl cinnamate 168).

Experiment XXIII

a) Examination of the infrared spectrum of (XXXV)

Results of examination of (XXXV) in the infrared region indicated the presence of a monosubstituted aromatic ring and a conjugated C=O bond (peak at 1716 cm.$^{-1}$) with a <u>cis</u> -C=C-conjugated to a C=O or -C=C- (peak at 1626 cm.$^{-1}$).

It was identical with authentic methyl cinnamate (Fig. XIII).

b) Examination of the ultraviolet spectrum of (XXXV)

The absorbance of (XXXIV) in the ultraviolet region was measured in alkali (0.05 <u>N</u>-sodium hydroxide solution). The peak found at 273 mμ was due to the cinnamoyl grouping.

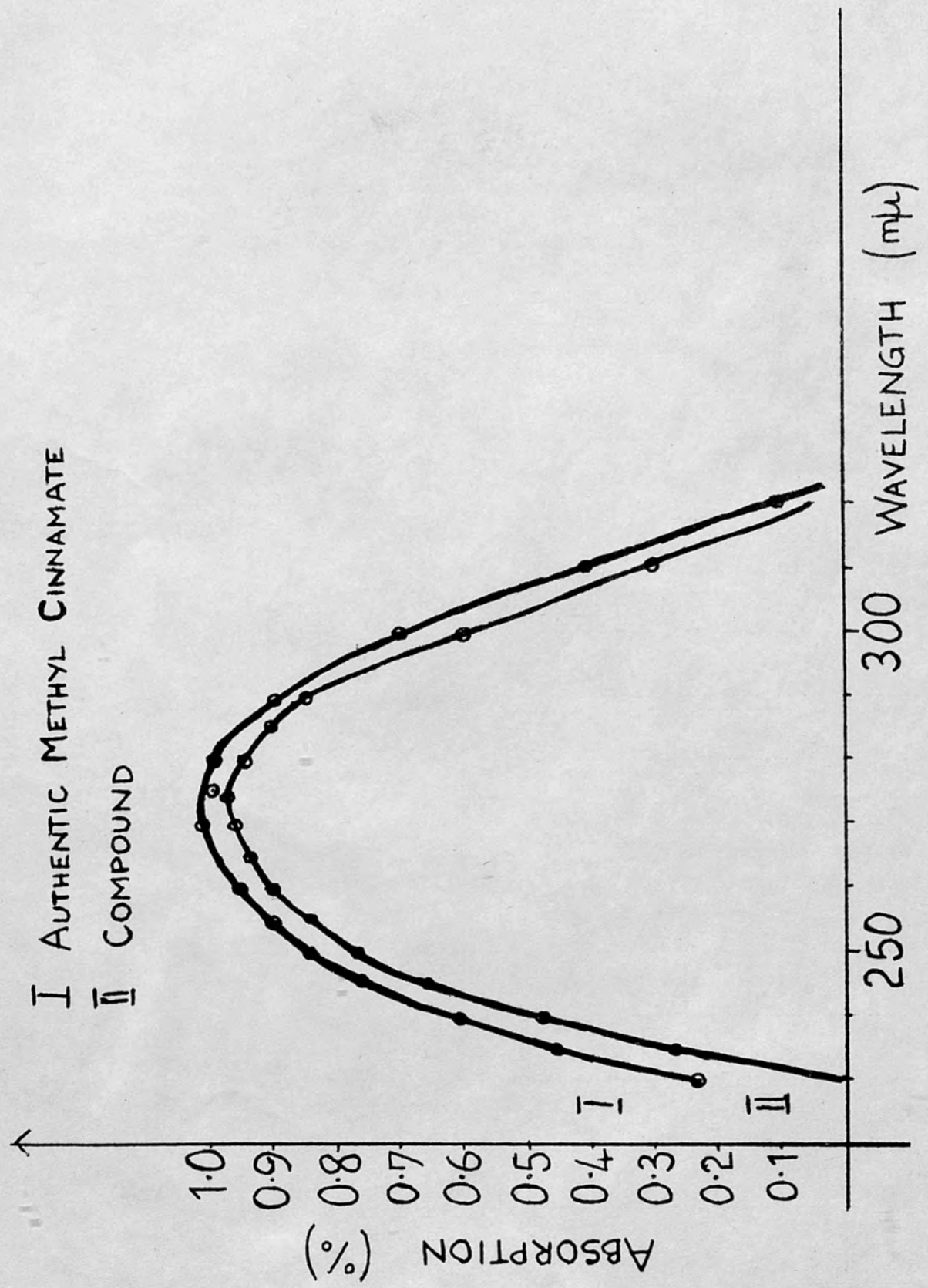It was identical with authentic methyl cinnamate (Fig. XIV).

AUTHENTIC METHYL CINNAMATE
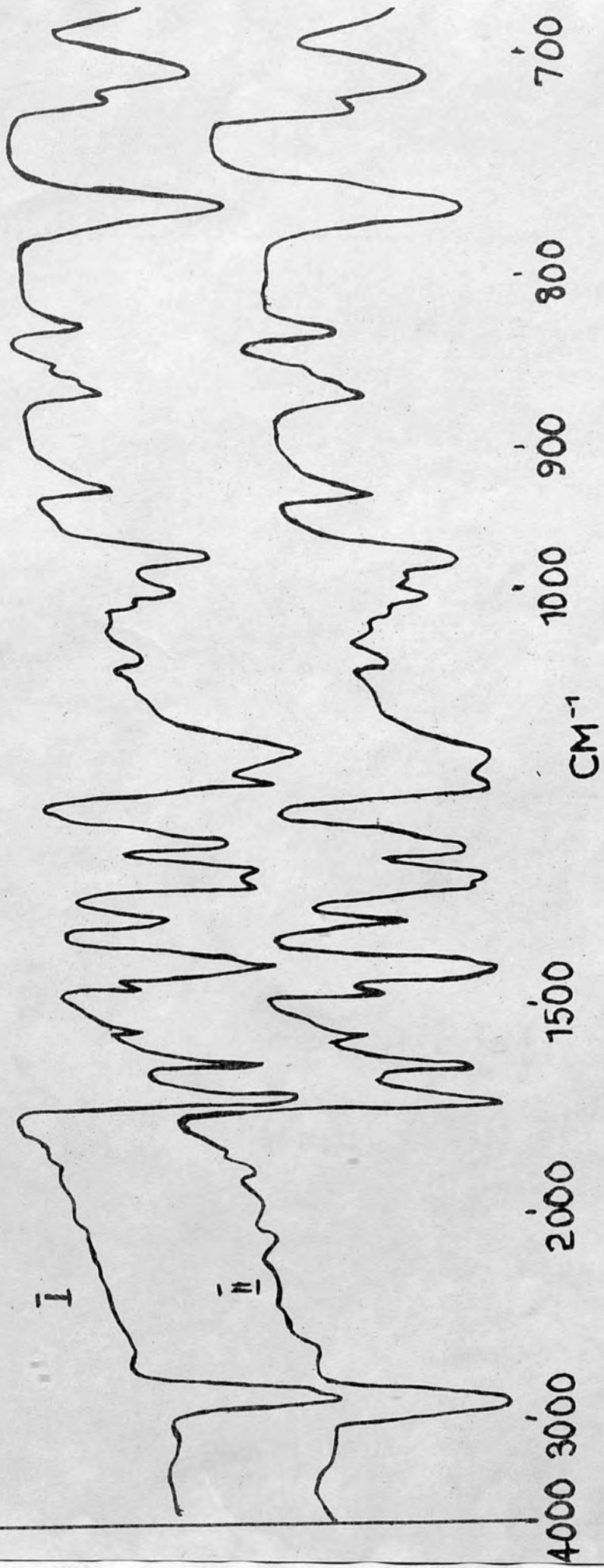
I   AUTHENTIC METHYL CINNAMATE
II COMPOUND XXXV

FIG XIII

-109-

Fig. XIV

Experiment XXIV

Hydrolysis of Methyl Cinnamate (XXXV)

(XXXV) (0.1 g.) was dissolved in aqueous sodium hydroxide
solution (10% w/v, 30 ml.) and heated on a boiling waterbath for 2 hr.
The solution was distilled and a colourless liquid (XLI) resulted.
The 3,5-dinitrobenzoyl derivative (XLII) of (XLI) had m.p. 108° (lit.[127]
m.p. 108°).

The white solid (XXI) remaining after removal of methyl alcohol
(XLI) was dissolved in warm water and 2N-sulphuric acid added. Cinnamic
acid (m.p. and mixed m.p. 133°) crystallised out.

Experiment XXV

Preparation of the Dibromide derivative of (XXXV)

(XXXV) (0.15 g.) was shaken with a saturated solution of bromine
in carbon tetrachloride until the reaction mixture retained its orange
colour. The solution was carefully concentrated under reduced pressure
and a white crystalline solid (XLIII; 0.08 g.) resulted. It was recrystallised
from petroleum ether (b.p. 60°-80°) and dried at 50° (m.p. 117°).

(Found: C, 38.75; H, 3.48; Br, 51.89.

Calculated for $C_{10}H_{10}O_2Br_2$: C, 38.63; H, 3.54; Br, 51.43)

lit.[122] m.p. 117°.

FIG. $\overline{XV}$

I METHYL CINNAMATE
II COMPOUND ~~XXXV~~ $\underline{XLV}$

Experiment XXVI

Examination of the infrared spectrum of (XLV)

This showed the disappearance of the peak at 1626 cm.$^{-1}$

(cf. methylcinnamate spectrum) and indicated that addition across

the double bond had taken place. (Fig.XV).

Experiment XXVII

Action of Boron Trichloride on hydroxy- and methoxy-cinnamic acid

derivatives

Experiment XXII was repeated using:-

p-Coumaric acid (4.9 g.)   ⎫   (80 ml.)
Caffeic acid (1.02 g.)   ⎬ in methylene chloride   (20 ml.)
Sinapic acid (2.13 g.)   ⎭   (40 ml.)

The products were either distilled (XXXVI) or isolated
chromatographically (XLVI) & (XXXVI)

(XXXVI) methyl p-coumarate from p-coumaric acid (3.65 g., m.p. 137.5°).

(Found: C, 67.25; H, 5.63; -OCH$_3$, 15.91%.

Calculated for C$_{10}$H$_{10}$O$_3$: C, 67.40; H, 5.66; -OCH$_3$, 15.96%).

lit.^{123} m.p. 137°.

(XLVI) methyl caffeate from caffeic acid (0.62 g., m.p. 152°).

(Found: C, 61.73; H, 5.00;

Calculated for C$_{10}$H$_{10}$O$_4$; C, 61.85; H, 5.19)

lit.^{124} m.p. 152°.

(XXXV) 3,4,5-trihydroxy methyl cinnamate from sinapic acid

(1.01 g., m.p. 174° with decomposition).

Experiment XXVIII

Acetylation of (XXVII) 3,4,5- trihydroxy methyl cinnamate.

(XXVII) (0.5 g.) was suspended in acetic anhydride (5 ml.).
Zinc chloride (0.05 g.) was added and the mixture left to stand on
a water bath (60°) for 2 hr.   The reaction product was poured into
iced water (100 ml.) and the resulting oil left at 0° overnight.
The oil solidified and was filtered off, purified by repeated
crystallisation from glacial acetic acid and finally crystallised
from a chloroform-petroleum ether (b.p. 60°-80°) mixture
(0.34 g. m.p. 170°). lit.[125] m.p.   168°.

(Found: C, 57.44; H, 4.75

Calculated for $C_{16}H_{16}O_8$;   C, 57.14; H, 4.80).

Experiment XXIX

Action of Boron Trichloride on an extract of Coffea sp.

Green beans of Coffea sp. (10 g.) were frozen hard in
liquid nitrogen and ground to a fine powder in a ball-mill.

A methanolic extract of the powder was examined by paper
chromatography in solvents A, B and G and shown to be rich in chlorogenic
acid.

The powder was dried at 60° under vacuum and then suspended
in methylene dichloride and treated with boron trichloride and methanol
as in the previous experiments (Experiments XXII and XXVII). The product
was evaporated to dryness on a rotary evaporator and then taken up
in chloroform for chromatographic examination. The coloured complexes

formed in the reaction, and tended to streak down paper chromatograms, were insoluble in chloroform.

Experiment XXX

Action of Boron Trichloride on an Extract of Antirrhinum majus

Flowers (12) of Antirrhinum majus were macerated in aqueous methanol (80% w/v; 50 ml.) and the extract evaporated to dryness under vacuum at 60°.

The resulting solid was suspended in methylene dichloride and treated with boron trichloride as before (Experiment XXIX). As in Experiment XXIX the resulting methyl esters were dissolved in chloroform for chromatographic examination.

Experiment $\overline{XXXI}$

Electrophoresis of Benzeneazo-p-carboxylates

Excess, freshly prepared diazotised p-amino benzoic acid (made by the addition of aqueous sodium nitrite solution (5% w/v in 2N-HCl; 5 ml.) followed by addition to 30 ml. with distilled water) was added to an aqueous solution of the phenol.

The dye solution was then streaked on Whatman 3MM paper which was developed with Solvent E. The chromatogram was sprayed with N-NaOH and the coloured band eluted from the chromatogram with aqueous methanol (80% v/v; 5 ml.). It was then examined on paper electrophoretograms using Buffers A and C. The paper electrophoretograms were again sprayed with N-NaOH solution to intensify the colour. The parent phenols were examined on the same electrophoretograms.

Experiment $\overline{\text{XXXII}}$

Electrophoresis of Benzeneazo-p-sulphonate

Freshly prepared diazotised sulphanilic acid was added to a solution of the phenol. The resulting reaction mixture was streaked on Whatman 3MM paper which was developed with Solvent E. The main coloured band was eluted from the chromatogram with aqueous methanol (80 v/v) and concentrated under reduced pressure. It was then examined on paper electrophoretograms together with the parent phenol using Buffers A and C. Spraying of the electrophoretograms with N-NaOH solution intensified the colour.

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