THE METABOLIC STUDY OF β-(3,4 dihydroxyphenyl)-L-alanine (L DOPA) BY PLANTS

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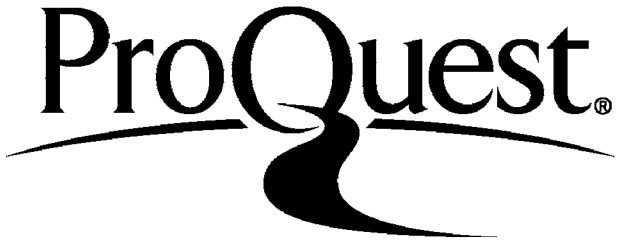

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The broad bean (Vicia faba major)

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ABSTRACT.

Methods for the detection and characterisation of DOPA derivatives in plant tssues have been investigated. In relation to this study, several methods for the production of possible naturally occurring derivatives were explored. The most success was achieved by feeding L-DOPA to Pisium sativum seeds which were then allowed to germinate for a short period. Five compounds were isolated from the treated seedlings and one of them was shown to be identical to the DOPA glucoside which had previously been reported in the testas of broad been (Vicia faba) seeds (5). The same compound was later identified in the cotyledons of dormant bean seeds, and its structure shown to be β - (3-O-β - D - glucopyranosyloxy) - 4 - hydroxyphenyl) - L - alanine. The chemical nature of the other compounds was also investigated and tentative deductions made for their structures.

A number of O and N - methylated derivatives of DOPA have been synthesised chemically. Their properties have been recorded and whereby isomeric O - substituted DOPA derivatives may be distinguished are outlined.

Using the information from the preliminary studies, the distribution of DOPA and its derivatives in about 200 different species of plants have been investigated. This has revealed that DOPA derivatives are rare in plants, although free DOPA has been detected in a number of leguminous species and in species grouped within the order of Centrospermae.

This latter discovery adds weight to the suggestion that DOPA is a precursor of the characteristic red-violet and yellow pigments which are found within the Centrospermae.

A detailed study of the distribution of DOPA and related compounds in the broad bean has been undertaken. Variations in the concentrations of the compounds throughout the growth cycle have been studied qualitatively using chromatographic and electrophoretic techniques, and quantatively, using an iodine colourimetric procedure and by ion exchange chromatography.

The presence of DOPA in the tissues of leguminous species is often associated with the formation of dark brown or black pigments. Observations on the biogenesis, histology and chemical nature of these pigments are described. Comparison of the chemical and physical properties of plant pigments with those of biosynthetically prepared melanins showed that there are considerable differences. The plant pigments do not appear to be similar to animal melanins.

The enzyme in dandelion (Taraxacum officinale) leaves which catalyses the deamination of DOPA has been studied. This appears to be labile, particulate and distinct from the phenylalanine ammonia lyase which is also present in the leaves.

CONTENTS.

PART I

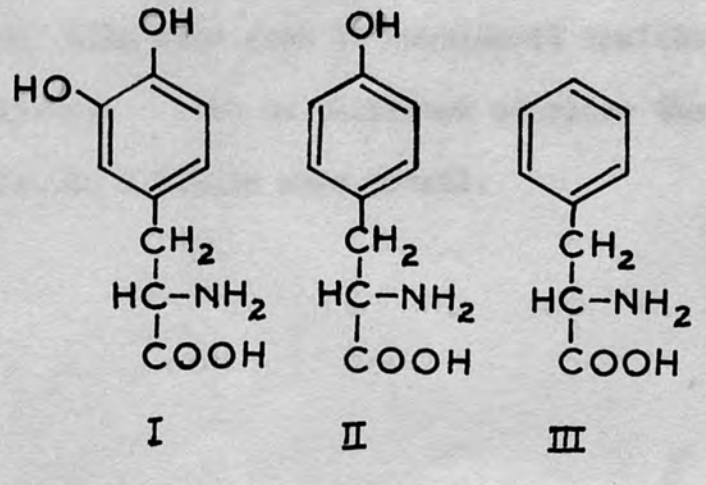
Introduction

INTRODUCTION.

I.1. General Introduction.

In 1915 Torquati (1) isolated a white crystalline solid from protein-free extracts of broad-bean pods, but although he demonstrated the presence of nitrogen he was unable to identify it. Later that year Guggenheim isolated the same compound (2), and realised that it was chemically identical to an hydroxylated aromatic acid, namely 3, 4-dihydroxyphenylalanine, which had been synthesised two years previously by Funk (3). But whereas the synthetic product was, of course, optically inactive, the one from bean pods was later found to be the L-isomer (4).

This new compound, β - (3,4 dihydroxyphenyl)-L-alanine (L-DOPA) (I), was obviously closely related to the common protein amino acids tyrosine (II) and phenylalanine (III), which appear to be ubiquitous in living organisms including the tissues of higher plants, and which are very important precursors of a wide range of secondary substances.



By contrast, DOPA has never been detected in any naturally occurring protein. There have been few accounts of its occurrence in plant tissues, and only one derivative, an O-β-D-glucoside in the seed coat of Vicia faba, has ever been reported (5).

Nevertheless, DOPA has been suggested as a metabolite in the biogenesis of diverse groups of substances ranging from alkaloids and the red and yellow nitrogen-containing pigments of the Centrospermae, to melanins and other polymeric products.

The purpose of the present study was to consider some aspects of the metabolism of DOPA summarised in Fig.1. Of particular interest was the extent to which DOPA is found free (or as simple derivatives) in the tissues of higher plants.

Attempts were made, therefore, to produce such derivatives, to determine their structure, and to devise general methods whereby the derivatives might be recognised and characterised.

Other facets of this problem which have been considered have included the blackening of leguminous tissues and the enzymic deamination of DOPA by preparations from Hordeum vulgare and Taraxacum officinale.

However, this work must be considered against the background of previous knowledge. Let us therefore consider the individual aspects of DOPA metabolism in a little more detail.

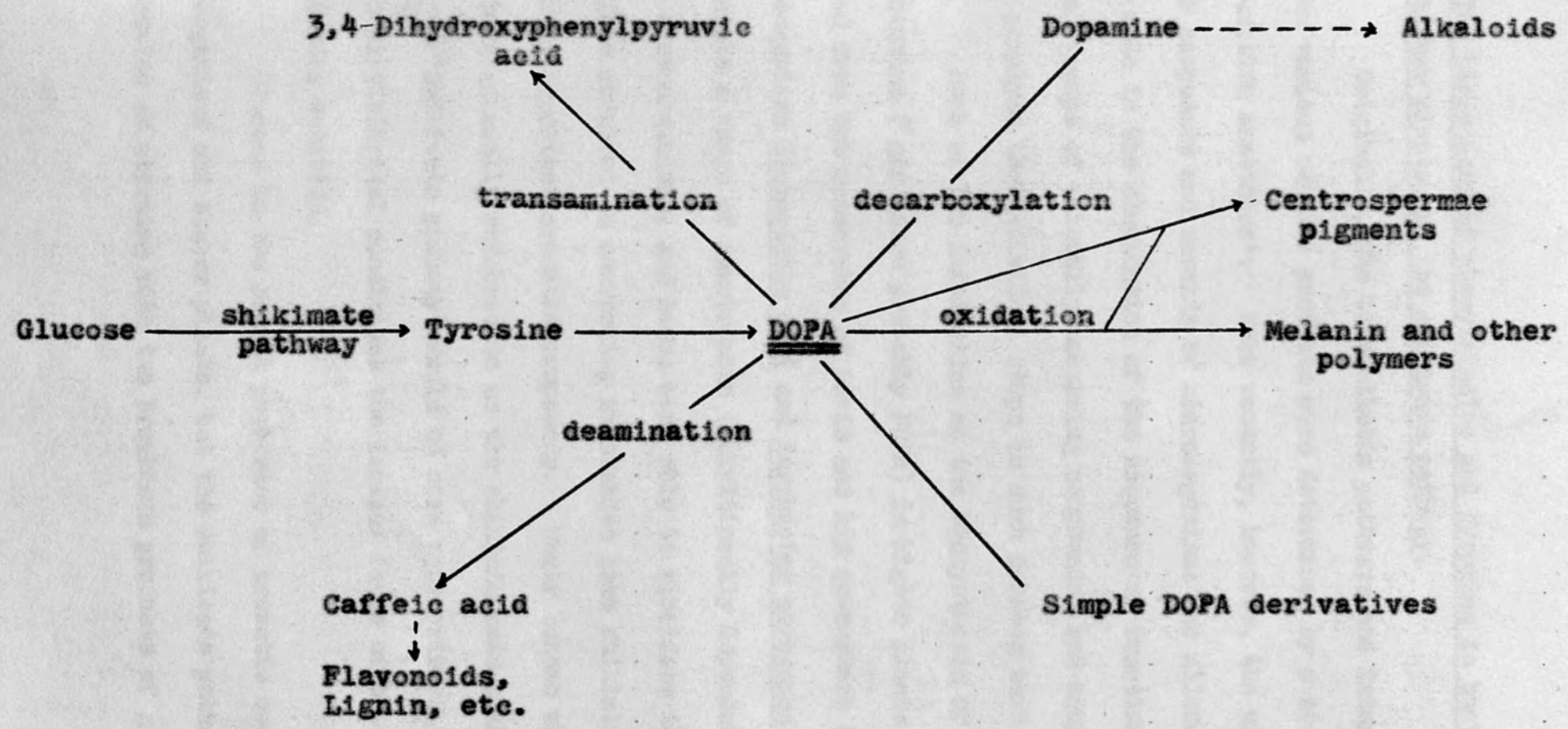


Fig. 1. **Possible metabolic pathways involving DOPA in higher plants.**

I.2. The biogenesis of phenylalanine and tyrosine in bacteria and higher plants; by the shikimate pathway.

Originally, the biosynthetic pathways and inter-relationships between various natural products were determined by a study of their 'comparative anatomies'. More recently, however, the use of radioactive tracer compounds and mutants of microorganisms has allowed great progress to be made in the elucidation of the sequence of reactions leading to several groups of naturally occurring compounds, and many of the enzymes which catalyse the individual steps in such schemes have been characterised.

Much of the information on the biosynthesis of phenylalanine and tyrosine (and hence probably DOPA) in higher plants was originally derived from the researches of Davis and his co-workers (6,7) with the microorganisms Escherichia coli and Aerobacter aerogenes. They managed to isolate a range of auxotrophic (nutritionally dependent) mutants of these microorganisms, and hence were able to elucidate the sequence of reactions involved in converting substances like shikimic acid into a number of important aromatic compounds. Their scheme which is shown in Fig. 2 is generally referred to as the shikimic acid pathway, although the term 'shikimate pathway' would be more appropriate, since under normal physiological conditions the ionised forms of the acids are the functioning entities.

Glucose is the actual precursor of aromatic compounds in both microorganisms and higher plants, but the shikimate pathway is usually represented as starting with two breakdown products of glucose,

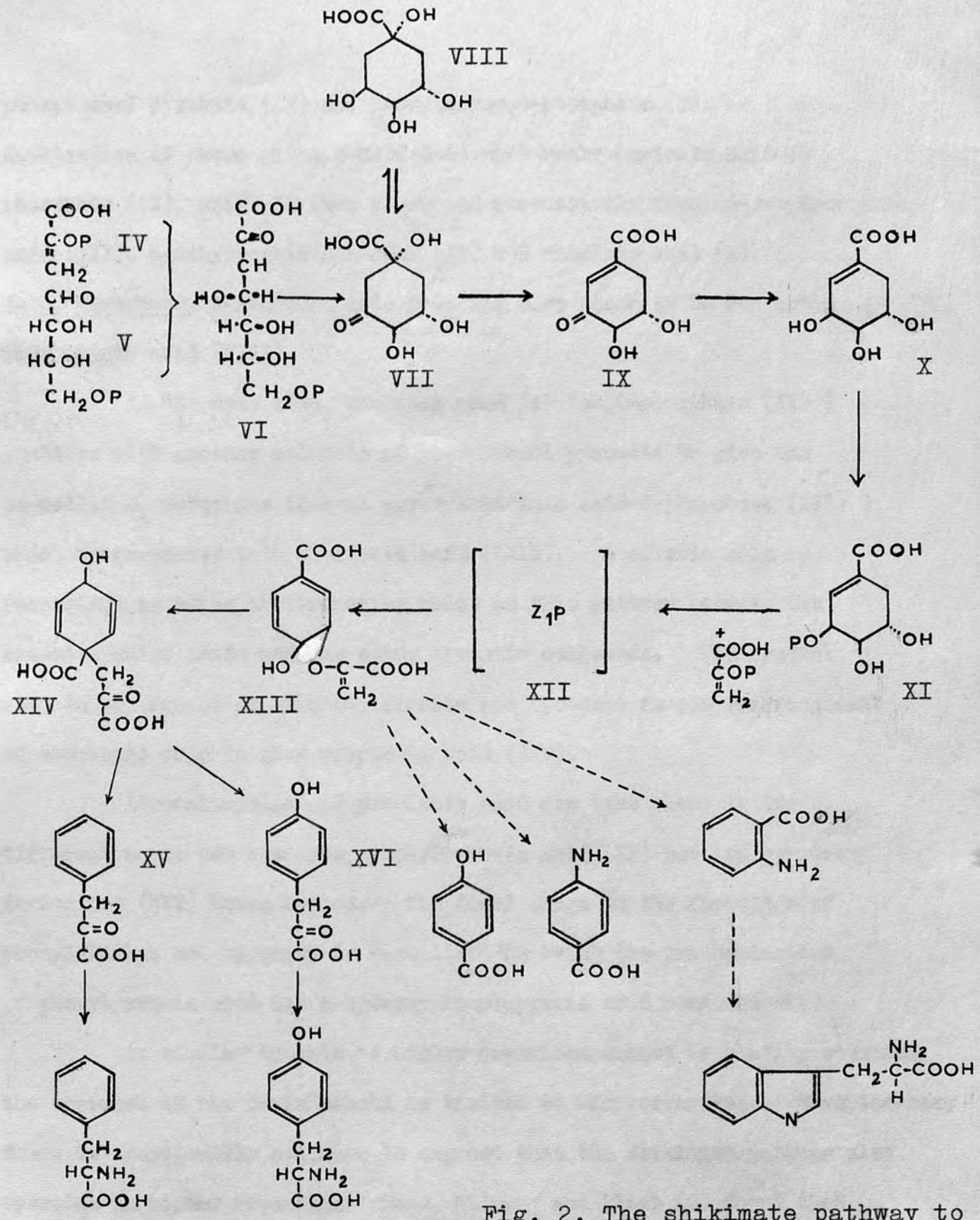


Fig. 2. The shikimate pathway to aromatic compounds in plants.

phosphoenol pyruvate (IV) and D-erythrose-4-phosphate (V). Combination of these gives 2-keto-3-deoxy-D-arabo-heptonic acid-7-phosphate (VI), which is then converted successively into 5-dehydroquinic acid (VII), 5-dehydroshikimic acid (IX) and shikimic acid (X). In A. aerogenes, 5-dehydroquinic acid was also shown to be converted into quinic acid (VIII).

In the next step, shikimic acid (as the 5-phosphate (XI)) combines with another molecule of phosphoenol pyruvate to give the so-called Z_1 phosphate (3-enol pyruvylshikimic acid-5-phosphate (XII)), which is converted into chorismic acid (XIII). Chorismic acid is recognised as being the branching point in this pathway between the aromatic amino acids and the other aromatic compounds. The crucial step in the formation of phenylalanine and tyrosine is the rearrangement of chorismic acid to give prephenic acid (XIV).

'Aromatisation' of prephenic acid can take place in two different ways; two products, phenylpyruvic acid (XV) and its p-hydroxy derivative (XVI) being formed. The final stage in the formation of phenylalanine and tyrosine is visualised as being the transamination of phenylpyruvic acid and p-hydroxyphenylpyruvic acid respectively.

As similar mutants of higher organisms cannot be readily obtained, the approach of the Davis school is limited to microorganisms. Nevertheless, there is considerable evidence to suggest that the shikimate pathway also operates in higher organisms. Thus, Gilvarg and Bloch (8) found that certain yeasts were able to utilise D-glucose-U-^{14}C to synthesise both

phenylalanine and tyrosine, and higher plants representing three different families have been shown to convert shikimic acid-^{14}C also into phenylalanine and tyrosine. The species in question were Salvia splendens (Labiatae) (9), wheat (Triticum vulgare, Graminae) and buckwheat (Fagopyrum tataricum, Polygonaceae) (10).

Shikimic acid itself appears to be widely distributed in plant tissues (11), and it is now generally accepted that in such tissues it occupies a central role in the biosynthesis of a wide range of imprtant aromatic compounds. Much of the evidence for this belief, and the general background information on the shikimate pathway as a whole has been reviewed by Bohm (11), Sprinson (12), Neish (13) and Pridham (14).

The comprehensive nature of these reviews makes further detailed comment unnecessary, but the experimental findings of Gamborg and Neish (10) and Weinstein, Porter and Laurencot (15) are perhaps worthy of mention. The former workers found that phenylpyruvic acid and phenyllactic acid were incorporated into the bound phenylalanine of young wheat and buckwheat shoots as readily as phenylalanine itself. Similarly, the p-hydroxy compounds were incorporated into the bound tyrosine by both species. There was no conversion of the p-hydroxy compounds into phenylalanine and only a relatively small utilisation of phenylpyruvic and phenyllactic acids for tyrosine synthesis.

Weinstein et al found that quinic acid -^{14}C was converted to shikimic acid, phenylalanine and tyrosine in young rose blooms, thus providing evidence that quinic acid could be utilised in the shikimate pathway in a manner which had been shown for A. aerogenes.

If the shikimate pathway operates in higher plants, one would expect that tyrosine would be formed more readily from p-hydroxy phenylpyruvic acid than phenylalanine, and this was found in the experiment just described. There is evidence, however, for the direct convertion of phenylalanine to tyrosine. Nair and Vining (16), for example, have demonstrated that spinach leaves contain an enzyme system capable of catalyzing the hydroxylation of phenylalanine.

Their purified enzyme preparation had a pH optimum of 4.2, and required supplementation with two electron donors for maximum activity. This requirement was met by adding tetrahydrofolic acid and a reduced pyridine nucleotide. Of a number of test substances studied, only L-phenylalanine and L-p-fluorophenylalanine were acted upon, both being converted to tyrosine.

In these respects the spinach enzymes appear to closely resemble the phenylalanine hydroxlase reported in animal liver (17).

I.5. The biogenesis of DOPA in higher plants.

By analogy with the scheme suggested above for the biogenesis of tyrosine in plant tissues, DOPA might be supposed to be formed from

3,4-dihydroxyphenylpyruvic by a process of transamination. However, there is no practical evidence to suggest that this is the case, and it seems far more likely that DOPA is formed from tyrosine by hydroxylation.

The enzyme system responsible for the hydroxylation step could be either a specific hydroxylase, analgous to the phenylalanine hydroxylase of spinach leaves or a more general oxidase of the 'tyrosinase' type. An enzyme of the former type was discovered in mitochondrial fractions from brain and adrenal medulla (18). It was suggested that this preparation involved Fe^{++} and required a cofactor for maximum activity.

It is more likely that the oxidation is catalysed by a tyrosinase, as these enzymes are known to be widely distributed in higher plants, and they have also been isolated from mammalian and

ADDENDUM

A detailed review by L. L. Ingraham of oxidative enzymes and their mode of action appears in Comprehensive Biochemistry, Vol. 14. (Ed. M. Florkin and E. H. Stotz) p. 424, Elsevier, London (1966).

(3). They contain copper.

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Tyrosinases from different sources show qualitative differences in their substrate specificities, and in their stability towards physical and chemical treatments, but all have three characteristics in common:

(1). They are able to catalyse the insertion of an hydroxyl group into monohydric phenols, ortho to the one already present.

(2). They are also able to catalyse the oxidation of o-dihydric phenols to the corresponding quinones (i.e. the removal of two hydrogen atoms).

(3). They contain copper.

Tyrosinases from mammalian sources have been widely studied because of the role they occupy in the biosynthesis of adrenaline and melanin pigments. They appear to be true tyrosinases since they are relatively specific for the oxidation of tyrosine and DOPA. The fact that these enzymes are capable of catalysing two distinct reactions raises the question as to whether one enzyme possessing two active sites or two separate enzymes are involved in the oxidation.

The available evidence favours the former alternative for the following reasons:

(1). No tyrosinase preparation yet studied has ever been satisfactorily demonstrated to catalyse the oxidation of monohydroxy compounds but not o-dihydroxy compounds.

(2). Conversely, all enzyme systems catalysing the oxidation of o-dihydroxy compounds to the corresponding o-quinones, also catalyse the oxidation of the monohydroxy compound under the proper conditions.

(3). The ability of tyrosinase preparations to catalyse both reactions is directly proportional to their copper content.

At one time the view was expressed that a separate enzyme existed in mammalian tissues, a DOPA-oxidase. This enzyme was thought to catalyse the oxidation of DOPA to melanin, but not of tyrosine to DOPA. The reason for this view, was a failure to appreciate that when tyrosine and tyrosinase react in the presence of oxygen, there is often a long induction period before oxygen uptake occurs. The addition of

small amounts of DOPA to the system, however, rapidly brings about the reaction, its function being to reduce the redox potential of the system (19). It is interesting to note that DL-DOPA was found to be only about 75 per cent as effective as the L-isomer, and that DOPA was fairly specific in regulating the induction period, although structurally related compounds did show some reaction. (19).

Tyrosinases of insect origin seem to be relatively less specific in their action (20). In the insects their function is intimately concerned with the formation of melanin and the sclerotisation of the exoskeleton. Insect tyrosinases are often in a latent or inactive form and need some form of activation before they are able to function. The current information on the role of tyrosinases in insect tissues has been comprehensively reviewed (20).

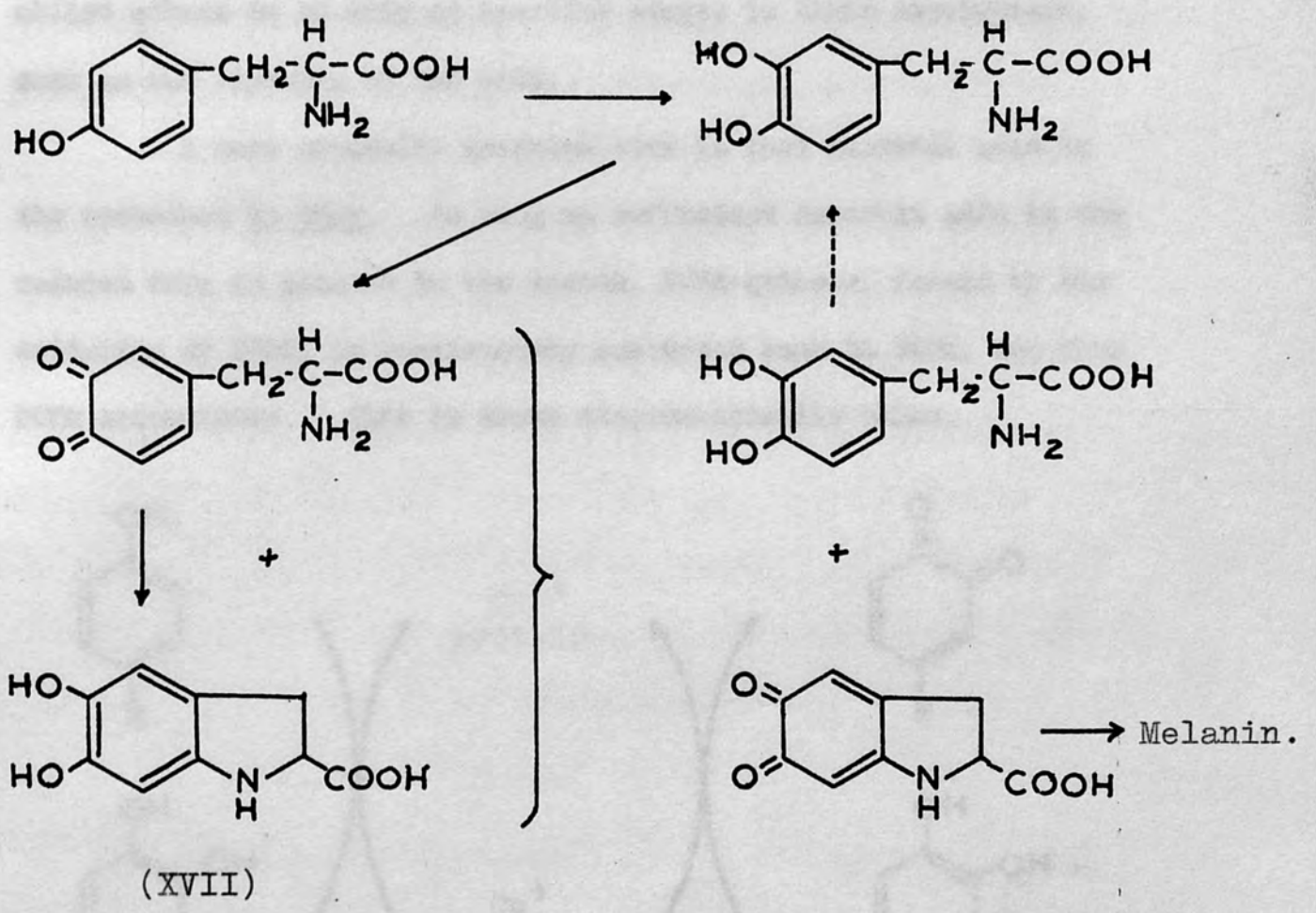
Plant 'tyrosinases' are less specific than their mammalian counterparts. Many of the plant enzymes catalyse the oxidation of simple phenol and catechol derivatives at a much greater rate than tyrosine and DOPA. Like mammalian tyrosinases, they are copper-containing enzymes, but in most cases their function in a particular tissue does not appear to be concerned with either the oxidation of tyrosine or the formation of melanin. These enzymes are therefore usually referred to simply as phenolases, although the terms phenol oxidases and polyphenoloxidases are also in common use to describe

the same enzymes. The advent of more precise terminology must await a fuller knowledge of their distribution in plant cells, and the extent and range of the oxidative reactions which they catalyse.

Both plant phenolases and mammalian tyrosinases are intra-cellular enzymes, but whereas disruption of mammalian tissues gives preparations in which the enzyme is retained on cytoplasmic particles, those of plant origin can usually be obtained in colloidal solution.

Evans and Raper (21) have demonstrated that tyrosine can be converted into DOPA in quite good yield by the in vitro action of tyrosinase preparations derived from mealworms, potatoes and wheat bran. Accumulation of this type is unusual, since it has been shown that o-dihydroxyphenols such as DOPA are acted upon at a much faster rate than the corresponding monohydroxy compounds (22). Evans and Raper pointed out that selective inactivation towards DOPA and not towards tyrosine was most unlikely, but nevertheless they were able to isolate DOPA, in yields of 10-20 per cent of the tyrosine oxidised, from reaction mixtures containing tyrosine and mealworm tyrosinase after 2-5 hours incubation. They were unable to detect any enzyme system which could account for any reducing action, and this led them to believe that one of the products of the oxidative reaction itself acted as a reducing agent. They therefore proposed the scheme shown in Fig.3. to explain the in vitro accumulation of DOPA. Support for this hypothesis was furnished

by the fact that the addition of leuco compound (5,6-dihydroxyindoline-2-carboxylic acid;XVII) to the system increased DOPA accumulation still further.

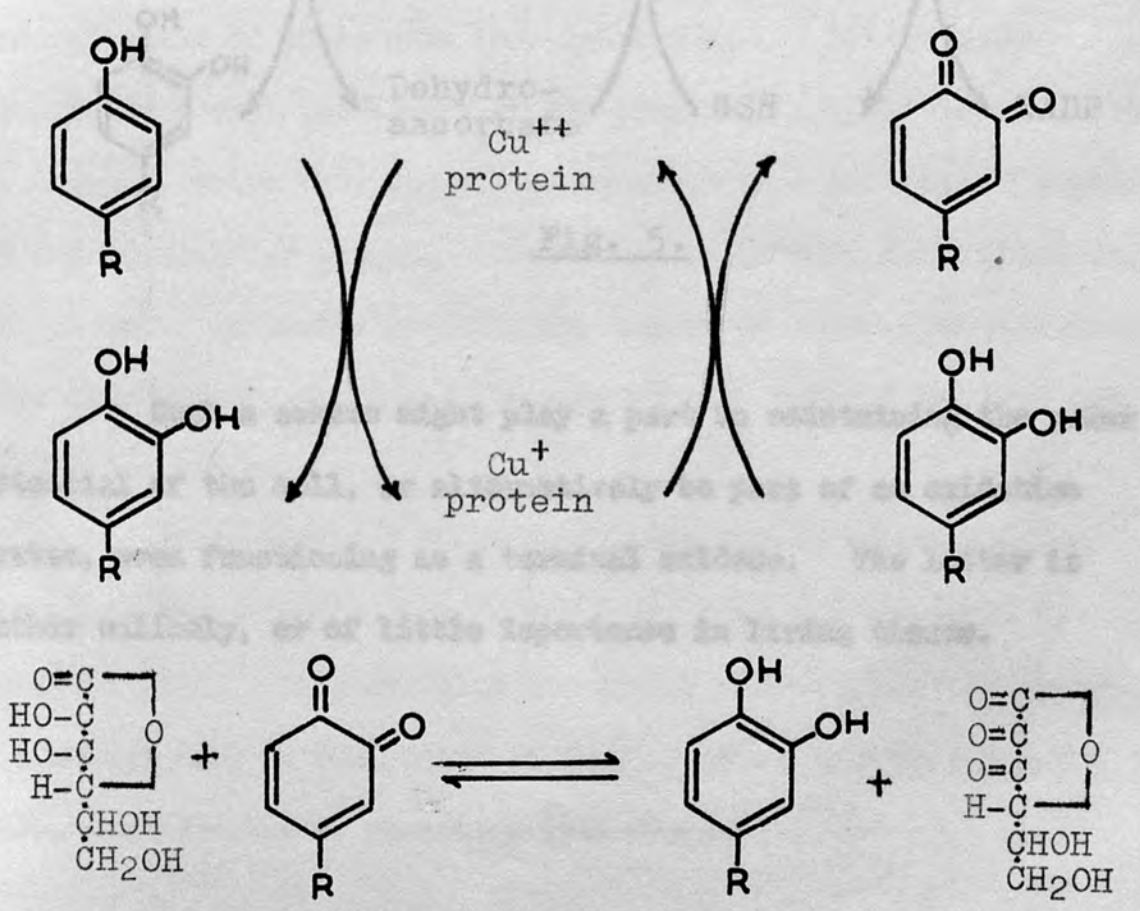


(XVII)

Fig. 3. A scheme to explain the in vitro accumulation of DOPA.

However, it is unlikely that this scheme explains the *in vivo* accumulation of DOPA in plant tissues, for it would suggest that DOPA accumulation is accompanied by reddening or blackening of the tissue concerned. Our own observations and those of others show quite clearly that many DOPA-rich tissues never show any pigmentation, whilst others do so only at specific stages in their development, such as the ripening of the pods.

A more generally accepted view is that ascorbic acid is the reductant *in vivo*. As long as sufficient ascorbic acid in the reduced form is present in the tissue, DOPA-quinone, formed by the oxidation of DOPA, is continuously converted back to DOPA, and thus DOPA accumulates. This is shown diagrammatically below.



There is some evidence that in the presence of a reducing agent such as ascorbic acid, quinones can act as intermediate hydrogen carriers, transferring hydrogen from a metabolite to molecular oxygen. It is possible, therefore, that the tyrosinase-DOPA-ascorbate system could play a part in plant respiration. As glutathione reductase and dehydroascorbate reductase are of widespread distribution in plants, the scheme might be expanded further as shown in Fig.5.

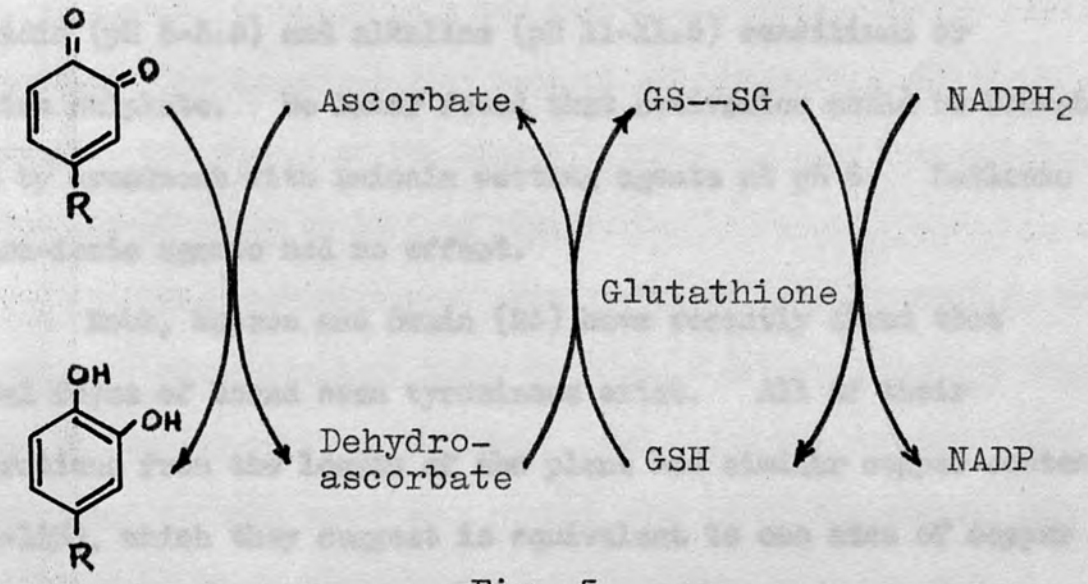


Fig. 5.

Such a scheme might play a part in maintaining the redox potential of the cell, or alternatively be part of an oxidation system, even functioning as a terminal oxidase. The latter is rather unlikely, or of little importance in living tissue.

Although little is known of the systems of the type just discussed, the available information suggests that they are often relatively complex. The 'tyrosinases' in Vicia faba have perhaps received more attention than others in higher plants. Thus, Kenten (22) found that aqueous extracts of broad bean leaves contained large quantities of a latent tyrosinase which could be converted into the active form by brief exposure of the extracts to acidic (pH 5-5.5) and alkaline (pH 11-11.5) conditions or ammonium sulphate. He later found that activation could be brought about by treatment with anionic wetting agents at pH 6. Cationic and non-ionic agents had no effect.

Robb, Mapson and Swain (23) have recently shown that several forms of broad bean tyrosinase exist. All of their preparations from the leaves of the plant had similar copper content (0.14-15%), which they suggest is equivalent to one atom of copper to one molecule of enzyme. They were not able to distinguish between the forms by substrate specificity, degree of latency or sedimentation. They also briefly discussed the relationship between the bean and fungal tyrosinases.

An interesting function for the latent tyrosinase-DOPA (or other phenolics) system in the broad bean was suggested by Deverall (24). This was that the system played a part in minimising the damage done by the attack of fungi such as Botyris fabae, the causative organism of chocolate spot disease.

Attack by such organisms involves the initial release of a polygalacturonidase to break down the pectin of the plant cell-wall. Deverall found that the anionic oligosaccharides so formed were able to activate the latent tyrosinase, which in turn catalysed the oxidation of DOPA to melanin-like pigments. These pigments are able to combine with proteins such as the fungal polygalacturonidase, inactivating them, and thus limiting the damage.

Deverall's hypothesis was that interactions of this sort could explain why chocolate spot lesions are generally limited in size and why the tissue of the lesion remains coherent for some time.

I.4 The distribution of DOPA in plants.

Although latent tyrosinases are known to be present in some tissues, many more have been shown to contain active phenolase systems. Most of these also contain tyrosine. One might suppose therefore, that the formation of DOPA might be a common step in higher plants, and that DOPA might be of widespread distribution. However, only a limited number of reports of the occurrence of DOPA can be found in the literature and these have generally been in leguminous plants.

Miller (25) observed that protein-free aqueous and ethanolic extracts of the seeds of the velvet bean (Stizolobium deeringianum) darkened considerably in contact with air. This led him to isolate the compound responsible using lead acetate precipitation.

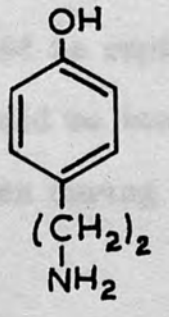
Melting point determinations on the compound itself and its tribenzoyl derivative, together with colour reactions showed that it was DOPA. On the basis of colour reactions alone, Miller also identified DOPA in twenty-six other varieties of the velvet bean and in S. hassjoo (Yokohama bean) and in S. niveum (Lyon bean). No quantative data were given.

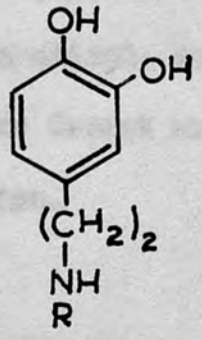
Hattori and Shiroya (26) confirmed the presence of DOPA in S. hassjoo seeds and noted that it also occurred in most other parts of the plant.

Two related species have been shown to contain large amounts of DOPA. Damodaran and Ramaswamy (27) were able to isolate 30 g of pure L-DOPA from 2 kg of Mucuna (Stizolobium) pruriens seeds, and Yoshida and Tokuku (28) isolated 19 g from 1 kg of M. capitata seeds. The isolation was again by lead acetate precipitation.

DOPA has also been reported in the common broom (Cytisus scoparius). Correale and Cortese (29) demonstrated that acetone extracts of the roots, trunk, twigs and flowers of this plant contained tyrosine, DOPA, tyramine (XVlll), dopamine (XlV), N-methyl dopamine (XX) and an unknown catechol derivative.

(XVIII)





(XIX R=H)
(XX R=CH_3)

DOPA appears to be less well distributed in non-leguminous species. Feng *et al* (30) reported the co-occurrence of DOPA and related compounds in *Portulaca oleracea* (Portulacaceae). They detected equal amounts (approximately 2.5 mg/g tissue) of noradrenaline and dopamine in the plant together with smaller amounts of DOPA.

Another rich source of DOPA was found to be the latex of *Euphorbia lathyrus*. Liss (51) isolated 1.62 g of crystalline L-DOPA from 100 ml. of latex using lead acetate precipitation.

DOPA has also been reported in *Aristolochia clematilis* (32), *Morus alba* (33), and in warm-zone sugar beet (*Beta* sp.(54)), but these reports are less well authenticated.

It appears, therefore, that DOPA is not widely distributed in higher plants, and one of the aims of the present work was to investigate its' distribution further. Three reasons, however, may be advanced to explain the failure to detect DOPA in any given tissue.

(1). That DOPA is not in fact formed. Either the appropriate enzymes or tyrosine are not present, or alternatively that the oxidase (tyrosinase) system and its substrate are separated in the cell.

(2). DOPA is present in the form of a glycosyl or similar derivative.

(3). DOPA once formed is rapidly metabolised, so that only a small pool exists which could be too small to detect or could even be destroyed by oxidation during extraction.

Which of these alternatives operates in any particular tissue depends upon the species and the tissue in question, and our knowledge at the present time is so scanty that it is difficult to distinguish between them. Some of the more important metabolic pathways of DOPA metabolism have been summarised already in Fig.1. These are:-

(1). Decarboxylation to yield dopamine; dopamine being thought to be a possible precursor of a number of alkaloids.

(2). Deamination of DOPA gives caffeic acid which is known to be a common plant constituent.

(3). Transamination.

(4). Oxidation of DOPA is known to result in the formation of melanin in animals, insects, etc., and a similar reaction might explain the formation of dark coloured pigments in some plant tissues. It has also been suggested that partial oxidation of DOPA followed by condensation gives rise to the red-violet pigments which are characteristic of species belonging to the Centrospermae.

I. 5 The formation of dopamine in higher plants.

Dopamine appears to be of limited distribution in higher plants. Mention has already been made of its occurrence in Portulaca oleracea and Cytisus scoparius (together with related compounds) and in the latter case Correale and Cortese (29) noted that the dopamine content of the plant increased throughout the growth cycle, from about 150 γg/g tissue in winter to about 500 γg/g in the following autumn. The concentration of related compounds also followed a similar pattern.

Later experiments (55), revealed that the seeds only contained traces of tyramine and DOPA but no free tyrosine, although this was present in the bound form. The early seedlings had tyrosine, tyramine, a trace of DOPA, dopamine and N - methyl dopamine (epinine), whilst later seedlings had a decreased amine content.

In the mature plant it was demonstrated that initially the tyramine and dopamine concentration increased with ripening, and then the tyramine content decreased whilst the dopamine increased. Both compounds had disappeared in the blackened pod. It appears, therefore, that dopamine plays some part in the formation of the black pigment in this tissue.

Dopamine also occurrs in high concentration in banana (Musa sp.) peel, and to a lesser extent in the pulp of the fruit and other parts of the plant (36). It was found that the dopamine content varies with the origin of the cultivated variety.

Two wild species, M. acuminata and M. balbisiana, are thought to be the ancestors of the commercial banana plants. As the former has a much higher dopamine content; varieties developed from it show this same characteristic (37).

There is some doubt as to whether dopamine originates in the same way in both animals and plants. In mammalian tissues dopamine appears to be formed from DOPA by decarboxylation. Enzyme preparations have been isolated from kidney and adrenal medulla which specifically catalyse this reaction. The formation of dopamine is of interest because it is the precursor of a number of physiologically important amines, the biosynthesis of which is illustrated in Fig.6.

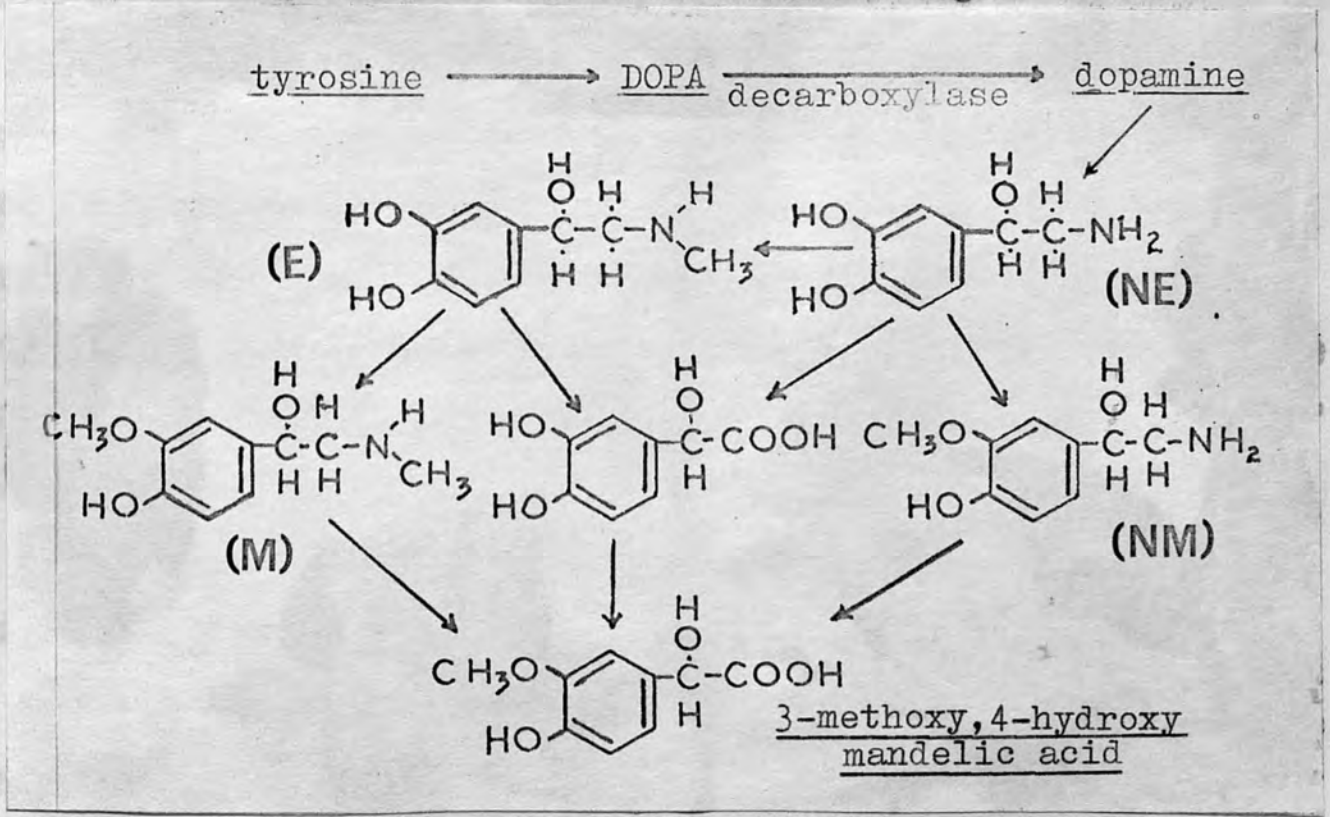


Fig.6 The biosynthesis of physiologically active amines in animals (38).

E = Epinephrine (Adrenaline) M = Metanephrine

NE = Norepinephrine NM = Normetanephrine

By contrast, the formation of dopamine in plants has received much less attention and there are conflicting opinions on the matter. The co-occurrence of tyrosine, tyramine, DOPA and dopamine in C. scoparius, for example, suggests two possible routes for the formation of dopamine.

(1) Tyrosine ——→ tyramine ——→ dopamine

(11) Tyrosine ——→ DOPA ——→ dopamine.

In the case of C. scoparius, it was found that the addition of L-phenylalanine-U-^{14}C to the nutrient solution of the plants at various stages of growth, led only to the formation of radioactive phenylethylamine. Labelled tyramine and dopamine could not be detected (39).

In a series of in vitro experiments, aqueous homogenates of the plant were shown to contain tyrosine decarboxylase activity, but no DOPA decarboxylase activity. 5 per cent conversion of tyrosine was found over 50-56 hrs. Another enzyme in the suspension oxidised tyramine to dopamine with about 2 per cent conversion (40). The formation of dopamine in this plant appears therefore to be by route (1) above.

Buckley and Towers (41), also think that this is the route in the banana plant. Most of their studies were with tissue disks.

A contrary view was expressed by Picinelli (42), after investigating the compounds present in embryos and young roots and shoots which developed from Vicia faba seeds germinated on filter paper.

Two monophenolic compounds and four dihydroxyphenolic compounds were detected on paper chromatograms, and these included tyrosine, DOPA and epinine, but not tyramine. He estimated the amount of each compound on paper, and found that the concentration of DOPA was always low, i.e. 40γg/g tissue, whilst the amount of epinine was consistently high. On this evidence it was suggested that epinine was formed via DOPA and not via tyramine.

Picinelli's findings have been checked in the present work, but attempts to detect epinine in young V. faba plants were without success, although tyrosine and large amounts of DOPA were detected. The low values for DOPA, quoted by Picinelli may have been due to his method of extraction, which was with acetone.

The available evidence which suggests that dopamine is formed from DOPA in plants, as it is in animals, is extremely tenuous. Nevertheless, it is widely accepted that DOPA is the precursor of dopamine in the biogenesis of a variety of alkaloids.

The above evidence, of course, was gained with monocotyledons and leguminous species, but it may well be that an alternative pathway operates in alkaloid-producing species.

I. 6 DOPA as an alkaloid metabolite.

A number of nitrogen- containing organic compounds, which are grouped together under the general term alkaloids, appear to be widely distributed in plant tissues. They form a heterogeneous group, the different classes of which show such a diversity of structural types, that

it is impossible to suggest a common function for them, nor to define a single biogenetic scheme to account for their formation.

A large number seem to be formed from aromatic amino acids. Radioactive tracer experiments have shown, for example, that phenylalanine is a precusor of ephedrine (XXI) in Ephedra distachya (45), and of d-nor-ephedrine (XXII) in Catha edulis (44). Similarly, tyrosine has been shown to be involved in the production of hordenine (XXIV) and N-methyl tyramine (XXIII) by germinating barley (45).

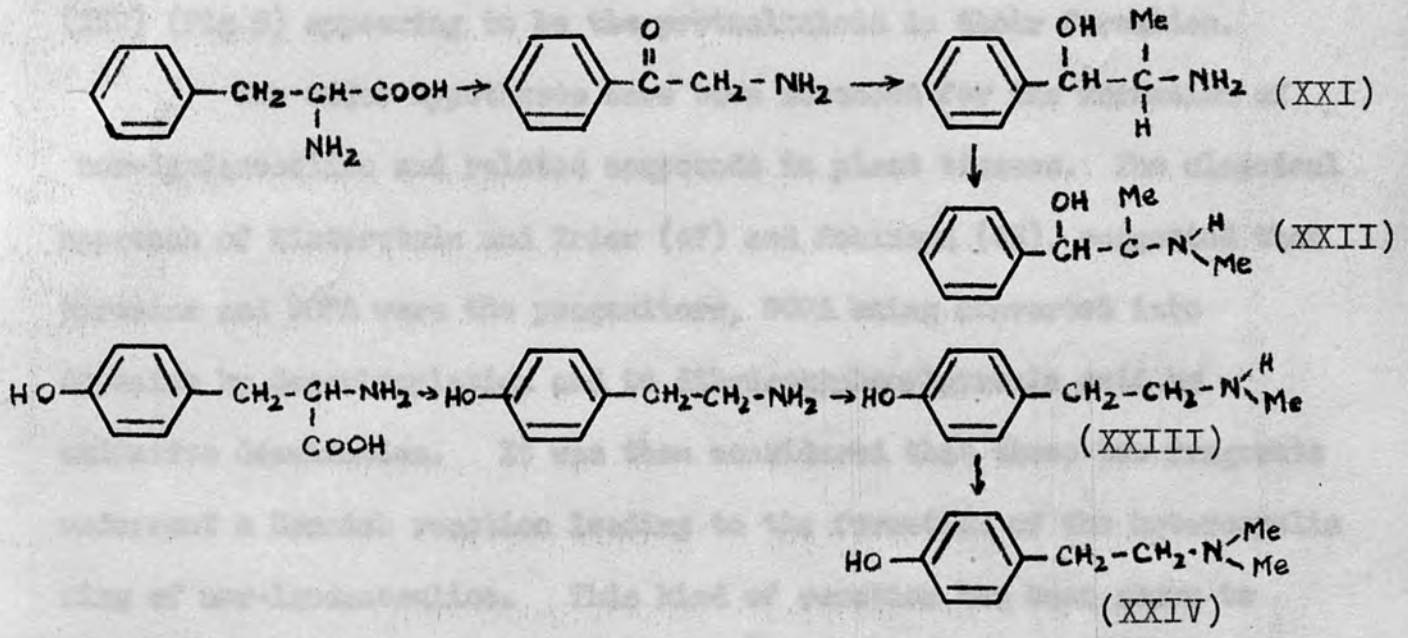


Fig.7

The role of tyrosine and DOPA in the formation of alkaloids is not fully understood, but it seems possible that one or both of these compounds are precursors of the isoquinoline, bis-benzylisoquinoline, Amaryllidaceae and Erythrina groups of alkaloids together with many others.

Much of the evidence for this view has been reviewed by Leete (46).

Two aspects of alkaloid biogenesis may be distinguished. Firstly, the formation of the basic alkaloidal skeleton (proto-alkaloid) from simple precursors such as tyrosine and DOPA, and then the secondary modifications to the skeleton producing the derived alkaloids.

A number of workers have shown that labelled tyrosine and dopamine are readily and specifically incorporated into the molecules of hydrastine, chelidonine, sanguinarine, berberine and the thebaine-codeine-morphine group of alkaloids in opium poppy, nor-laudanosoline (XXV) (Fig.8) appearing to be the protoalkaloid in their formation.

Two major hypotheses have been advanced for the formation of nor-laudanosoline and related compounds in plant tissues. The classical approach of Winterstein and Trier (47) and Robinson (48), suggested that tyrosine and DOPA were the progenitors, DOPA being converted into dopamine by decarboxylation and to dihydroxyphenylpyruvic acid by oxidative deamination. It was then considered that these two fragments underwent a Mannich reaction leading to the formation of the heterocyclic ring of nor-laudanosoline. This kind of reaction has been shown to occur readily *in vitro* under physiological conditions [illegible] and it has been suggested that this step is not enzyme catalysed.

The alternative hypothesis of Wenkert (50) differed from the above in suggesting that prephenic acid rather than tyrosine was involved in some of the biogenetic steps.

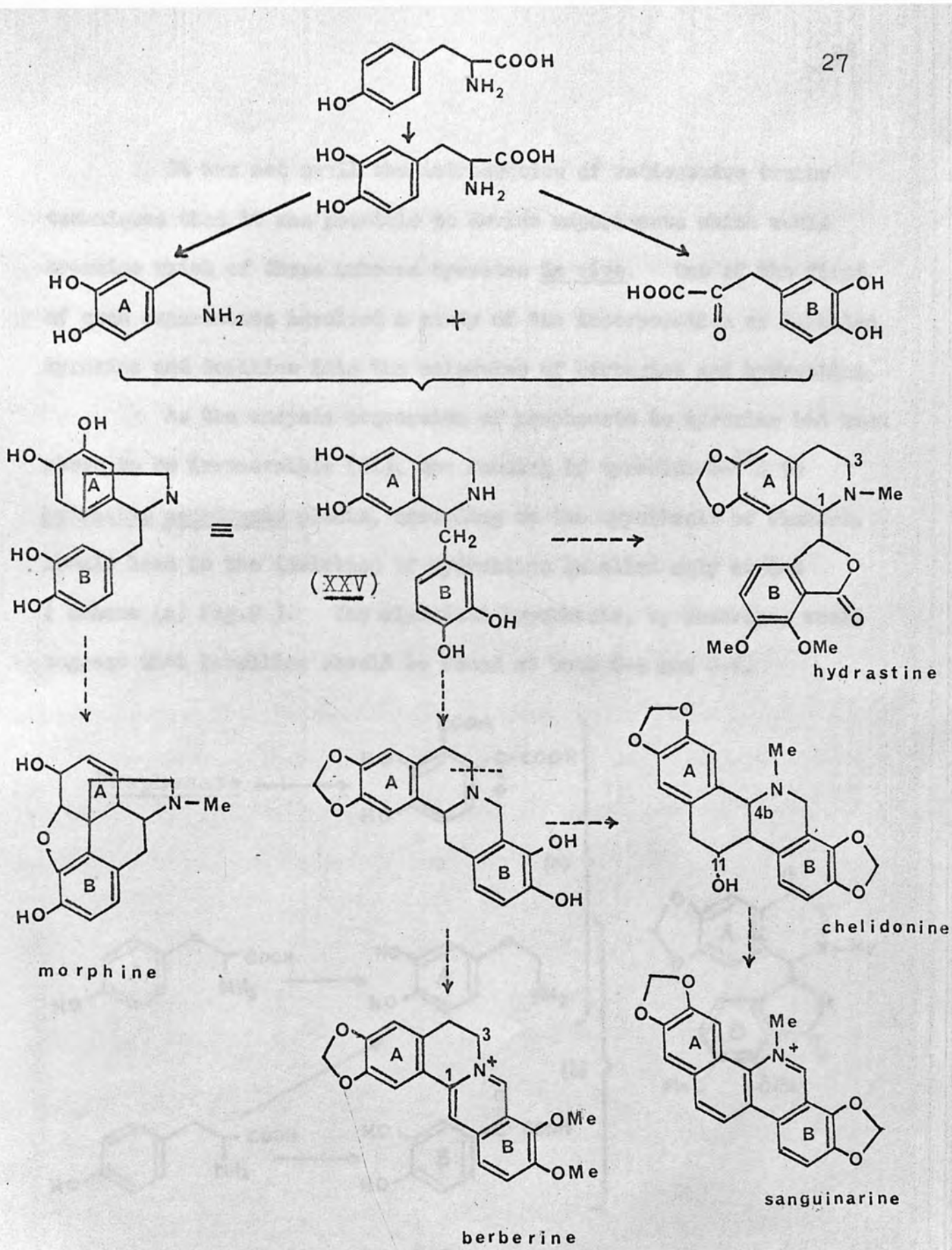


Fig. 8. DOPA as an alkaloid metabolite

It was not until the introduction of radioactive tracer techniques that it was possible to devise experiments which would determine which of these schemes operates *in vivo*. One of the first of such experiments involved a study of the incorporation of labelled tyrosine and dopamine into the molecules of berberine and hydrastine.

As the enzymic conversion of prephenate to tyrosine had been shown to be irreversible (51), the feeding of tyrosine-5-^{14}C to *Hydrastis canadensis* plants, according to the hypothesis of Wenkert, should lead to the isolation of hydrastine labelled only at C-4 (Scheme (a) Fig.9). The classical hypothesis, by contrast, would suggest that labelling should be found at both C-4 and C-7.

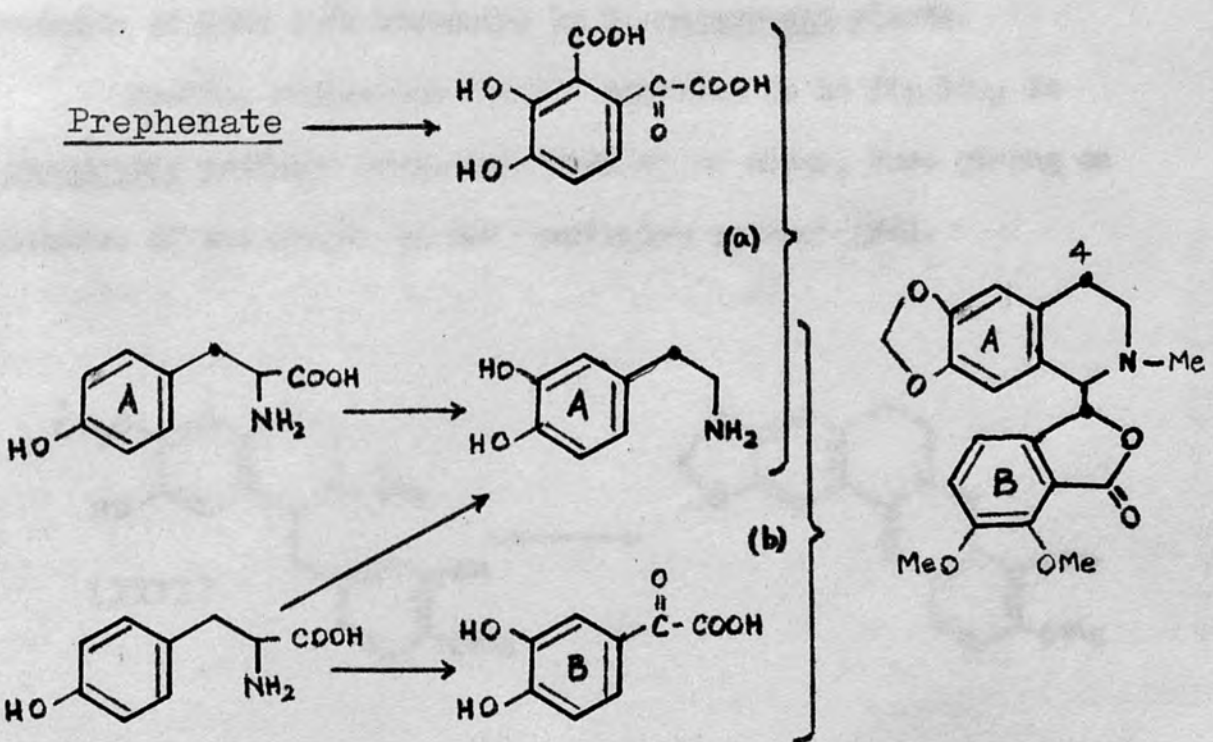


Fig. 9.

Evidence in support of the classical view was provided by the experiments of Gear and Spencer (52, 53, 54), who found that hydrastine was derived from two tyrosine units which were not, however, incorporated with equal efficiency. They later found that tyrosine-3-^{14}C gave rise to hydrastine labelled at C-4 and C-7; tyrosine-2-^{14}C gave hydrastine labelled at C-1 and C-3, whilst dopamine-1-^{14}C gave hydrastine labelled only at C-3. The same incorporation pattern was found in berberine after feeding labelled tyrosine, by Monkovic and Spencer (55), thus confirming that dopamine-1-^{14}C gave rise to berberine labelled only at C-3.

The only direct evidence that DOPA is involved in alkaloid biosynthesis was provided by Barton *et al* (56), who found a 0.9 % conversion of DOPA into berberine by *H. canadensis* plants.

Feeding reticuline (XXVI), labelled as in Fig.10., to *H. canadensis* produced berberine labelled as shown, thus giving an indication of the origin of the 'berberine carbon' (56).

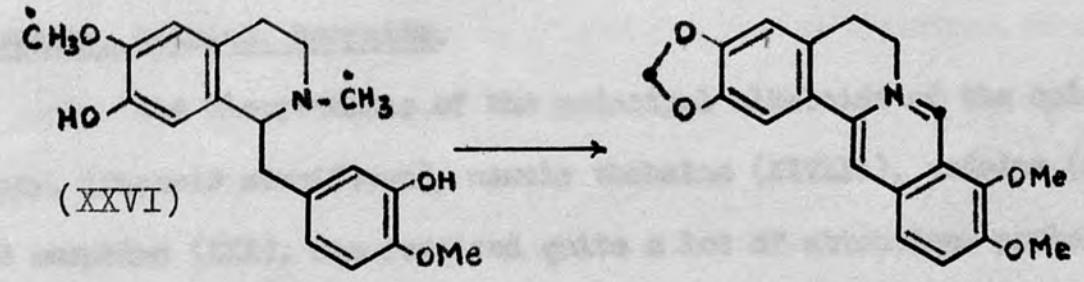


Fig. 10. The origin of the 'berberine carbon'.

Chelidonine.

Leete postulated that this alkaloid is formed in an analgous manner to the previous two, biosynthesis taking place via an intermediate such as stylopine (XXVll) by bond fission and rearrangement as shown below:

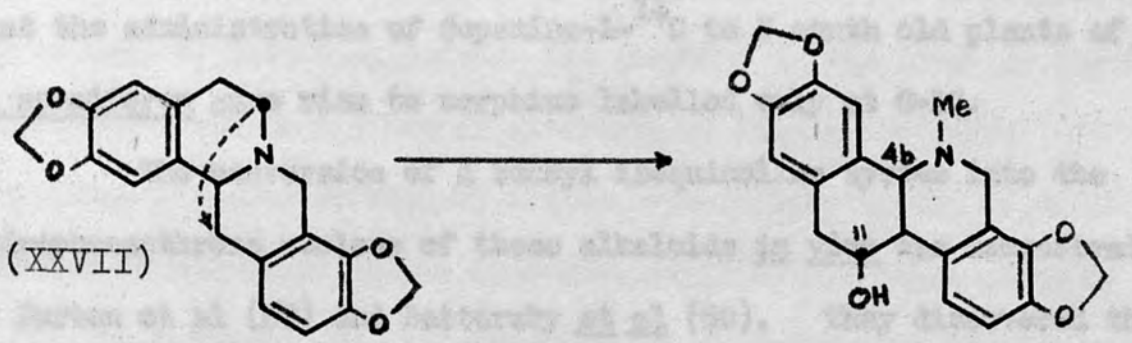
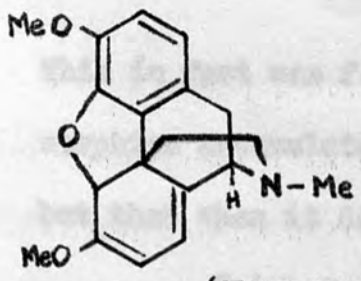


Fig. 11.

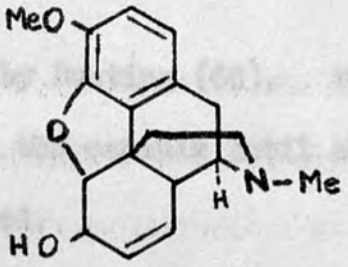
Leete tested this hypothesis by feeding tyrosine-2-^{14}C and dopamine-1-^{14}C hydrobromide to mature Chelidonium majus plants. Isolation of the chelidonine showed that with tyrosine, labelling was confined to C-4b and C-11 (57), whilst dopamine gave labelling only at C-11 (58). In the latter case he was able to isolate labelled stylopine.

Thebaine, Codeine, Morphine.

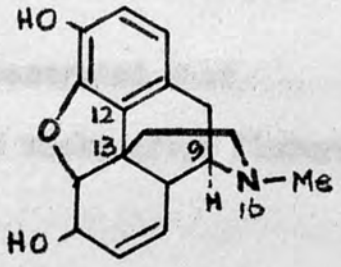
The biosynthesis of the principal alkaloids of the opium poppy, (Papaver somniferum), namely thebaine (XXVlll), codeine (XXlX), and morphine (XXX), has received quite a lot of attention, probably due to the commercial importance of these compounds. Leete (58) demonstrated



(XXXVIII)



(XXIX)



(XXX)

that the administration of dopamine-1-^{14}C to 5 month old plants of P. somniferum gave rise to morphine labelled only at C-16.

The conversion of a benzyl isoquinoline system into the hydrophenanthrene nucleus of these alkaloids in vivo was demonstrated by Barton et al (69) and Battersby et al (60). They discovered that the incorporation into the alkaloids increased as the precursor was changed from nor laudanosoline, to the di-O-methyl ether, and to the N.O.O.-trimethyl derivative (reticuline (XXVI)). However, tetrahydropapaverine, which possesses no phenolic hydroxyl groups was not significantly incorporated.

It was found, by the comparative rates of incorporation of $^{14}CO_2$, that thebaine is the first of the P.somniferum alkaloids to be formed, and this is converted by successive O-demethylations to codeine and morphine as shown in Fig.12 (61). It was also later shown that this sequence is irreversible (62).

As morphine appears to be the last alkaloid formed in this series, one might expect an accumulation of morphine in some tissues.

This in fact was found by Bunting (65). He demonstrated that morphine accumulated in the capsule until about 6 weeks after flowering, but that then it declined.

Fairbairn and Wassel (64), however, found the situation to be more complex than this, and that in fact, marked variations in the content of thebaine, codeine and morphine in the latex of the developing fruit took place at intervals of only a few hours. They found evidence to suggest the irreversibility of the sequence thebaine to morphine, so the fact that morphine decreased markedly at certain times of the day, was taken as an indication that it must be converted into an unknown non-alkaloid substance. The rapid changes in the content of these alkaloids suggests that they play an active part in the metabolism of the plant, rather than being simply waste-products.

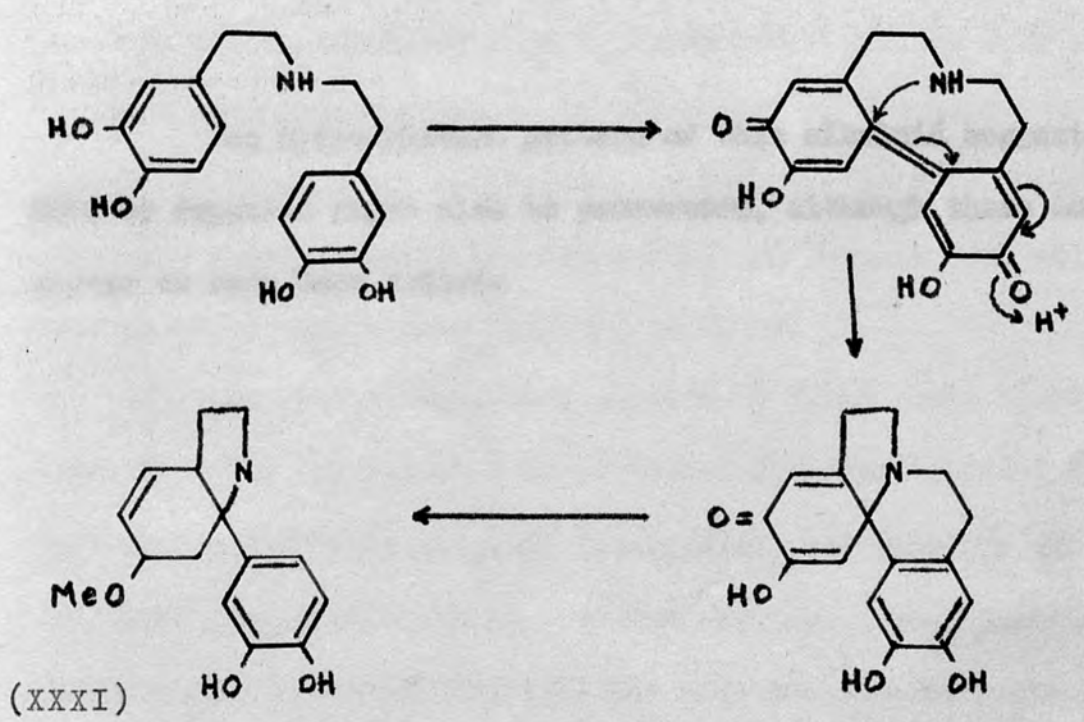
The earlier radioactive tracer experiments with simple precursors such as phenylalanine and tyrosine (65), showed that phenylalanine was incorporated into the morphine structure to a much lesser degree than tyrosine. Unfortunately, DOPA was not tested.

Examination of the plant itself revealed that phenylalanine, tyrosine and their biochemical equivalents phenylpyruvic and p-hydroxy phenylpyruvic acid were present in some of the organs but only in trace amounts. DOPA was found to be absent (66).

Acetone powders prepared from the roots of the plant, however, were shown to be able to catalyse the conversion of tyrosine to DOPA (67). Tyrosinase activity was later demonstrated in other organs of the plant, highest activity being recorded during the early stages of development (68). The authors suggested that this demonstrated the probable function of the enzyme in the biogenesis of the alkaloid skeleton. Further work is required to confirm this hypothesis.

Erythrina alkaloids.

The erythrina alkaloids, of which erysopine (XXXI)is an example, could also be plausibly derived from two molecules of DOPA via nor-laudanosoline as shown in Fig.13 (69)

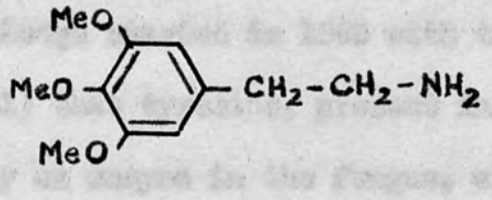


(XXXI)

Fig. 13.

Mescaline.

Mescaline (XXXII) is one of the main alkaloids present in the cactus Anhalonium lewinii, and is well known for its property of inducing halucinations. Leete (70) demonstrated that radioactive tyrosine and phenylalanine were both incorporated into the mescaline skeleton, tyrosine being more actively incorporated than phenylalanine. There was little randomistion of labelling, thus suggesting that tyrosine is a direct precursor or mescaline.



(XXXII)

The hydroxylation pattern of this alkaloid suggests that DOPA or dopamine might also be precursors, although these do not appear to have been tested.

1.7 DOPA as a melanin precursor and its possible function in the formation of some dark pigments in plants.

It is now generally accepted that one of the commonest functions of DOPA in animal tissues is in the formation of dark brown or black melanin pigments. These are frequently present in skin, hair, feathers; the iris of the eye, liver and peritoneum and also in the inks from cuttlefish, squid and octopus and in a variety of insects.

Although these pigments have been recognised for centuries, our modern knowledge started in 1895 with the discovery by Bourquelot and Bertrand (71) that tyrosine, present in the toadstool Russula nigrans was blackened by an enzyme in the fungus, which they named tyrosinase.

The term melanin, which is derived from the Greek word melas meaning black, was first used by Furth and Schneider (72) to describe a black precipitate obtained by the action of a tyrosinase of insect origin on tyrosine. Examination of this precipitate showed that it had the same general properties and analytical data as some animal pigments, and so these also became known as melanins.

The animal melanins, because of their great biological interest, have been the subject of a large number of investigations by workers covering a very wide range of disciplines, but even now the chemistry of these compounds is still somewhat obscure. The function and distribution of animal pigments has been reviewed comprehensively (73, 74, 75).

Pigments closely related to melanins are present in a number of tissues, such as hair and feathers, which are pale brown or yellow in colour. To distinguish between the various pigments it is usual to call those which are black and dark brown eumelanins and the paler ones phaeomelanins. Very little is known about phaeomelanins, although it has been suggested that they are similar to eumelanins, but in a lower oxidation state. The relationship is probably more complex than this.

In contrast to melanins of animal origin, those of vegetable origin have received very little attention. Nevertheless, it is well known that tyrosine and tyrosinase are widely distributed, and that black pigmentation is not uncommon. Dark brown and black pigmentation, for example, is commonly observed in the spores and hyphae of higher fungi, and as markings on petals, leaves, seed-coats, and pods of higher plants.

Mention has already been made of the co-occurrence of tyrosine, DOPA, tyramine and dopamine in the pods of Cytisus scoparius (29), the concentration of these being greatest in the autumn just before blackening takes place. Vicia faba and Stizolobium hassjoo also contain tyrosine and DOPA and show blackening at some stage of their growth, and it seemed likely therefore that the pigments produced may be closely related to the eumelanins of animal origin.

The structure of animal and synthetic melanins.

Animal melanins are usually associated with proteins, and being insoluble, they are very difficult to obtain in a pure state. They are also resistant to degradative procedures. Much of the original work on the structure of melanins therefore, involved a synthetic approach, using melanins prepared in the laboratory by the enzymic oxidation of tyrosine or DOPA under physiological conditions, or by the autoxidation of DOPA, dopamine and related compounds.

With a certain amount of care, the initial stages of melanogenesis can be visualised, and the intermediates islated. It was this approach which enabled Raper and co-workers (76) to elucidate a sequence of reactions leading to melanin formation. The scheme which he put forward, which is now called the Raper scheme, is shown on Fig 14.

Although Raper's work provided a very useful stimulus to melanin studies, later chemical and radioactive tracer experiments have shown that melanin formation is an extremely complex process. However, it is generally agreed that the final stages of melanogenesis involve the oxidative polymerisation of indole-5,6-quinone (XXXIII), although the actual details are still somewhat obscure.

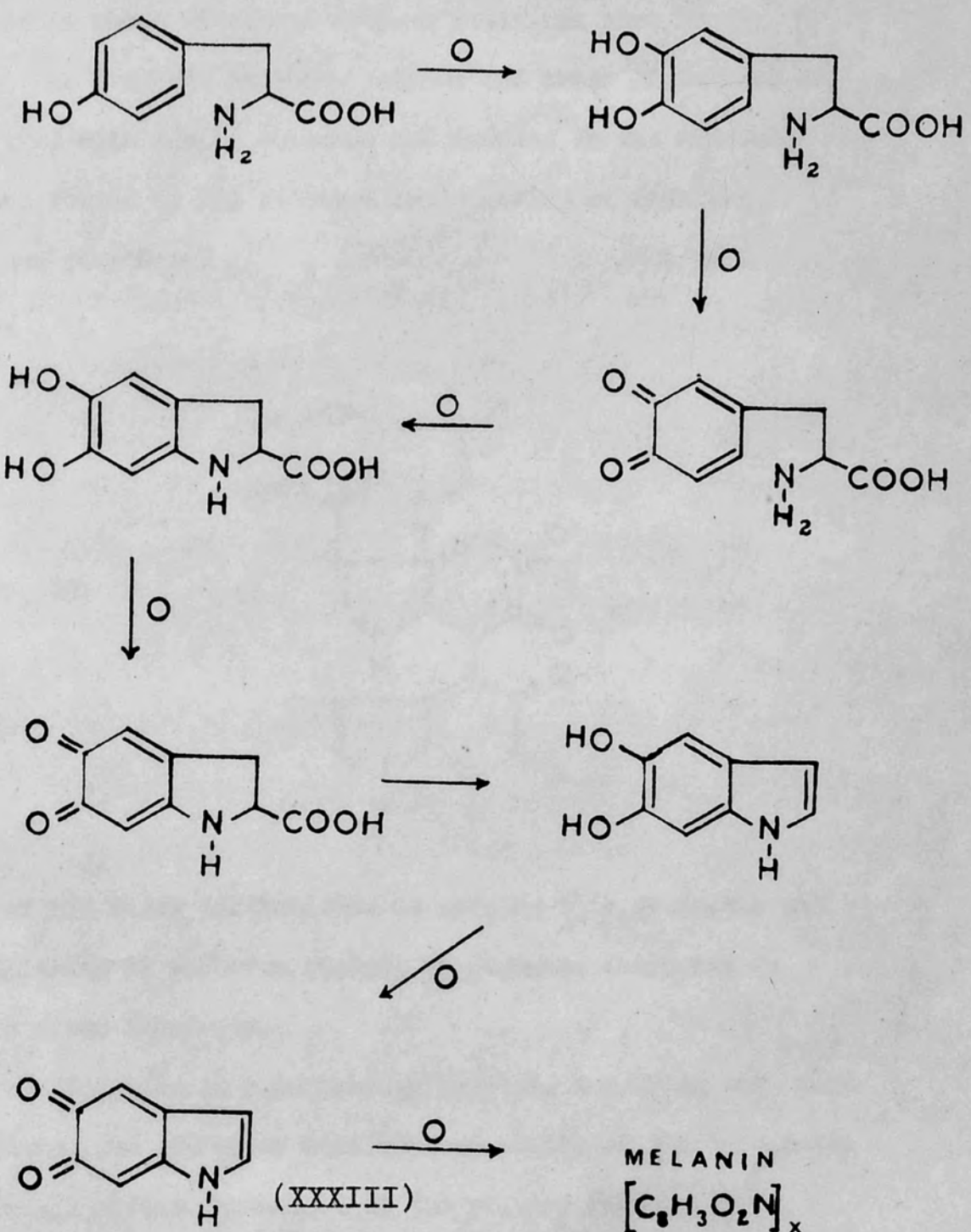


Fig. 14. Raper's scheme for melanogenesis

Oromartie and Harley-Mason (77) studied the products which resulted from the autoxidation of a series of 5,6 dihydroxyindole and DOPA derivatives in which different nuclear positions were blocked by methyl groups. As a result of these studies and those of Bu'Lock and Harley-Mason (77a) with simple quinones and indoles it was concluded that melanin was formed by the repeated condensation of indole-5, 6-quinone between positions 3 4 or 7 as shown below.

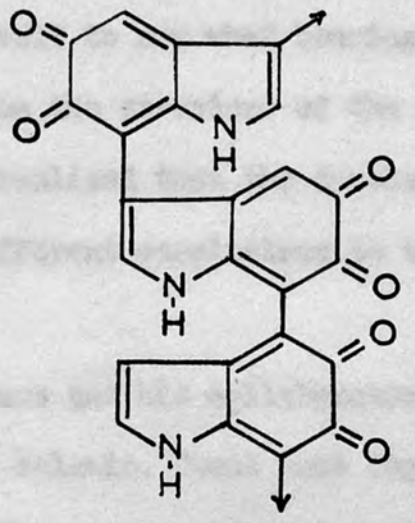


Fig.15.

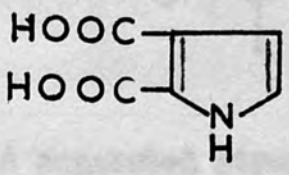
One of the major difficulties in working with synthetic and autoxidation melanins is the wide variety of pigments which can be obtained from a given substrate.

As melanogenesis is a multistage process, involving both fast and slow reactions, the source of activity and purity of the tyrosinase preparation can all affect the nature of the polymer formed.

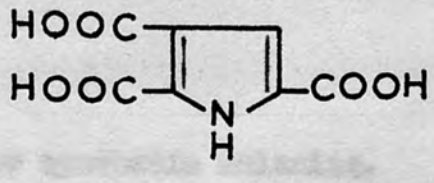
This difficulty can be overcome by autoxidation, but even here there are several pitfalls. Cromartie and Harley-Mason (77), for example, obtained a stable red solution by the autoxidation of 2,3-dimethylindole-5,6-quinone at pH 6.85, whilst Robertson et al (78) obtained a high molecular weight pigment by conducting the oxidation at pH 8. Even more surprisingly Swan et al (79), found that the products obtained by the autoxidation of dopamine at pH 8 differed according to whether oxygen or air was bubbled through the solution.

It is rather difficult to say what bearing the studies on the synthetic melanins have on the structure of the naturally occurring pigments. Already it is realised that the synthetic approach to melanin structure lead to different conclusions to those reached by a degradative approach.

For example, Nicolaus and his collaborators (80), working with a biosynthetic tyrosine melanin, found that degradation of the melanin by oxidation with hydrogen peroxide led to the production of the two pyrrole carboxylic acids, (XXXIV) and (XXXV).



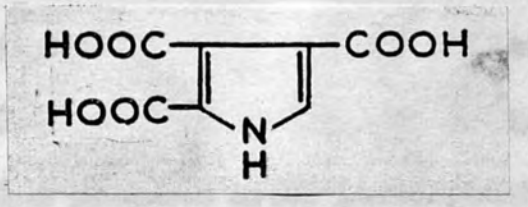
(XXXIV)



(XXXV)

Similar results were also obtained by Binns and Swann with autoxidation melanins produced from tyrosine, DOPA, dopamine and 5,6-dihydroxyindole (81).

The significant feature of these findings was the failure to detect pyrrole 2,3,4, tricarboxylic acid (XXXVI),



which would have been the expected product had the melanins possessed a 3-7 linked backbone structure.

This evidence led to the suggestion that melanins might have a 4-7 linked backbone with some links through position 2 as shown in Fig.16.

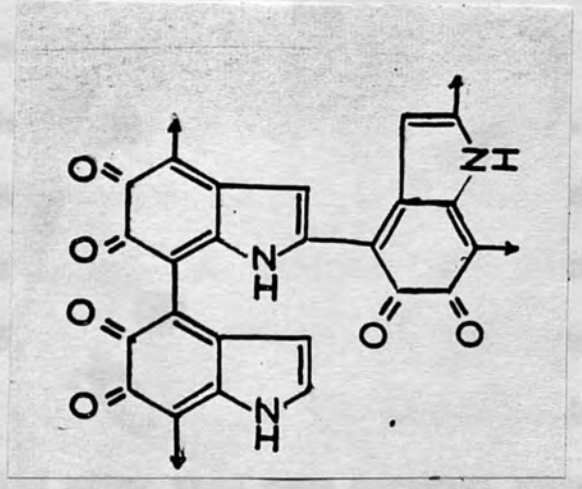


Fig. 16. A suggested structure for synthetic melanins.

To test these suggestions on a natural melanin, Nicolaus et al (82), next turned their attention to the black pigment present in the ink sac of the cuttlefish, Sepia officinalis, which they named sepiomelanin. In this case oxidation was performed with both hydrogen peroxide and potassium permanganate and they were able to show the production of (XXXIV), (XXXV) and small amounts of (XXXVI).

In a later, more comprehensive study (85), they compared the pyrrolic acids produced after permanganate oxidation of a number of melanins and pyrrole black, either on the original pigment or after decarboxylation by heating the pigment to 190-200°. From their results they concluded that sepiomelanin resembled DOPA and 5,6-dihydroxyindole melanins far more closely than pyrrole black. They also found evidence for the presence of a carboxyl group in position 2 of the indole nucleus of sepiomelanin, but not in the synthetic pigments.

Further important work followed (84). Alkali fusion of sepiomelanin was found to yield 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, 4-methyl catechol and a substance tentatively identified as 5,6-dihydroxyindole-4,7- dicarboxylic acid. This was the first concrete evidence that indole units existed as such in melanins, and it gave added proof that some carboxyl groups were retained by the natural pigment.

The technique of alkali fusion proved to be of great value. Nicolaus and collaborators (85) used it on a variety of eumelanins, including those from animal hairs, ox choroid, squid and octopus ink, feathers and axolotl livers. 5,6-dihydroxyindole and 2-carboxylic acid were amongst the products on each occasion.

Different results, however, were obtained when they studied the black pigment isolated from the fungus, Ustilago maydis (86).

Alkali fusion of ustilagomelanin resulted in the production of catechol, protocatechuic acid and salicylic acid, and not the indole derivatives produced by the animal eumelanins. As the same products were formed when a catechol melanin, prepared by the enzymic oxidation of catechol at pH 6.8, was subjected to the same procedure, it was assumed that the two pigments were similar.

Further evidence for this belief was provided when it was found that catechol was the only detectable phenol present in alcoholic extracts of the spores.

Piatelli and Nicolaus (87) continued their studies by conducting alkali fusions on a variety of plant and fungal pigments, with the results which are shown overleaf in Table 1.

Their findings led them to suggest that plant pigments may be distinguished from those of animal origin, in that the former yield catechol derivatives on alkali fusion, whereas the latter yield indole derivatives.

Table 1. The products from the alkali fusion of some black plant pigments.(after Nicolaus et al (87)).

Source of pigment	Products obtained by alkali fusion.
1. Helianthus annuus (seeds)	catechol
2. Citrullus vulgaris (seeds)	"
3. Ipomea purpurea (seeds)	"
4. Luffa cylindrica (seeds)	"
5. Martynia proboscidea (seeds)	"
6. Yucca Aloefolia (seeds)	"
7. Asparagus plumosus nanus (seeds)	"
8. Aquilegia (seeds)	"
9. Ustilago maydis (spores)	"
10. Daldinia concentrica (fruits)	1,8 dihydroxynaphthalene, catechol
11. Aspergillus niger (spores)	unidentified products
12. Capnodium nerii	unidentified products.

It is to be emphasised, however, that this school did not examine pigments obtained from leguminous species, which might be expected to be of the indole type in those species containing high concentrations of DOPA.

The terminology of dark vegetable pigments.

One of the difficulties of describing work with dark brown or black pigments of vegetable origin, is the rather confused state of terminology which exists in the literature. The term melanin has been so indiscriminately used, that it now has very little chemical significance.

Before undertaking a study of leguminous pigments, it seemed profitable to come to some decision regarding the nomenclature to be used.

At the present time there are two main schools of thought on nomenclature. One group, expressing the classical viewpoint, limits the term melanin to nitrogen-containing pigments derived from tyrosine and DOPA (88), whereas others, notably the Nicolaus school, also include catechol polymers.

In view of the important contributions made by the Nicolaus school, and the widespread use of the term catechol melanin in the literature, it would seem appropriate to classify black pigments derived from catechol as melanins.

Equally important is the observation made by Thomson (89) who pointed out, "More attention should perhaps be paid to the fact that

DOPA and 5,6-dihydroxyindole are catechols. It is possible that all melanins are essentially catechol melanins, the pyrrole rings in tyrosine melanin, being perhaps only secondary factors which provide cross linkages and a greater degree of complexity and irregularity".

If one accepts that catechol polymers are melanins, and if one accepts that the black autoxidation products of DOPA, catechol and the like are also melanins, then one must include these in the definition, so for the present work melanins have been defined as : High molecular weight, insoluble pigments, formed by the action of oxygen, usually in the presence of a tyrosinase, on tyrosine, DOPA, catechol and related substances.

Unfortunately, any simple definition such as this does not help in distinguishing between the various types of melanin. It was, therefore, decided to devise a system of classification and in undertaking this task, certain criteria were considered to be of importance.

(1) Individual pigments should be classified with regard to the most readily available information. It seems more sensible, for example, to distinguish natural melanins by means of the compounds they produce on alkali fusion and other degradative procedures, rather than by their precursors as these are often fairly difficult to establish.

(11) In devising a system of terminology, consideration was given to terms already in the literature, used in their original sense wherever possible.

(111) The environmental conditions under which pigments are formed.

Bearing these criteria in mind, it became apparent that three types of pigments exist; melanins produced in vitro, melanins of animal origin and those of plant origin.

This distinction between animal and plant melanins is suggested, partly because of the findings of Nicolaus and his co-workers, but mainly because of the wide variety of phenolic substances present in plant tissues, and the unspecific nature of their oxidases.

These conclusions resulted in the following suggested system of classification for melanins.

Laboratory produced melanins.

A biosynthetic melanin is a dark brown or black insoluble pigment formed in vitro by the action of oxygen, in the presence of a tyrosinase, on tyrosine, DOPA, catechol or related substances. Similar pigments formed in the absence of a tyrosinase are autoxidation melanins.

Animal melanins.

A eumelanin is a dark brown or black pigment of animal origin, usually occurring in association with protein, and yielding 5,6-dihydroxy-indole or derivatives on alkali fusion.

Phaeomelanins are similar to the above, but are paler in colour.

Plant melanins.

It is suggested that dark brown or black pigments from plant and fungal sources, which yield 5,6-dihydroxyindole and/or catechol derivatives on alkali fusion, should be termed phytomelanins.

1.8. The role of DOPA in the formation of Centrospermae pigments.

It has been recognised for nearly a century that two groups of nitrogen-containing pigments were present in species which are normally placed in the order Centrospermae. One group, which Dreiding (90) suggested should be called betacyanins, are red-violet in colour, whilst the other group, which Dreiding calls the betaxanthins, are yellow. Like the more common, and more widely distributed nitrogen-free anthocyanin and flavonoid pigments,(Fig.21), betacyanins and betaxanthins occur in the cell sap (vacuoles) and are therefore dassified as chymochromes.

Work on these compounds was hampered owing to the difficulty in obtaining them in a pure, crystalline state, and it is only in the last few years that significant progress has been made in elucidating their structures. Much of the earlier work has been reviewed by Dreiding.

The first pigment to be isolated in the crystalline state was betanin from Beta vulgaris (91,92). Enzymic hydrolysis of betanin gave glucose and the aglucone, betanidin, which was itself crystallised as salts with bases and as the hydrochloride (93,94).

All of the betacyanins isolated so far have been shown to be derivatives of betanidin or the diastereoisomer isobetanidin.

Alkaline degradation of either betanidin or isobetanidin in the absence of oxygen was found to give 5,6-dihydroxyindole-2-carboxylic acid, 4-methyl pyridine and ammonia (95). Further work (95,96,97,98,99) on

chemical structure and stereochemistry of these compounds has shown that isobetanidin only differs from betanidin in the configuration at C-15. The structure of betanidin (XXXVII R=H) and isobetanidin (XXXVIII R=H) are shown in Fig.17.

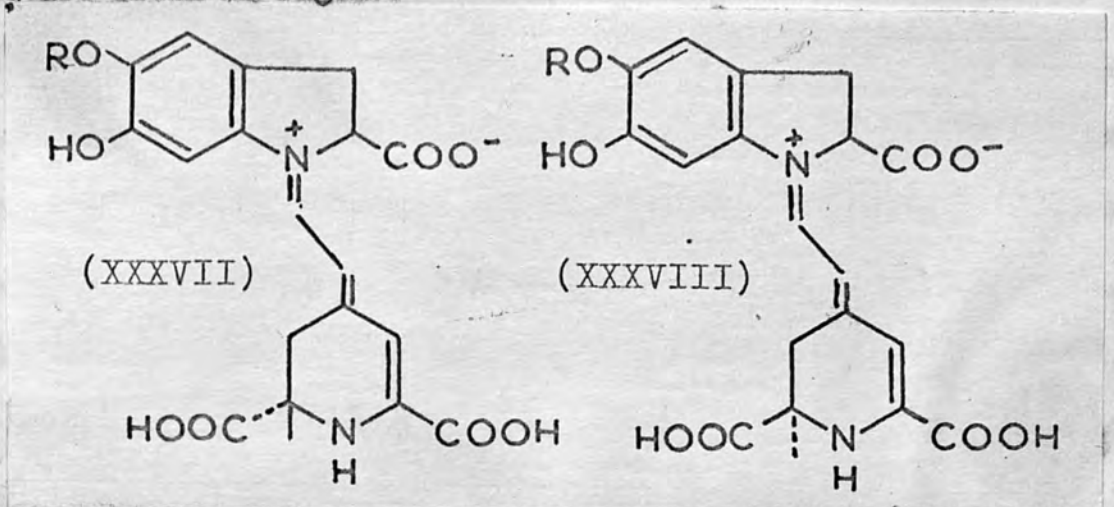


Fig.17.

Betanin was shown to be 5-O-β-D-glucosylbetanidin (XXXVII R - Glu.) by Piattelli et al (100). Their structural determination, which involved methylation of betanin, followed by mild alkaline fusion, and the isolation of 5-hydroxy-6-methoxyindole-2-carboxylic acid from the products, is summarised in Fig.18.

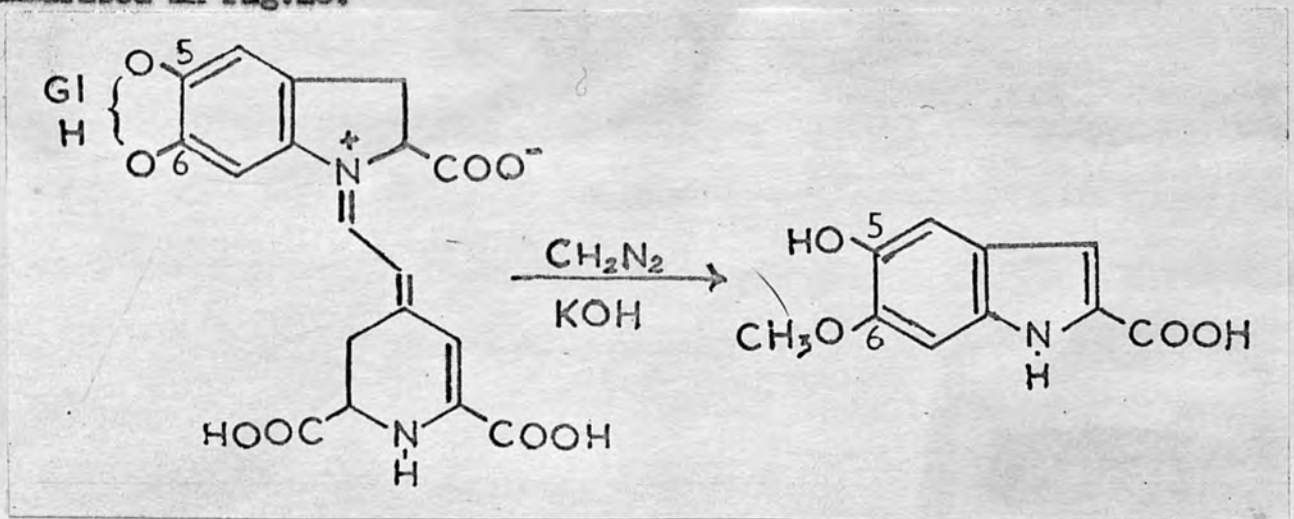


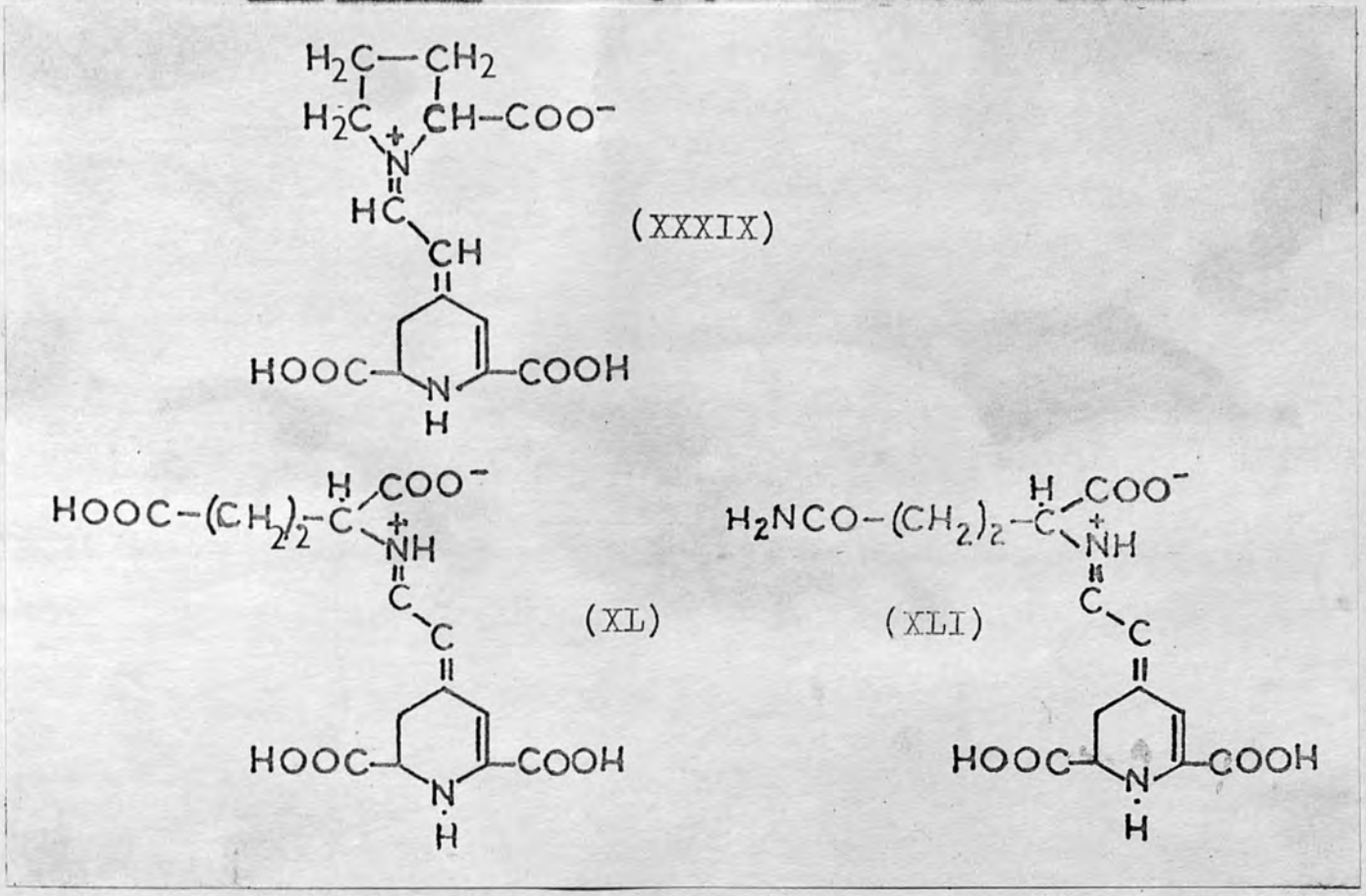
Fig.18. The structure of betanin (100).

A detailed survey of plant tissues for betacyanins and betaxanthins by a number of workers (101,102,103) has brought to light several interesting facts. One which has already been mentioned, is that all the betacyanins studied produced either betanidin or isobetanidin on hydrolysis (104,105). Another, and perhaps a more important observation, is that betacyanins and anthocyanins apparently never co-exist in the same plant or even within the same family, even though other classes of flavonoid pigment occur with betacyanins in the same plant. This mutual exclusion, and the limited distribution of betacyanins, was thought to be of considerable taxonomic significance by Mabry et al (106). They suggested that the order Centrospermae should be reserved for betacyanin-containing families such as the Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didieraceae, Ficoidaceae, Mesembryanthemaceae (Aizoaceae), Nyctaginaceae, Phytolaccaceae, Portulacaceae and Stegnospermaceae. They considered that those anthocyanin-containing families such as the Caryophyllaceae and Illecebracaceae should be treated as a separate phyletic group.

It is perhaps unusual that a single chemical character should be accorded such major taxonomic importance, but as Mabry et al point out, the totally different structures of the anthocyanin and betacyanin pigments suggests an entirely different biogenetic pathway, and hence a different evolutionary background.

The survey of plant material also led to the discovery of other Centrospermae pigments.

A betaxanthin (indicaxanthin), for example, was isolated from Opuntia ficus-indica (the "prickly pear", Cactaceae), and has since been shown to have structure (XXXIX). Two other betaxanthins were isolated from Beta vulgaris with the proposed structures XL and XLI.



(XXXIX)

(XL)

(XLI)

Dreiding (106) has suggested that betanidin might be formed from two molecules of DOPA according to the scheme shown in Fig.20. Horhammer et al (107), confirmed that labelled DOPA was incorporated into the betanin produced in the root and hypocotyl of Beta vulgaris, but the distribution of radioactivity was not determined, so that they were not able to confirm or disprove Dreiding's hypothesis.

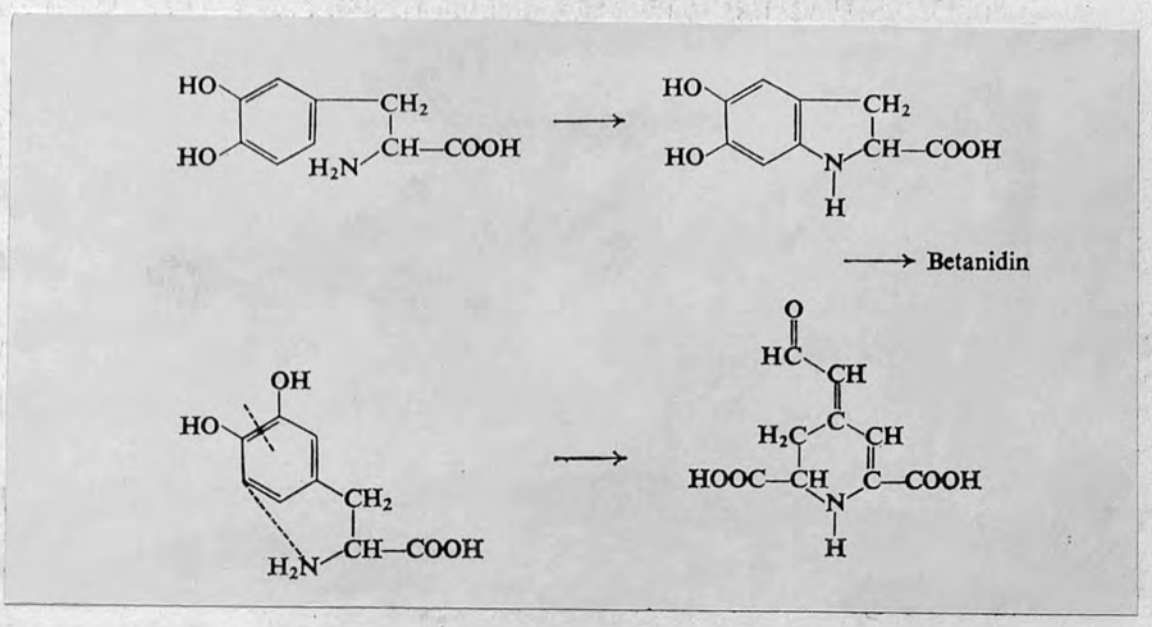


Fig.20. Dreiding's scheme for the biosynthesis of betanidin.

Minale et al (108) incubated the pulp from Opuntia ficus-indica fruits with labelled proline or DOPA, and isolated the betanin and indicaxanthin formed. They found up to 0.8% incorporation of proline into the indicaxanthin, 95% of the radioactivity, not surprisingly was confined to the proline moiety. The incorporation of DOPA into this pigment (up to 0.6%) was almost entirely into the hydrogenated pyridine moiety. Unfortunately, they did not determine the distribution of radioactivity in the betanin, but their work provides the first experimental evidence in support of Dreiding's hypothesis. As much as 1% of the DOPA was incorporated into betanin in these experiments.

1.9. Aromatic amino acids as precursors of cinnamic acids and flavonoid compounds.

Of the numerous phenolic secondary products of metabolism present in plant tissues, the cinnamic acids and flavonoids are the most widely distributed.

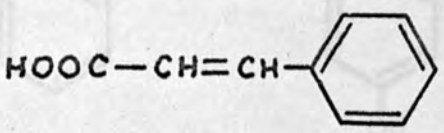
The cinnamic acids can be regarded as being C_6-C_3 compounds, whilst the flavonoids possess the C_6-C_3-C_6 structure, where each C_6 unit is a benzene ring, the state of oxidation which together with that of the connecting C_3 portion determines the physical properties of the compound. Typical examples are shown in Fig.21.

p-Coumaric acid (XLIII), caffeic (XLIV), ferulic (XLV) and sinapic (XLVI) acids are the most commonly occurring of the cinnamic acids, and anthocyanidins, flavonols, and leucoanthocyanidins the commonest of the flavonoids. These compounds appear to serve a variety of functions (110,111,112). Many of them are present in plant tissues as glycosides or esters.

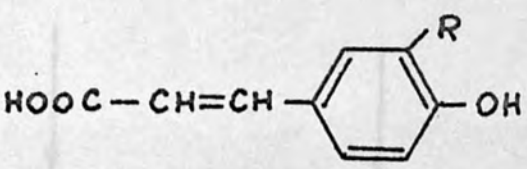
Numerous radioactive tracer experiments have established that the cinnamic acids, the B ring of flavonoid compounds and the individual units of lignins are formed from aromatic amino acids, whilst the A ring of flavonoids is formed by the head-to-tail linkage of acetate units followed by ring closure. The possible pathways in the formation of cinnamic acids and the pathways to the various secondary growth substances are summarised in Fig. 22.

Fig. 21. Cinnamic acid and flavonoid constituents of plants.

Cinnamic acids



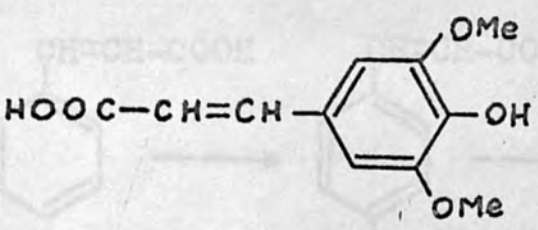
XLII Cinnamic acid



XLIII R = H p-Coumaric acid

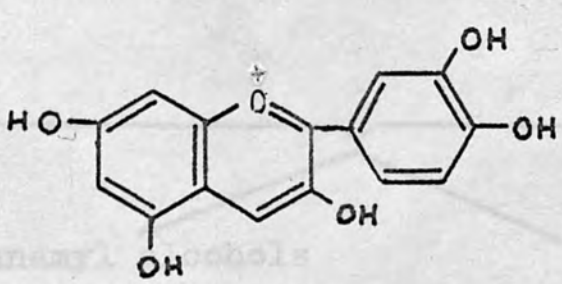
XLIV R = OH Caffeic acid

XLV R = OMe Ferulic acid



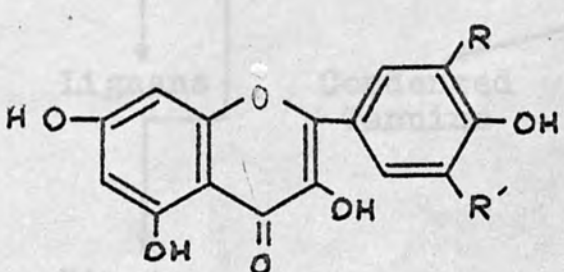
XLVI Sinapic acid

Anthocyanidins



XLVII Cyanidin

Flavonols



XLVIII R = R' = H

Kaemferol

XLIX R = OH; R' = H

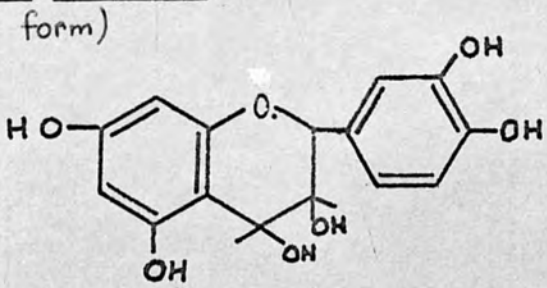
Quercetin

L R = R' = OH

Myricetin

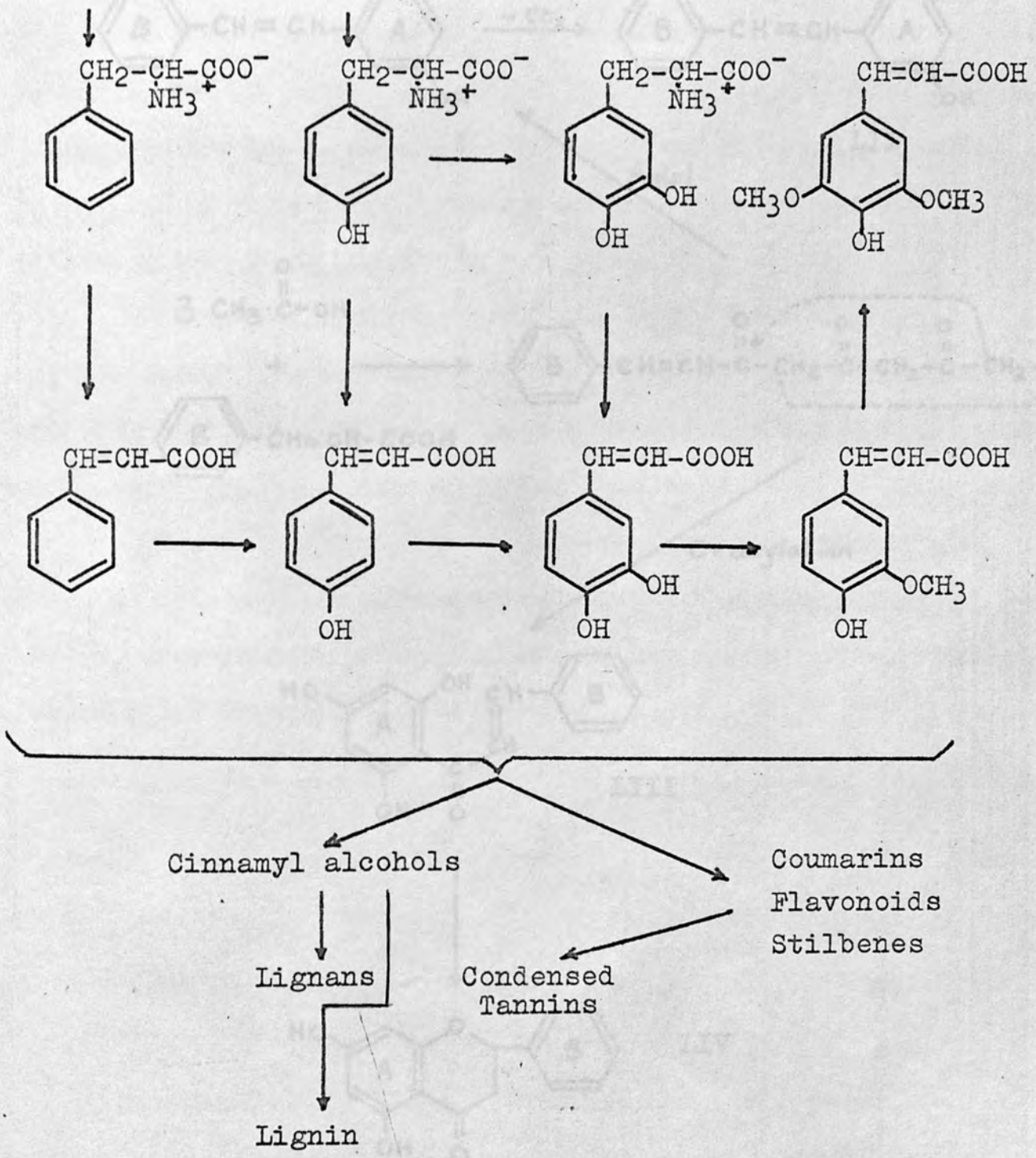
Leucoanthocyanidins

(Monomeric form)



LI Leucocyanidin

Fig. 22. The formation and metabolism of cinnamic acids in plants



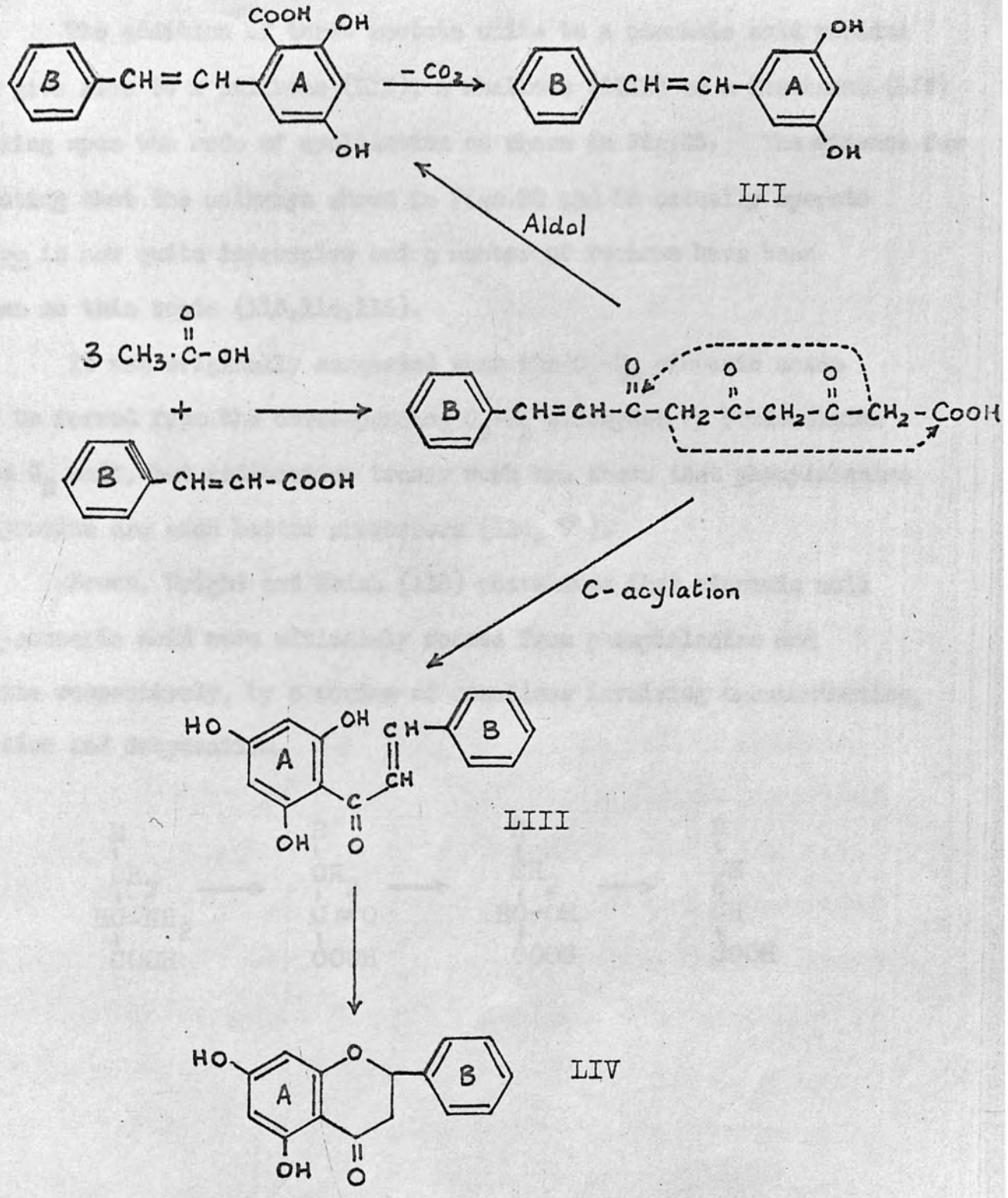


Fig. 23. The acetate pathway.

The addition of three acetate units to a cinnamic acid residue could give rise to a stilbene (LII), a chalkone (LIII) or a flavanone (LIV) depending upon the mode of cyclisation as shown in Fig.23. The evidence for suggesting that the pathways shown in Figs.22 and 23 actually operate *in vivo* is now quite impressive and a number of reviews have been written on this topic (113,114,115).

It was originally suggested that the C_6-C_3 cinnamic acids might be formed from the corresponding C_6-C_1 aldehydes by condensation with a C_2 unit, but radioactive tracer work has shown that phenylalanine and tyrosine are much better precursors (116, 9).

Brown, Wright and Neish (118) postulated that cinnamic acid and *p*-coumaric acid were ultimately formed from phenylalanine and tyrosine respectively, by a series of reactions involving transamination, reduction and dehydration.

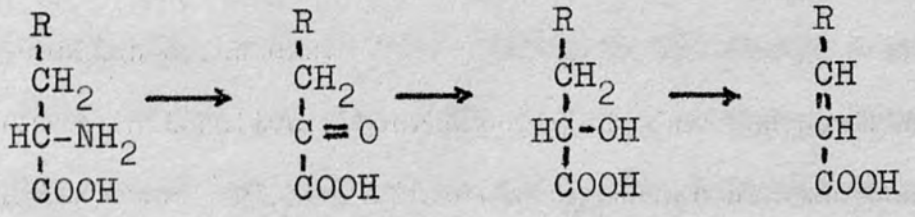


Fig.24.

Neish (119), however, pointed out that this could also be brought about by a single deaminating step.

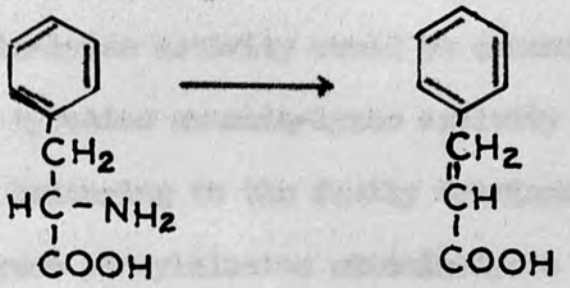


Fig.25.

If this was so, then one might suppose that cinnamic acid, p-coumaric acid and caffeic acid could be formed by the deamination of phenylalanine, tyrosine and DOPA respectively as shown in Fig.22, but this does not appear to be generally true.

Neish and co-workers (13), for example, found that although radioactive phenylalanine was a good precursor of the phenyl propanoid units of lignins from 11 species of plants (representing 10 families), tyrosine was only effective in the case of Triticum vulgare (wheat) and Calamognostis inexpansa, both of which belong to the family Graminae.

In 1961, Koukol and Conn (121) isolated an enzyme from barley (Hordeum vulgare) which was able to convert L-phenylalanine to cinnamic acid, thus confirming Neish's original postulate. They named the enzyme L-phenylalanine ammonia-lyase.

Later, Neish isolated another enzyme from the same source which was able to deaminate tyrosine (119) and established the individuality of the tyrosine ammonia-lyase (tyrase) by means of inactivation studies.

Some explanation for the limited incorporation of tyrosine into lignin became apparent when it was found, that although phenylalanine ammonia-lyase activity could be demonstrated in all the plants studied, tyrosine ammonia-lyase activity could only be detected in species belonging to the family Graminae.

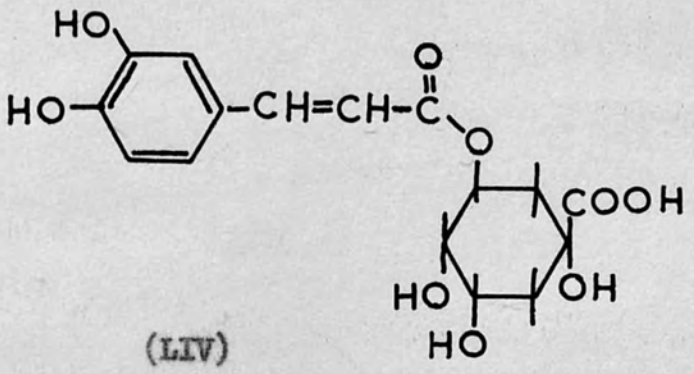
Also, whereas phenylalanine ammonia-lyase was found to be specific for L-phenylalanine, the tyrase preparation catalysed the deamination of L-tyrosine and DL-m-tyrosine, the latter twice as effectively as tyrosine itself.

The tyrase preparation also was found to have some activity on L-DOPA , deamination being established by demonstrating that ammonia was produced in the reaction. The activity, however, was much less than with tyrosine as the substrate.

The widespread distribution of caffeic acid (122,123) and compounds which could possibly have been derived from it in plant tissues has led workers in these laboratories to investigate whether DOPA could be a possible intermediate in its formation.

MacLeod and Pridham (124) isolated the product from the reaction of DL-DOPA with a crude preparation from the leaves of Taraxacum officinale, and by means of colour reactions and chromatographic and electrophoretic behaviour,were able to prove that it was trans-caffeic acid.

The dandelion preparation, as expected from Neish's observations, showed considerable L-phenylalanine ammonia-lyase activity, but no activity with respect to tyrosine. The activity with respect to DOPA was therefore of interest, particularly as dandelion leaves were found to contain high concentrations of chlorogenic acid (LIV).



(LIV)

A study of the enzymes present in both barley and dandelion leaves has therefore been included in the present work.

RESULTS AND DISCUSSION

PART II

The formation and characterisation of derivatives of DOPA and related compounds

II.1 Preliminary investigations.

It is fairly clear from the introductory remarks that much remains to be discovered about the metabolism of DOPA in plants. One aspect, in particular, which has received very little attention, is the extent to which DOPA occurs naturally in the combined form, the nature of the derivatives themselves and their possible function.

Attempts were made at the outset, therefore, to prepare or isolate suitable derivatives and to record their properties so that a survey for their presence in plant tissues could be undertaken.

Paper chromatography and paper electrophoresis were chosen as being the most suitable techniques for the separation and characterisation of the derivatives, but it was necessary to undertake a survey of locating reagents to determine the most suitable one for routine use.

As DOPA and many of its derivatives possess both phenolic and amino functions, a variety of reagents can be employed for their location. Certain techniques, such as those which depend upon the reducing or complexing properties of the o-dihydroxyphenyl residue, and those, such as ninhydrin, which produce a coloured product with amino acids appeared to be somewhat limited in their scope, as they do not locate derivatives in which either one of the phenolic hydroxyl groups or the amino acid residue was substituted.

A better choice of reagent seemed to be one which would react with phenolic groups, as this would locate all DOPA derivatives except those in which both hydroxyl groups were blocked, and this is most unusual in naturally occurring compounds.

After conducting a series of trials with various reagents using paper chromatograms of plant extracts, the diazotized p-nitroaniline/NaOH location procedure was accepted as being the most satisfactory. In this modified procedure (125), dried papers were sprayed with an acidic solution of diazotized p-nitroaniline, and then oversprayed with an aqueous solution of sodium hydroxide.

This procedure is relatively sensitive but its greatest attribute is the wide spectrum of colours which are produced by phenolic compounds. As the colour of the azodye depends upon the substituents around the aromatic nucleus of the phenol, it seemed likely that it would prove of great value in distinguishing between DOPA derivatives which were unsubstituted in the aromatic ring or substituted at the C_3-C_4 hydroxyl.

Although at the beginning of the survey it was known that unsubstituted derivatives gave a characteristic blue colouration with the reagent, the colour produced by 3 - and 4 - substituted derivatives was unknown because suitable standards were not available.

One of the first tasks to be undertaken, therefore, was to obtain such derivatives and determine their colour reactions and chromatographic and electrophoretic behaviour.

The preparation of DOPA derivatives.

As a DOPA-O-β- D-glucoside had already been detected in the testas of broad-bean seeds, this tissue was the first to be examined. Unfortunately, English translations of the original Japanese work[5] were not available at the outset, and a trial and error approach had to be adopted. This proved to be extremely arduous and alternative methods were sought to produce suitable O-substituted derivatives.

The first of such methods was a synthetic approach as outlined by Irvine and Gilmour (126) and Irving and Hynd (127). DL-DOPA and anhydrous D-glucose were intimately mixed and heated under reflux in a variety of aqueous and alcoholic solutions, and the products examined on chromatograms and electrophoretograms. Although a number of products could be detected on electrophoretograms with diazotised p-nitroaniline/ NaOH, after some of the procedures, they all gave the blue colour typical of compounds unsubstituted in the ring.

Two enzymic glycosylations in vitro were also tried. It had previously been shown in these laboratories that extracts of Aspergillus niger were capable of catalysing the transfer of glucose from a donor such as maltose to a phenolic hydroxyl group (128,129). When this was tried with DOPA, however, no O-glucosyl derivatives could be detected in the digests. Similarly, when DOPA was incubated with E.coli β-galactosylase and lactose(cf 130) the appropriate O-β-D-galactosyl derivatives could not be detected.

By far the most successful of the earlier attempts to produce O-substituted derivatives was a biosynthetic approach in which DOPA was fed to pea seeds which were then allowed to germinate.

II.2 The biosynthesis of DOPA derivatives using pea seeds.

Seeds of Pisum sativum var 'Early Onward' were soaked in a solution of L-DOPA and then allowed to germinate in the dark for 4 days. Concentrated extracts prepared from the seedlings were examined on paper chromatograms, and this revealed that five compounds in addition to DOPA were present in the treated seeds which were not in control seeds. For convenience these compounds were designated P_1-P_5 in order of increasing Rf values in ethylacetate/acetic acid/water solvent (C).

By careful fractionation of the extracts on paper chromatograms and electrophoretograms, small quantities of each were obtained in the form of freeze-dried powders. The colour reactions, chromatographic and electrophoretic properties of the compounds are summarised in Table 2 ; the U.V.spectra are shown in Figs. 26,27 and 28.

It was soon apparent that the majority of the biosynthetically produced compounds were not simple DOPA derivatives, as only two, namely P_2 and P_5 produced DOPA on hydrolysis with dilute mineral acid . In view of the fact that a compound chromatographically and electrophoretically identical to P_2 had been detected in the cotyledons of untreated broad-bean seeds it was decided to concentrate attention on this material.

Table 2. Colour reactions and chromatographic and electrophoretic behaviour of compounds isolated from Pisum sativum seeds fed with L-DOPA

(1) Corrected for electroendosmosis (2) Uncorrected values

		P_1	P_2	P_3	P_4	P_5	
1. Colour reactions							
Diazotised p-nitroaniline / NaOH		pink	plum	plum	lilac	blue	
After drying		pink	grey-green	grey-green	lilac	fades	* R_{DOPA} and M_{DOPA} values
Ninhydrin		- ve.	+ ve.	+ ve.	- ve.	- ve.	
Sodium molybdate		-	-	-	-	brown	+ $R_{\underline{N}\text{-Me DOPA}}$ $M_{\underline{N}\text{-Me DOPA}}$ values
2. Chromatographic behaviour *						* +	
in Solvent	A	0.46	0.46	0.53	0.82	- -	
	B	0.60	0.60	0.40	0.85	1.44 1.00	
	C	0.28	0.35	0.53	0.50	1.25 0.94	
	D	0.23	0.37	0.53	0.61	1.11 0.92	
	E	0.63	0.56	0.33	0.88	1.38 1.15	
	G	0.31	0.30				
3. Electrophoretic behaviour *							
in formic acid	(1)			0.50	0.72/ 0.62		
	(2)	0.54	0.85	0.71	0.83/ 0.78	0.65 0.88	
phosphate buffer	(1)		0.71	0.74	0.74		
molybdate buffer	(1)					0.96 0.90	

Fig. 26.

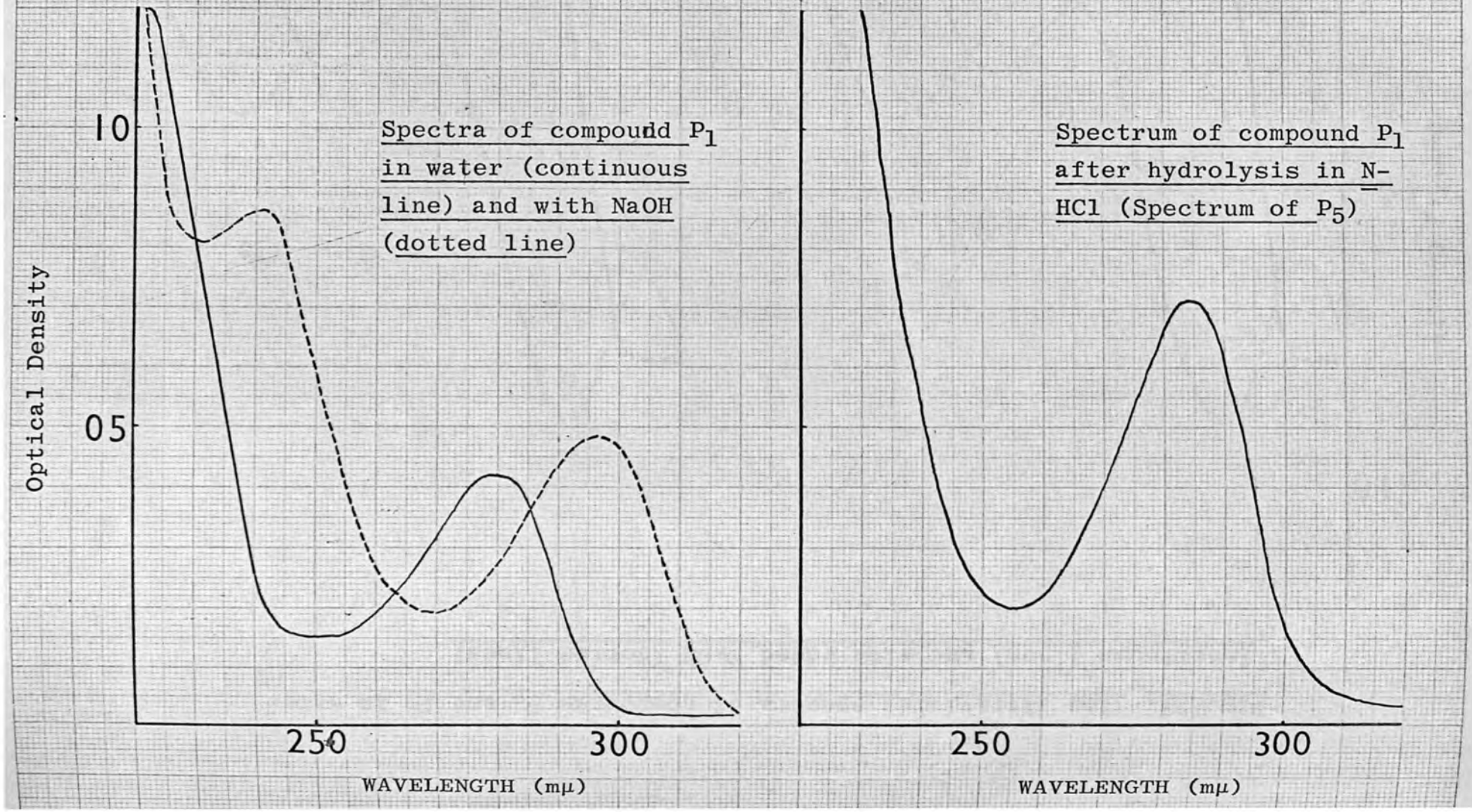

Optical Density
10
05
250
300
WAVELENGTH (mμ)
Spectra of compound P_1 in water (continuous line) and with NaOH (dotted line)
Spectrum of compound P_1 after hydrolysis in N-HCl (Spectrum of P_5)
250
300
WAVELENGTH (mμ)

Fig. 27. The ultraviolet spectra of compounds P_2 and P_3 in water (continous lines) and with added NaOH (dotted lines)

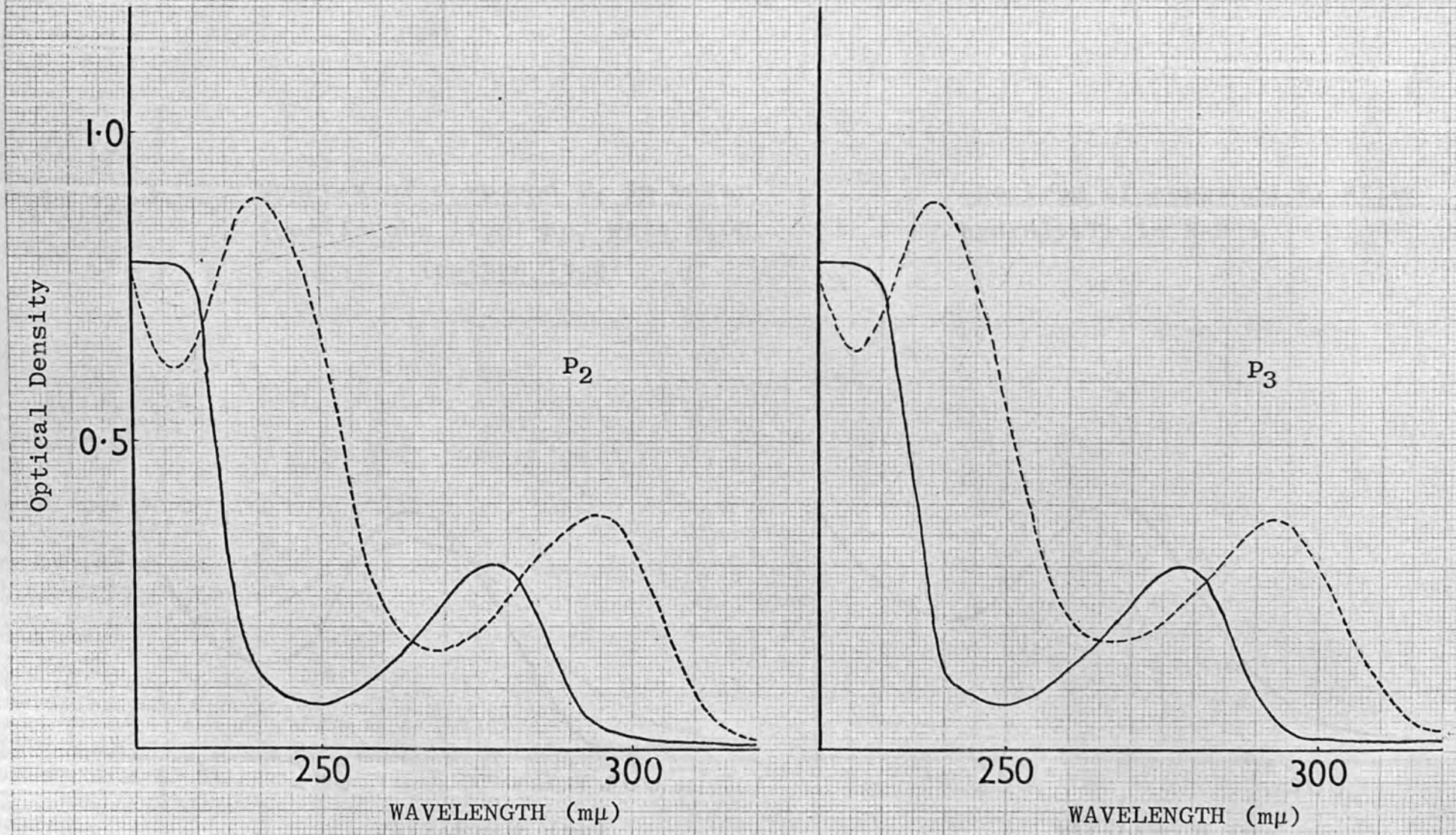
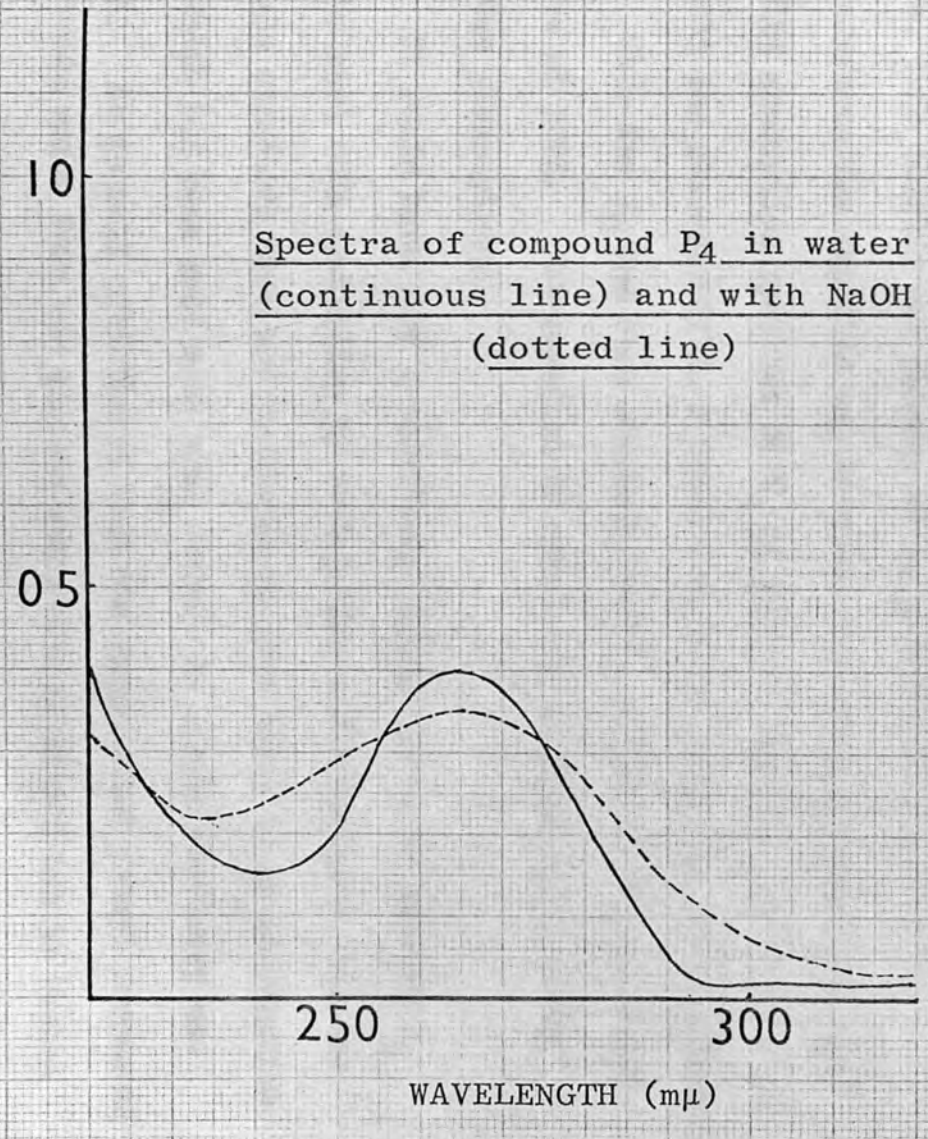
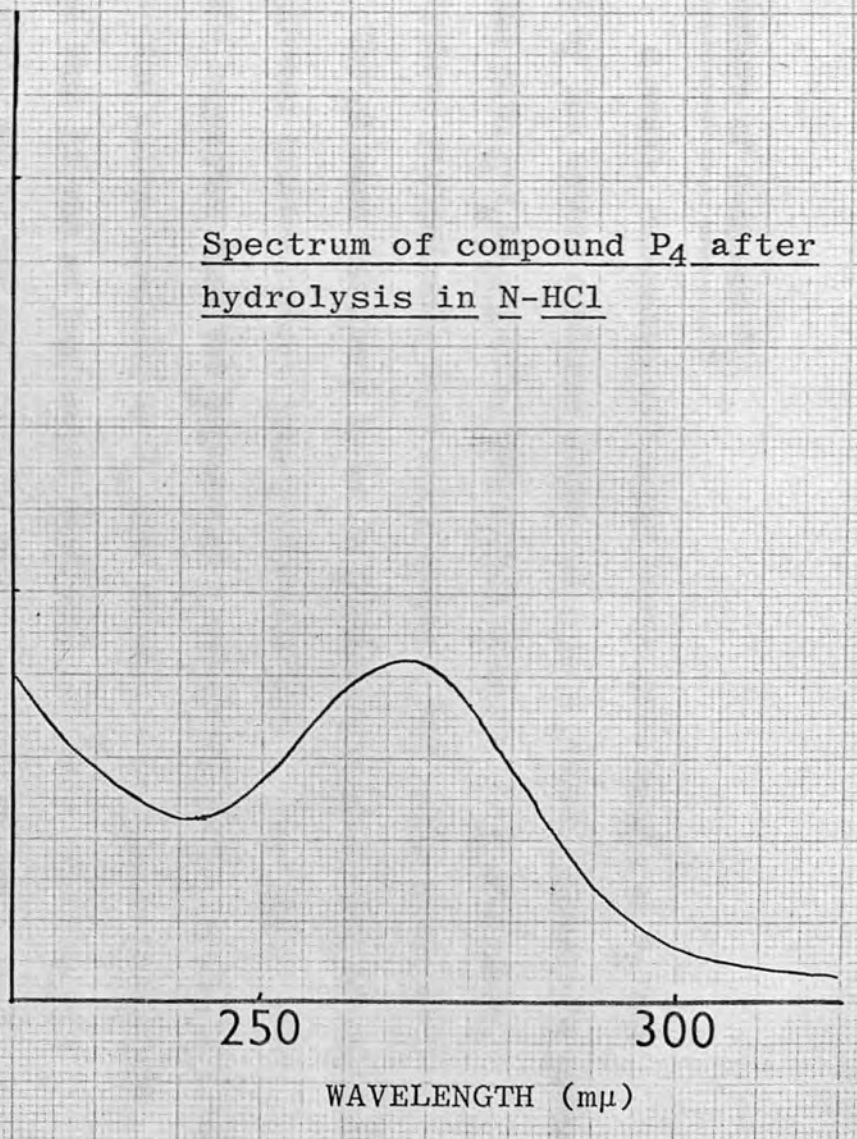


Fig. 28



Spectra of compound P_4 in water (continuous line) and with NaOH (dotted line)



Spectrum of compound P_4 after hydrolysis in N-HCl

The structure of compound P_2.

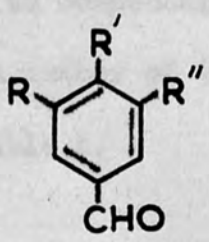
a small, chromatographically homogeneous sample of P_2 was obtained by the method outlined above, as a pale yellow hygroscopic powder. Hydrolysis with N-H_2SO_4, N-HCl, 50% formic acid or β- D-glucoside glucohydrolase yielded only DOPA and glucose in the molar ratio of 1 : 1. The glucose was shown to be in D-configuration by its conversion with D-glucose oxidoreductase into D-gluconic acid. The rate of acid hydrolysis suggested that the D-glucosyl residue was in the pyranoid form.

The colour produced by P_2 with diazotised p-nitroaniline/NaOH suggested that it was an O-glucosyl derivative of DOPA, and this was further indicated by the fact that it failed to complex with sodium molybdate, thus showing the absence of an o-dihydroxyphenyl group. Further confirmation for this was obtained when it was shown that the ultraviolet absorption spectrum of P_2 in aqueous alkaline conditions did not exhibit the characteristic hypsochromic shift when treated with borate.

Thus far P_2 had been characterised as an O-β-D-glucopyranosyl derivative of DOPA. It remained to establish which hydroxyl group of DOPA was glucosylated.

It would have been a relatively simple matter to convert P_2 to a methylated DOPA derivative by methylation and hydrolysis, but the appropriate standards were not available at that time, so an alternative approach which had been used with some success in lignin chemistry was attempted. It is well established that the unsaturated phenyl propanoid (C_6-C_3) units which form the complex structure of lignin can be broken

down by alkaline nirtobenzene oxidation to the appropriate C_6-C_1 aldehydes such as p-hydroxybenzaldehyde (LV), vanillin (LVI) and syringaldehyde (LVIII) (131).



LV R = H, R' = OH, R'' = H
LVI R = H, R' = OH, R'' = OMe
LVII R = H, R' = OMe, R'' = OH
LVIII R = OMe, R' = OH, R'' = OMe

An attempt was, therefore, made to determine the position of the glucosyl residue in P_2, by using the oxidative procedure on the O-methylated derivative of P_2. Thus, if the glucosyl residue was in the 4 position, the expected product would be vanillin, but if it was on the 3-hydroxy group the expected product would be isovanillin (LVII).

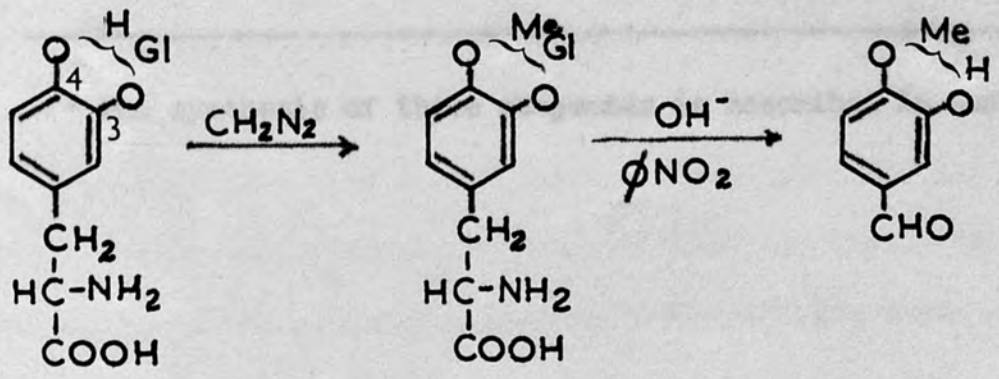


Fig.30. Procedure for determining the substitution pattern in O-substituted DOPA derivatives.

The experimental procedure used was a modification of that described by Stone & Blundell (151). It was first necessary to study the applicability of the method to compounds with saturated side chains as found in DOPA, by using a number of test substances. The results of the study are given in Table 5.

Table 5.

Compound studied	Aldehyde produced on oxidation
Tyrosine	p-hydroxybenzaldehyde
Ferulic acid	vanillin
* 3-O-methyl DOPA	vanillin
* N-methyl-3-O-methyl DOPA	vanillin
* 4-O-methyl DOPA	isovanillin
* N-methyl-4-O-methyl DOPA	isovanillin
Sinapic acid	syringaldehyde
Caffeic acid	-
DOPA	-

* The synthesis of these compounds is described in Section II 4.

The chromatographic systems suggested by Stone and Blundell (131) or electrophoresis in bisulphite-containing buffers (132) were not suitable for resolving mixtures of vanillin and isovanillin. They could however be readily separated by the electrophoresis in borate buffers (0.1M) at pH 8.7 and 10.0. The difference in the acidities of the hydroxyl groups of vanillin and isovanillin made them easily distinguishable from one another.

The mobilities are given in Table 4.

Table 4.

Aldehyde	M SA (pH 8.7*)	M SA pH 10.0*)
p-hydroxybenzaldehyde	0.86	0.89
vanillin	0.80	0.80
isovanillin	0.31	0.70
syringaldehyde	0.74	0.70

* Corrected for electroendosmosis.

The aldehydes were located on the electrophoretograms by the purple-brown colour produced with 2,4-dinitrophenylhydrazine/NaOH (133). This locating procedure produced a further distinguishing feature.

Spots of the vanillin changed to russet-brown on drying the paper, whilst those of isovanillin changed to yellow.

Methylation of P_2 was accomplished by three different methods; with silver oxide in dimethylformamide (134); with diazomethane; and by a combination of these two procedures. Each sample was then oxidised with nitrobenzene/ NaOH and the reaction products examined on paper electrophoretograms.

The only aldehydic product detected in each case was isovanillin, thus showing that P_2 was β- (3- (O-β-D- glucopyranosyloxy) -4-hydroxyphenyl)-L-alanine.

The structure of compounds resembling P_2 in the testas and cotyledons of broad bean seeds.

Compounds with identical chromatographic properties and giving similar colour reactions to P_2 were also found naturally, firstly in the cotyledons of dormant broad bean seeds and later in the testas of green seeds.

The compound from the cotyledons was isolated by preparative paper chromatography and subjected to the same analytical procedures described for P_2. It was thus possible to prove quite conclusively that the two compounds were identical.

The compound from the green testas was isolated by preparative paper chromatography and by the precipitation method described by Nagasawa (5). Again it was possible to establish the identity of this compound.

When the results of the work on methylated DOPA derivatives became available (see Section II.4) it seemed appropriate to confirm the findings using the testa compound.

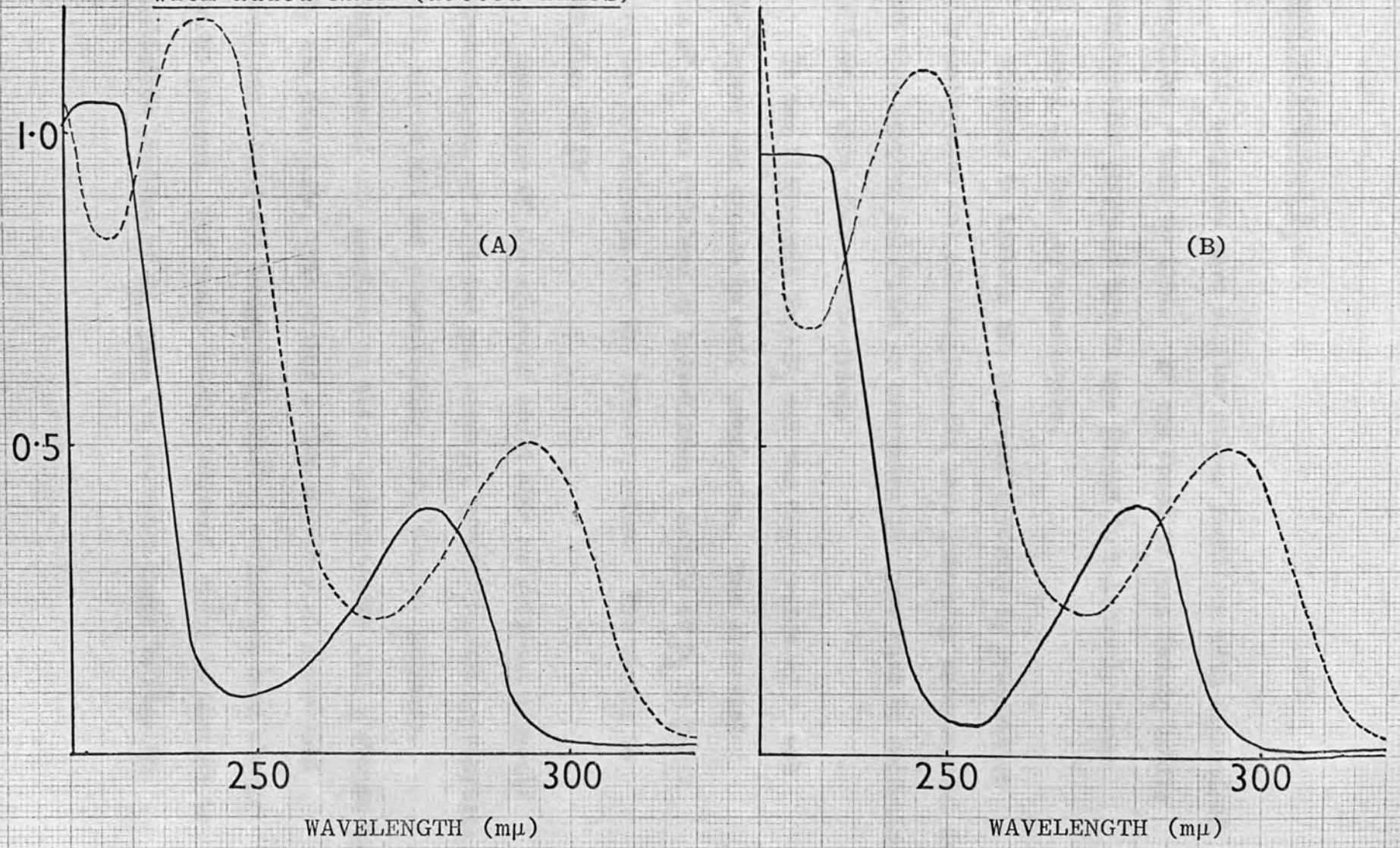
A sample of the compound was therefore methylated with diazomethane, and the product hydrolised with dilute mineral acid. This yielded a compound whose chromatographic and electrophoretic properties and colour reactions were identical to those of an authentic specimen of 4-O-methyl DOPA, thus establishing that the testa compound was substituted in the 3-position.

The ultraviolet spectra of the testa compound in water and with added alkali were also typical of DOPA derivatives substituted in the 3 position, as can be seen by comparison with the spectra of 3-O-methyl DOPA in Fig. 31. (see Fig. 35)

The structure of compound P_3

Compound P_3 was only formed in trace amounts during the feeding experiments. Hydrolysis with dilute mineral acids or β-D-glucoside glucohydrolase yielded DOPA and glucose. The colour reactions and ultraviolet spectra of this compound suggested that the 3 position of the aromatic ring was substituted, but as P_3 was never detected as a natural constituent of any plant tissues, no further examination was carried out.

Fig. 31. Comparison of the spectra of the DOPA glucoside isolated from broad bean testa (A) and 3-O-methyl DOPA (B). Measurements in water (continuous lines) and with added NaOH (dotted lines)



Structure of compound P_5.

This compound gave similar colour reactions with diazotised p-nitroaniline / NaOH to DOPA. It was found to be a fairly labile dihydroxyphenolic compound but it was formed in relatively large amounts during the feeding experiments.

Attempts to isolate the compound directly by preparative paper chromatography and electrophoresis were generally unsuccessful due to the fact that aqueous solutions on concentration gave rise to reddening and blackening even when freeze-dried.

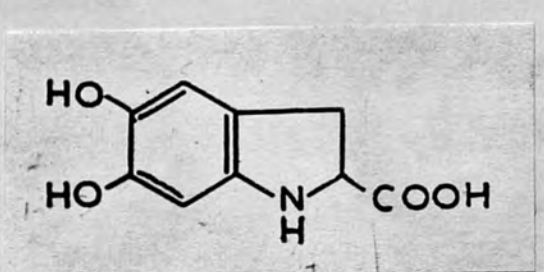
It was discovered that P_1 was the O-glucoside of P_5 and that the latter was stable to acid hydrolysis. The ultraviolet spectrum of P_5 was obtained by hydrolising a sample of P_1 in N-HCl and using the hydrolysate for the determination. The result is shown in Fig. 27 (b).

Specimens of the aglycone could also be obtained directly from chromatograms of DOPA-fed pea extracts by eluting the appropriate bands with dilute HCl and concentrating under reduced pressure. When such material was examined on electrophoretograms (0.1 M formic acid), however, two compounds, both giving a blue colour with diazotised p-nitroaniline / NaOH and having very similar mobilities were always found to be present. The faster moving compound, which is thought to be an oxidation product of P_5, gave the spectrum shown in Fig. 29.

P_5 showed the following reactions : It gave a blue colouration with diazotised p-nitroaniline / NaOH; formed a brown complex with sodium molybdate and gave a negative reaction with ninhydrin.

The colour reactions indicated the presence of an o-dihydroxyphenyl group and the absence of an-amino acid group. It was originally thought that P_5 was an N-alkyl derivative of DOPA such as N-methyl DOPA, but comparison with an authentic sample of this compound showed that although they had similar chromatographic and electrophoretic properties they were not identical. The comparison is made in Table 2.

β -3,4-Dihydroxyphenyl propane compounds such as DOPA, N-methyl DOPA, dopamine etc. also show an ultraviolet absorption maximum at 280 mμ in aqueous solution, whereas P_5 had a maximum at 284 mμ. This suggested that the latter has a different basic structure. Taking into account the ease with which it is oxidised and the fact that it does not fluoresce a possible structure seemed to be 5,6-dihydroxyindoline-2-carboxylic acid (XVII)



(XVII)

Structure of compound P_1.

Compound P_1 was formed in approximately equal amounts to P_2 during the feeding experiments, and it was later also found to be formed in other feeding experiments (Section II 3).

Hydrolysis of P_1 with dilute mineral acids, 50% formic acid, and β-D-glucoside glucohydrolase gave rise to P_5 and glucose in the molar ratio 1:1. The rate of acid hydrolysis and the fact that the liberated glucose could be converted to D-gluconic acid by D-glucose oxido-reductase confirmed that this compound was an O-β-D-glucopyranoside. The absence of an o-dihydroxyphenyl grouping was established as previously described.

If P_1 is a derivative of 5,6-dihydroxyindoline-2-carboxylic acid, then methylation followed by hydrolysis would give rise to the appropriate methyl derivatives. Attempts to produce such compounds synthetically were without success. There is thus no direct confirmation for these assumptions.

In a private communication Professor Piattelli stated that the treatment of betanin (R = H, XXXVII) in aqueous solution with sulphur dioxide, cleaves the molecule as shown in Fig.32 liberating 5-O-β-D-glucosyloxy-6-hydroxyindoline-2-carboxylic acid (LIX).

A sample of betanin was degraded in this manner and the products were examined on paper electrophoretograms (0.1 M formic acid).

The main product, although having similar electrophoretic properties to P_1, gave a greyish colour with diazotised p-nitroaniline / NaOH.

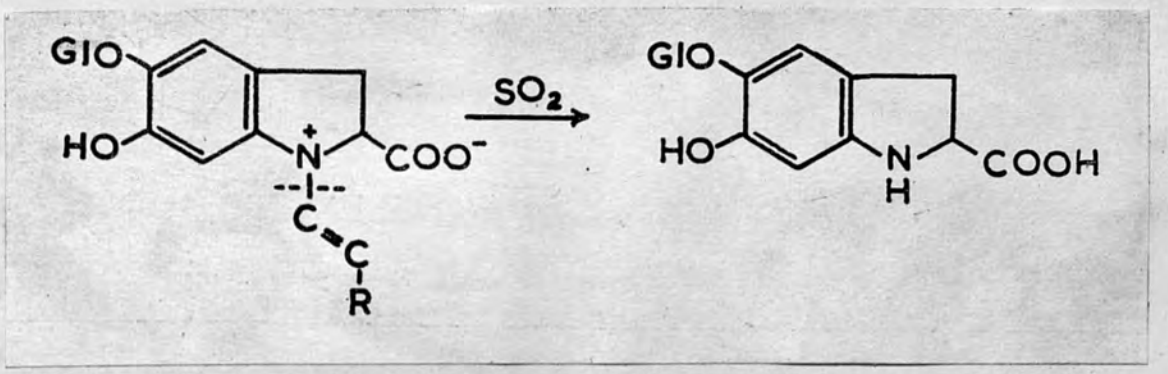


Fig.32. Degradation of betanin with sulphur dioxide.

In conlusion if P_1 has the suggested structure it must be the isomeric 6-O-β-D glucosyl derivative of indoline-2-carboxylic acid.

Structure of compound P_4.

The ultraviolet spectrum of P_4 in water and with added alkali (Fig.28) shows considerable difference to those produced by DOPA derivatives.

Hydrolysis of P_4 with mineral acids, 50% formic acid or β-glucosidase gave rise to D-glucose and two o-dihydroxyphenylic compounds having similar properties in most of the chromatographic systems used (^{R}DOPA ~ 2) and on paper electrophoretograms. Closer examination showed that the compound which had been designated P_4 also gave rise to two spots on electrophoretograms using formic acid as an electrolyte and is therefore possibly a mixture.

Some indication of the source of these compounds was given by the discovery that samples of P_5 stored overnight in sodium borate produced a compound, the spectrum of which was identical to the aglycone of P_4.

Although a compound with the same mobility as P1 was detected amongst the products, it gave a different colour reaction with diazotised p-nitroaniline / NaOH.

II. 3 Further feeding experiments.

The DOPA feeding experiments were continued with a variety of species. The general technique was to cut an actively growing section of the plant about 9"-1' in length, and to dip the stem immediately into a saturated solution of DOPA contained in a boiling tube. The cut section was allowed to absorb and metabolise the DOPA for 4-6 days, after which time extracts were prepared from the leaves and these were examined chromatographically.

For purposes of comparison, a similar section of the plant dipped into distilled water to act as control.

The results varied considerably depending on the species chosen. In some, the presence of DOPA appeared to have no effect, whilst in others, the presence of DOPA resulted in rapid death of the tissue. In the case of Menthapiperita, for example, sections cut from the top of the plant and immersed in distilled water eventually rooted and produced flowers. The sections immersed in DOPA solution, however, rapidly blackened and withered within 24 hours.

In other species, such as Aristolochia clematilis blackening was quite marked in a few days in the DOPA fed sections, but growth appeared to be normal, and flowers were produced in the same way as with the controls.

The presence of DOPA in tissues in which it is not normally to be found, therefore, produced a variety of effects. Infact, in one case, that of Lupinus polyphyllus, feeding of L-DOPA appeared to halt the rapid wilting exhibited by the controls.

DOPA was detected even in the controls of Galega officinalis, during these experiments. As it could not be detected in the uncut tissues it must have been formed as a result of tissue damage.

The full list of results is given in Table 5. The compounds most frequently detected were those which corresponded to P_1 and P_2. Other compounds giving the blue colouration with diazotised p-nitro-aniline / NaOH of o-dihydroxyphenolic compounds or the pink colour given be P_1 were also encountered. The colours quoted in Table 5 refer to this fact.

Table 5. Feeding experiments with plants

Species	Compound fed	Amount in tissue	Compounds formed	Comments
Alchemilla monticola	DL DOPA	-	-	
Amaranthus flavus	"	+++	P_1(+), P_2(+), and a pink spot ahead of DOPA *	
Amaranthus polygamus	"	++	P_1(+), P_2(+)	
Aristolochia clematilis	"	++++	P_1(+), P_2(+), 2 blue spots ahead of DOPA *	DOPA-fed cuttings blackened but growth normal.
Astragalus aloepecuriodes	"	-	-	
Astragalus glycyphyllus	"	-	-	
Chenopodium album	"	++++	P_1(++), P_2(+), a pink spot ahead of DOPA (++) *	
Gentiana asclepiadea	"	+++	P_1(±), P_2(+), 2 pink spots ahead of DOPA (+) *	

* in Solvents C, D + F

Table 5. (continued)

Species	Compound fed	Amount in tissue	Compounds formed	Comments
Galega officinalis	L DOPA	+	P_2 (+)	
	D DOPA	+	P_2	
	Control		DOPA	
Lathyrus rotundifolius	L DOPA	++	P_2 (++)	No blackening
	D DOPA	++	P_2 (++)	observed
Lupinus polyphyllus	L DOPA	++++	A number of pink spots behind DOPA	Control cutting quickly shrivelled, but DOPA-fed cutting robust.
Nasturtium sp.	DL DOPA	++	2 pink spots behind DOPA (+)	
Mentha piperita	"	++	P_1 (±) and P_2 (+)	
Veronica longifolia	"	++++	P_1 (++)	
Vigna cylindrica	"	++	P_2 (+) and a pink spot ahead of DOPA	
Vicia dumetorum	"	+	P_2 (+)	

II 4. Synthesis and properties of methylated DOPA derivatives.

The synthetic and biosynthetic studies just described only partially answered the questions which had been posed at the outset, so in order to shed further light on the problem it was decided to synthesize methylated DOPA derivatives with two objectives in mind.
(1) It was desirable to record the properties of these derivatives, as it seemed likely that this information would be of value in characterising the products from for example, O-glycosides after methylation and hydrolysis.
(11). The reported occurrence of N-methyl dopamine and 3-O-methyl caffeic acid (ferulic acid) in some plant tissues, made it likely that the corresponding derivatives of DOPA might also be found.

Although a number of syntheses have been reported since this work was initiated, at the time most profitable approach seemed to be that of Deulofen Guerrero (135) who prepared N-methyl-(3-methoxy-4-hydroxyphenyl) DL alanine by condensing vanillin with creatinine (LX.Fig.33 (a) R= CH_3 followed by reduction with sodium amalgam and hydrolysis with barium hydroxide.

It was thought that this method, with modifications, might be useful in preparing the other methylated analogues. For example, by replacing vanillin by isovanillin in the synthesis it was hoped that corresponding 4-O-methyl compound might be prepared.

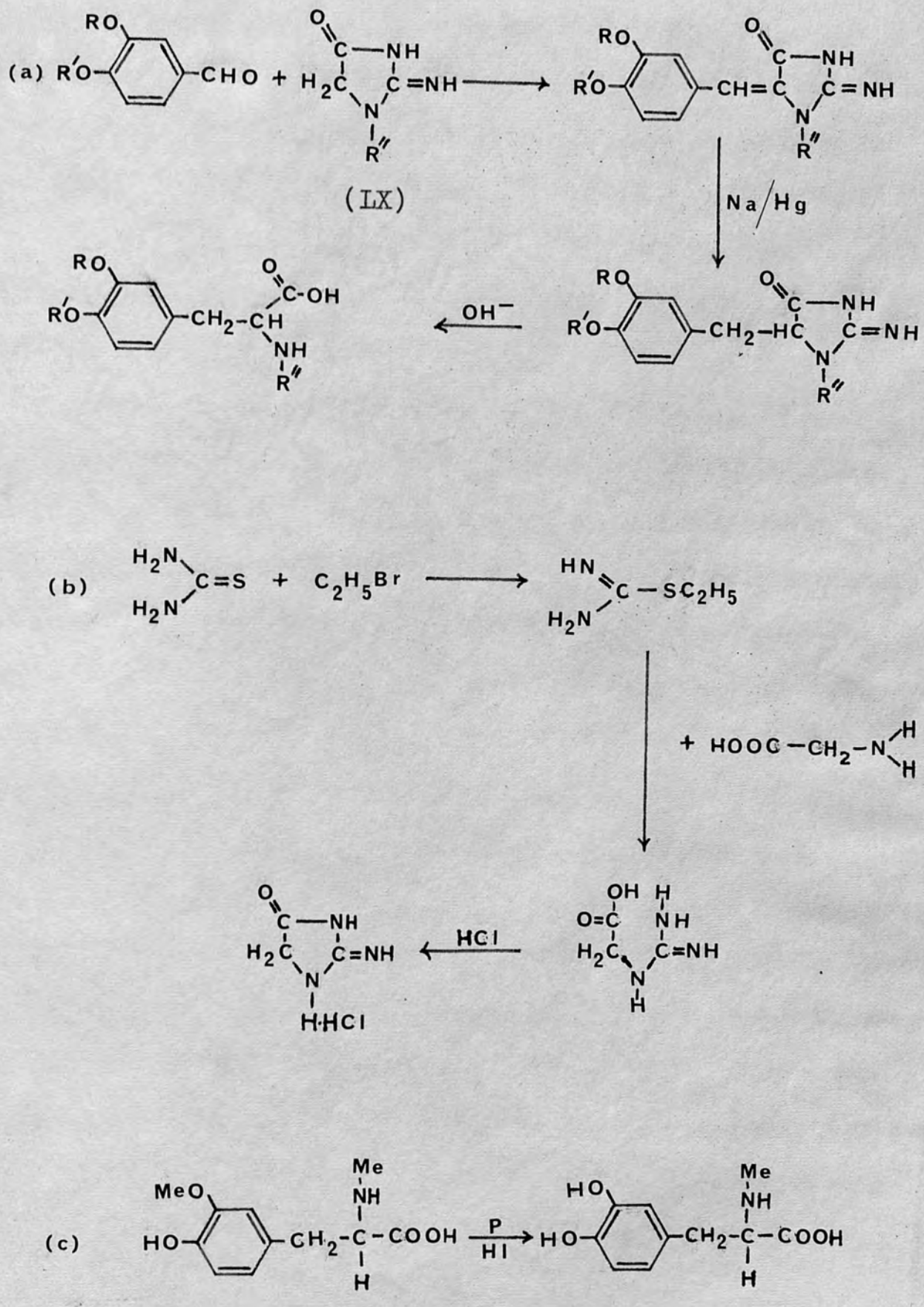


Fig. 33. The synthesis of methylated DOPA derivatives.

Similarly, by substituting creatinine in the condensation by glycocyamidine (LX,R" = H) it was hoped to prepare the amino acids unsubstituted on the nitrogen. The sequence of reactions involved in the synthesis of glycocyamidine hydrochloride are summarised in Fig.33 (b).

An alternative synthesis for 3-O-methyl DOPA had been described by Johnson and Bengis (136), but as this involved more intermediate steps it was not considered.

N-methyl DOPA was obtained by the partial demethylation of N-methyl-3-O-methyl DOPA with phosphorous and hydriodic acid (Fig.33 (c)).

By the above procedures it was possible to obtain samples of five methylated derivatives of DOPA. Each was obtained as a colourless, chromatographically homogeneous, crystalline solid. Two other 'model' compounds, the isomeric O-methyl dopamine derivatives, were generously donated by Messrs. Merck, Sharp and Dohme.

Colour reactions of the methylated compounds.

The colour reactions summarised in Table 6 confirm that 3-and 4-hydroxyl substituted DOPA derivatives can readily be distinguished from one another and from unsubstituted derivatives by means of the diazotised p-nitroaniline / NaOH reagent. A further distinguishing feature is the colour change which takes place when sprayed papers are allowed to dry. For example, the original plum-red colour given be 3-O-substituted compounds changes to grey-green under such circumstances. Features such as this proved to be very valuable in the later work with plant extracts.

Table 6. Colour reactions of DOPA, dopamine and their O- and N-methylated derivatives.

Compound	diazotised p-nitroaniline / NaOH		Ninhydrin	Molybdate
	Original colour	After drying		
DOPA	blue	fades	+ve.	brown
N-methyl DOPA	blue	fades	-ve.	brown
dopamine	blue	fades	-ve. *	brown
3-O-methyl DOPA	plum	grey-green	+ve.	-
N-methyl 3-O-methyl DOPA	plum	grey-green	-ve.	-
3-O-methyl dopamine	"	grey-green	-ve. *	-
4-O-methyl DOPA	blue-violet	blue	+ve.	-
N-methyl-4-O-methyl DOPA	blue-violet	blue	-ve.	-
4-O-methyl dopamine	"	blue	-ve. *	-

* brown colouration.

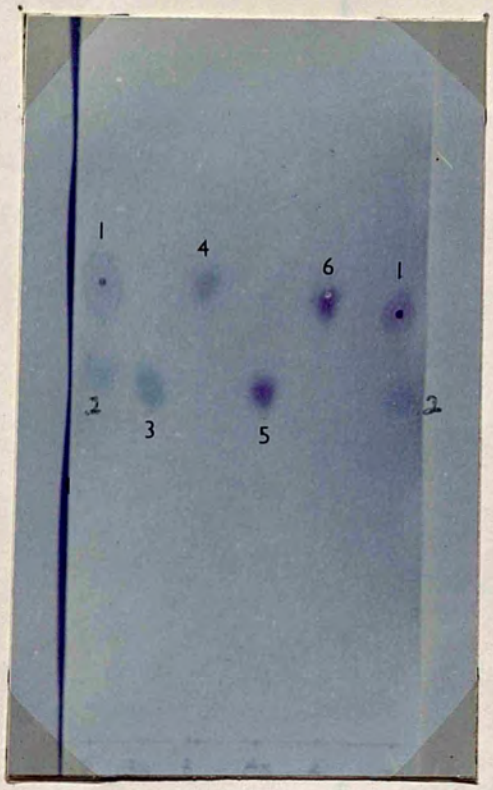


Fig. 34. Colour reactions of DOPA derivatives with diazotised p-nitroaniline / NaOH. Electrophoretogram run in 0.1 M formic acid.

1. DOPA
2. N-methyl DOPA
3. N-methyl-3-O-methyl DOPA
4. 3-O-methyl DOPA
5. N-methyl-4-O-methyl DOPA
6. 4-O-methyl DOPA

Table 7. Chromatographic behaviour of methylated DOPA derivatives.

R_{DOPA} values

Compound	Solvent B	Solvent C	Solvent F
3-O-methyl DOPA	1.64	1.44	1.12
N-methyl-3-O-methyl DOPA	2.07	1.70	1.47
4-O-methyl DOPA	1.54	1.34	1.04
N-methyl-4-O-methyl DOPA	2.00	1.60	1.40
N-methyl DOPA	1.50	1.29	1.39

Ultraviolet spectra.

The ultraviolet spectral data for the O-methylated compounds are given in Table 7 and Fig.35. The spectra of the isomeric compounds are similar, but there is quite a large difference in the relative absorbance in the regions around 240 and 295 mμ in alkaline solution.

The bathochromic shift from 280 mμ which occurs on adding alkali, is due to the increased interaction of the O- (compared with the -OH) with the benzene ring; the magnitude of shift being a measure of this increase and the intensity of absorbance being proportional to the transition probability.

In the case of 4-O-methyl derivatives there is no interaction between the $_{}O-$ and the $-CH_2-$, and there is thus an increased absorption at around 290 mμ. In the case of 3-O-methyl derivatives, however, the $-CH_2-$ is para to the $_{}O^-$ and therefore depresses the electron donating tendencies to a small extent. This reveals itself by a decreased absorption.

These properties which were exhibited, not surprisingly, by all the model compounds, were thought to be of value for distinguishing between compounds belonging to either of the isomeric O- substituted series. This hypothesis was tested by studying the ultraviolet spectral properties of phenol, o-, m- and p-cresols and the two hydroxy-methoxy toluenes in water and with added alkali. The general validity of the approach was thus established.

Table 8

Ultraviolet spectral properties of methylated derivatives

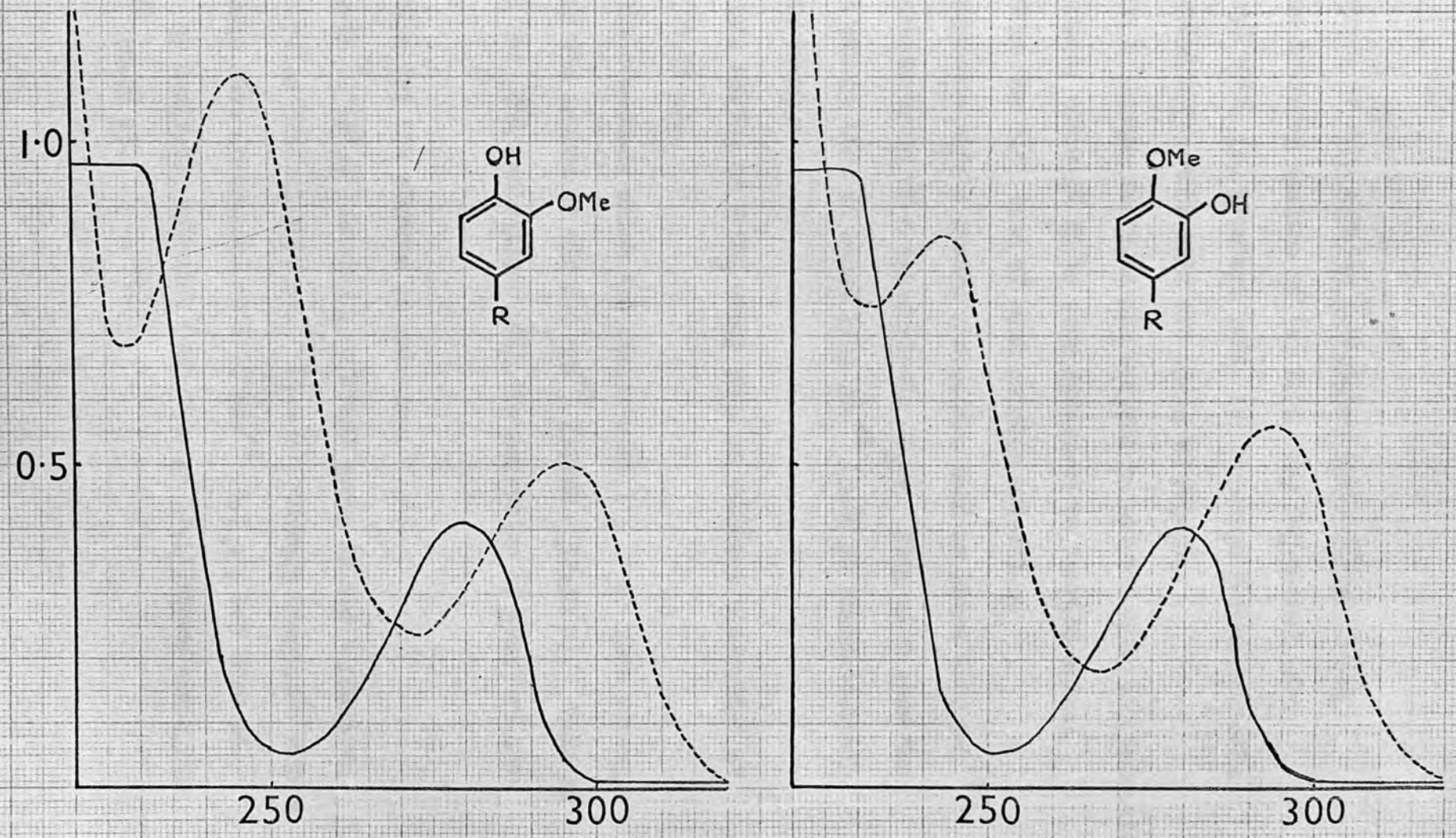
(Figures in brackets denote the bathochromic shift)

	DOPA				N-Me-DOPA				3-O-Me-DOPA				N-Me-3-O-Me DOPA			
	Max.		Min.		Max.		Min.		Max.		Min.		Max.		Min.	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
A. in water	-	280	-	251	-	280	-	252	227	280	218	251	227	280	218	252
B. 5 ml.A. +1ml. $\frac{3M}{250}$ NaOH	240	295 (+15)	232	265	241	295 (+15)	232	267	244	294 (+14)	227	271	244	294 (+14)	227	270
C. Bx + 1 ml. $\frac{3M}{250}$ H_4BO_4	-	288	-	261	-	288	-	262								

	4-O-Me-DOPA				N-Me-3-O-Me DOPA				3-O-Me dopamine				4-O-Me dopamine			
	Max.		Min.		Max.		Min.		Max.		Min.		Max.		Min.	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
A. in water	226	280	218	251	227	280	218	252	227	279	216	250	-	278	-	250
B. 5 ml. A + 1ml. $\frac{3M}{250}$ NaOH	242	293 (+13)	231	267	242	293 (+13)	231	267	242	295 (+16)	227	270	242	292 (+14)	230	266

Table 8.

Fig. 35. Ultraviolet spectra of isomeric O-methylated DOPA and dopamine derivatives in water (continuous lines) and with added NaOH (dotted lines)



Infrared spectra of DOPA and its methylated derivatives.

The infrared spectra of (a) DOPA, (b) 3-O-methyl DOPA, (c) 4-O-methyl DOPA, (d) N-methyl DOPA, (e) N-methyl 3-O-methyl DOPA and (f) N-methyl 4-O-methyl DOPA are shown in Fig.36.

The following features are helpful in establishing the indentity of the compounds, although it is very difficult to ascribe actual structures from the I.R. data of such complex molecules.

(1) All compounds show a strong absorption band at about 1600 cm.$^{-1}$ (6.25 μ) characteristic of a zwitterionic CO_2^-. They also show the absence of a strong band at about 1700 cm.$^{-1}$ (5.88 μ) characteristic of C=O (as in COOH).

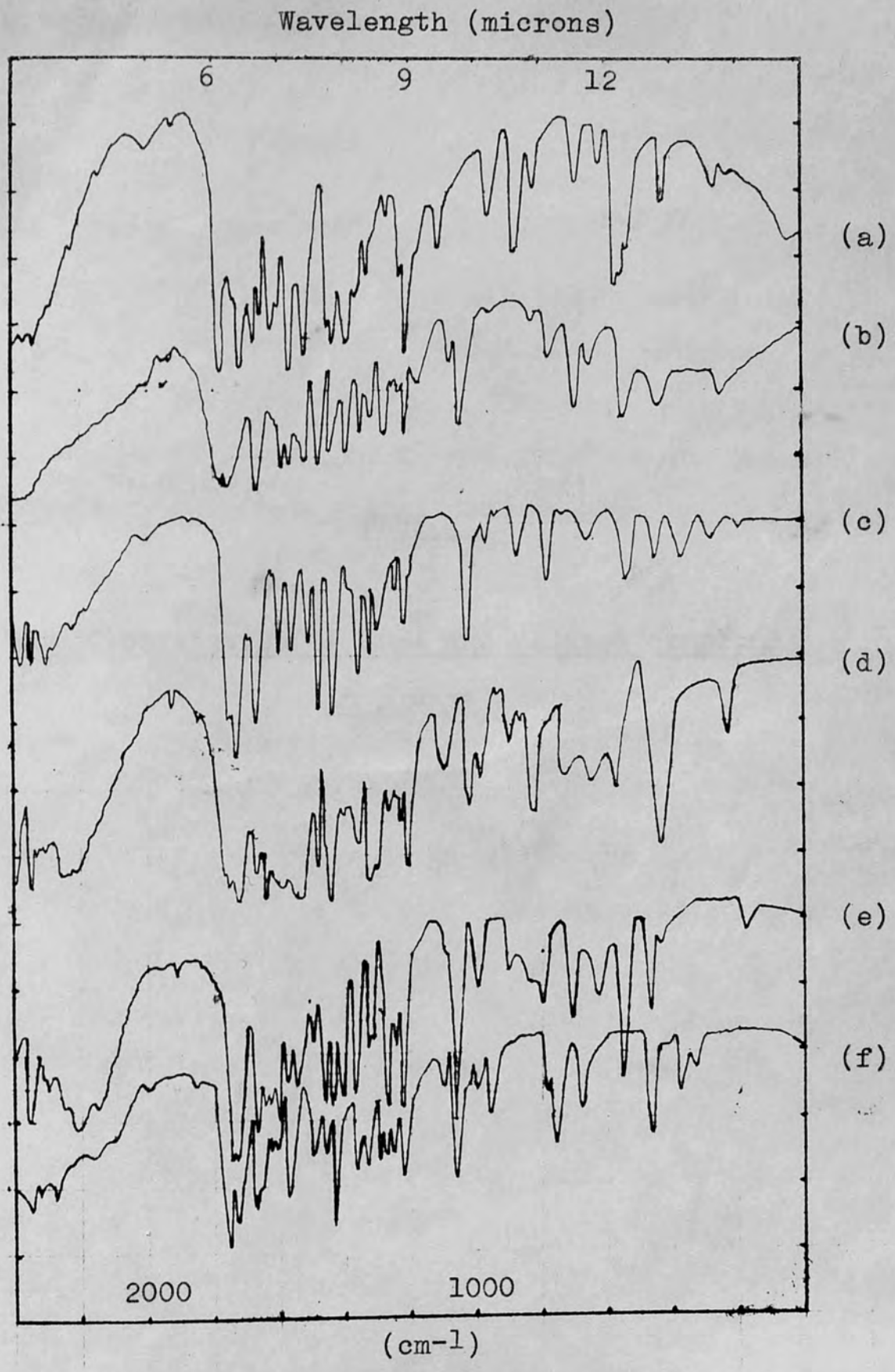
(2) Compounds having an N-CH_3 group show an enhanced absorption at 780 cm.$^{-1}$ (12.75 μ).

(3) Aromatic compounds which are substituted 1', 2' 4' show strong absorption in the region of 810-820 cm.$^{-1}$ (12.20 - 12.35 μ). This is shown by all the DOPA derivatives with the exception of N-methyl 4-O-methyl DOPA.

The explanation for its absence is unknown.

(4) Compounds (1',2',4') having a hydroxyl group in the 4 position show an absorption band at 1850 cm.$^{-1}$ (5.4 μ), whereas compounds with a 4-methoxy group show absorption at 1760 cm.$^{-1}$ (5.7 μ), but none at 1850 cm.$^{-1}$. By contrast, tyrosine (substituted 1',4')shows absorption at 1900 cm.$^{-1}$ (5.25 μ).

Fig. 36. The infrared spectra of methylated DOPA derivatives



PART III

The distribution of DOPA and related compounds in plants

III. 1. The distribution of DOPA and related compounds in plants.

The information gained during the experiments described in the previous section suggested means whereby derivatives of DOPA in plant tissue extracts might be located and also characterised. Using this information a survey of over 200 tissues was undertaken to discover just how common free DOPA and its derivatives were in higher plants.

One of the most surprising findings of this survey was the inability to detect simple carbohydrate derivatives. Despite considerable efforts to locate them, particularly the phenolic derivatives of DOPA, only the DOPA-O-β-D-glucoside in the broad bean was found. This therefore suggests that these derivatives are extremely uncommon in plants.

The results of this search given in Table 9 can be, by their very nature, only an incomplete guide to the general situation existing in higher plants. From them, however, the point emerges that DOPA is not a common constituent of plant tissues, even though in cases where it is found it is often one of the major phenolic constituents.

A few compounds giving the same colour reaction as DOPA with diazotised p-nirtoaniline / NaOH were detected in certain tissues. Chromatograms of extracts prepared from mature broad bean leaves in the Autumn following a relatively hot dry Summer, for example, showed quite large quantities of a compound with properties suggestive of a DOPA glycosyl ester. Attempts to isolate or even detect this compound a year later, following a cool, damp Summer were without success.

Observations such as this show quite clearly the difficulties and limitations inherent in surveys of the present type. In the majority of cases however, the occurrence of DOPA in any particular tissue was checked in at least two successive seasons.

Table 9 . The distribution of DOPA in plants.

Key to the Table

+++ Large amounts of DOPA present

++ Medium amounts of DOPA present

+ Small amounts of DOPA present

⁎ Possible trace of DOPA

- No DOPA detected

Family	Species and Variety	Tissue examined	Quantity of DOPA
A. Monocotyledons			
Agavaceae	Dracaena sp.	Leaves	-
	Sansevieria trifasciata	"	-
Amaryllidaceae	Galanthus mivalis	"	-
	Narcissus sp.	Stem sap	-
	Narcissus pseudonarcissus	"	-
Gramineae	Hordeum vulgare	Stems	-
Iridaceae	Iris sp.	Leaves and stem	-
Liliaceae	Cordyline australis	Leaves	-
	C. stricta	"	-
	Hyacinthus sp.	"	-
	Tulipa sp.	"	-

Musaceae	Musa sp.	Skin of ripe and unripe fruit, roots	-
Orchidaceae	Cymbidium sp.	Leaves, stem, flowers	-
Palmaceae	Cocos neucifera	Leaves	-
Pontederiaceae	Eichornia crassipes	Leaves	-
B. Dicotyoedons			
Actinidiaceae	Actinidia chinensis	Leaves and sap	-
Amaranthaceae	Amaranthus caudatus	Stem and leaves	-
	flavus	" " "	-
	hypochondiacus	" " "	-
	polygamus	" " "	-
Apocynaceae	Amsoniatabernaemontana	Latex	-
	Apocynum androsaemifolium	"	-
	Rhazya orientalis	"	-
	Vinca major	"	-
Araliaceae	Hedra helix	Leaves	-
Aristolochiaceae	Aristolochia clematitis	"	-
	liana ?	"	-
	Pararistolochia goldiaene	Leaves and stem	-
Campanulaceae	Adenophora lilifolia	Latex	-
	Campanula alliarifolia	"	-
	carpatica	"	-
	clomerata	"	-
	grosseki	"	-
	lactiflora	"	-
	muralis	"	-
	persicifolia	"	-
	rapuncloides	"	-
	Codonopsis clematidea	"	-
	ovata	"	-

	Jasione janka	Latex	-
	perennis	"	-
	Lobelia cliffortiana	"	-
	syphilitica	"	-
	Michauxia spp.	"	-
	Platycodon grandiflorum	"	-
	Spetularia speculum	"	-
	Symphyandra hofmanni	"	-
Chenopodiaceae	Atriplex hortensis	Leaves and stem	-
	Beta vulgaris	" " "	-
	Chenopodium album	" " "	-
	amaranticolor	"	-
	glaucum	"	-
	polysperma	"	-
	rubrum	Young green leaves	++
		reddened leaves	+
	salinum	Leaves and stem	-
	virgatum	" " "	-
	Halimione portulacoides	" " "	-
	Salsola kali	Young leaves and stem	++
		Mature " "	++
Compositae	Bellis perennis	Whole plant	-
	Eupatorium platyphyllum	Leaves	-
	Taraxacum officinale	Leaves and stem	-
Crassulaceae	Sempervivum anomalum	Concentrated sap	-
	arachnoideum	" "	-
	arvenense	" "	-
	doelsianum	" "	-
	dolomiticum	" "	-
	flagelliforme	" "	-
	grimmeri	" "	-
	pallida	" "	-
	rupicolum	" "	-
	rectorum	" "	-
	ruthenicum	" "	-
Euphorbiaceae	Euphorbia amygdaloides	Latex	-
	capitata	"	-
	characias	"	-
	epithymoides	"	-
	helioscopia	"	-
	lathyrus	"	+++
		Green pod	++
	myrsinites	Latex	-
	pulcherrima	"	-
	semivillosa	"	-

	Euphorbia sikkimensis	Latex	-
	spinosa	"	-
	wulfanii	"	-
Geraniaceae	Pelargonium sp.	Leaves	-
Gesneriaceae	Episcia fulgida	Leaves	-
	Saintpaulia ionantha	Leaves and stem	-
Hippocastanaceae	Aesculus hippocastanum	Leaves	-
Lauraceae	Persea gratissima	"	-
Moraceae	Ficus elastica	Latex	-
	indica	"	-
Myricaceae	Myrica gale	Leaves	-
Nymphaeaceae	Nymphaea alba	Leaves and stem	-
Papilionateae (Leguminosae)	Astragalus alopecroides	Leaves	-
	cicer	Leaves and stem	-
		Young pod	-
		Blackening pod	+
	glycophyllos	Leaves	-
	Baptisia australis	Young shoots	+++
		Mature leaves	+++
		Whole pod	+++
	Corogana arborescens	Leaves	-
	Colutea arborescens	Pods	-
	orientalis	Leaves	-
	Coronilla scorpioides	"	-
	Cytisus capitatis	Leaves	-
	hirsuitus	"	-
	purpurea	"	-
		Flowers	-
	scoparius	Leaves	+
		Flowers	+
		Pod	+
	sessifolius	Leaves	-
	Desmodium canadense	"	-
	nutans	"	-
	trifolium	"	-
	Galega orientalis	"	-
	Genista spartium	"	-
	Gleditschia delany	"	-

	Glycine soja	Leaves	-
	Glycorhiza foetida	"	-
	Hedisarum coronarium	"	-
	Indigofera gerardiana	"	-
	Laburnum vulgare	Stem	-
		Flowers	-
		Pod	±
		Seed coat	-
		Cotyledons	-
		Seed coat hydrolysate	-
	Lathyrus glymerum	Leaves	-
	montanus	Leaves	-
		Stem	-
		Pod	-
		Seed coat	-
		Cotyledons	-
	nissola	Leaves	-
	ochrus	Leaves	-
	uligonosus	Leaves	-
	Lens esculenta	Leaves	-
	Lotus tetragonalobus	Leaves	-
	Lupinus arborensis	Leaves	-
		Stem	-
		Pod	-
		Seed coat	-
		Cotyledons	-
	polyphyllus	Leaves	-
		Stem	-
		Flowers	-
		Young pod	-
		Blackening pod	+
	Maachia amurensis	Leaves	-
	Medicago arabatica	Leaves	-
	falcata	Leaves	-
	sativa	Leaves	-
	Melilotus alba	Leaves	-
	altissima	Leaves	-
	Mucuna deeringianum	Young leaves	+++
		Old leaves	+++
		Flowers	+++
		Pod	+++
		Funicle	+++
		Seed coat	+++
		Cotyledons	+++
	pruriens	Leaves	+++

	Onobrychii caput galli	Leaves	-
	Ononis natrix	Leaves	-
	Phaseolus mungo	Leaves	-
	Pisum jamardii	Leaves	-
	sativum	Young shoots	-
		Leaves	-
		Pod	-
		Seed coat	-
		Funicle	-
		Cotyledons	-
	Securigera caronilla	Leaves	-
	Spartum funiculum	Leaves	-
	Thermopsis montana	Leaves	-
	Trifolium pratense	Leaves	-
		Pod	-
	stellatum	Leaves	-
	Trigonella caerulea	Leaves	-
	cretica	Leaves	-
	foenum	Leaves	-
	Vicia angustifolia	Leaves	-
		Young pod	-
		Blackening pod	+
		Seed coat	-
		Cotyledons	-
	cracca	Leaves	-
		Pod	-
	disperma	Leaves	-
	dumetorum	Leaves	-
		Green pod	-
		Seed coat	-
	faba (see section ⅁ 2)	Cotyledons	-
		Seed coat	-
		SC Hydrolysate	-
	hirsuita	Leaves	-
		Young pod	-
		Blackening pod	-
	larythroides	Leaves	-
		Pod	-
	lutea	Leaves	-
		Pod	-
	narbonensis	Leaves	-
		Pod	-
		Funicle	-
		Seed coat	-
		Cotyledons	-

	Vicia sativa	Leaves	-
		Stem	-
		Pod	-
		Seed coat	-
		Cotyledons	-
		Blackening pod	+
	sepium	Leaves	-
		Stem	-
		Pod	-
		Seed coat	-
		Cotyledons	-
		Blackening pod	+
	Vigna cylindrica	Leaves	-
Polygonaceae	Rheum rhaponticum	Leaves and stem	-
	Rumex sp.	Leaves	-
Primulaceae	Lysimachia sp.	Leaves and Stem	-
Rubiaceae	Galium palustre	Leaves and stem	-
Salicaceae	Salix purpurea	Leaves and bark	-
Saxifragaceae	Saxifraga stolinifera	Leaves	-
Solanaceae	Hyosyamus niger	Juice from fruit	-
	Lycopersicon esculentum	Leaves, stem and juice from fruit	-
	Nicotiniana tabacum	Leaves	-
	Solanum nigrum	Juice from fruit	-
	S. quitoense	" " "	-
	S. tuberosum	Leaves	-
Umbellifereae	Foenicum vulgare	"	-
Urticaceae	Pilea cadera	"	-

The results quoted in Table 9 together with those of previous workers suggest that DOPA is mainly limited to species belonging to the Leguminosae or Centrospermae. A number of other features, however, emerged during the survey and it is worthwhile considering these in a little more detail.

Chenopodium rubrum and Salsola kali (Chenopodiaceae).

In view of the suggested role of DOPA in the biogenesis of the nitrogenous pigments in this group, the demonstration of free DOPA in the non-pigmented tissues of these two species is very interesting.

The DOPA concentration in Salsola kali tissues in which no pigment formation took place, remained constant throughout the year, whereas a fall in the DOPA concentration, paralleling the formation of red pigment was noted in Chenopodium rubrum.

The demonstration of DOPA in these species makes it reasonable to suppose that DOPA might also be found in related species and in related families.

Euphorbia lathyrus (Euphorbiaceae)

Liss (31) had demonstrated the presence of high concentrations of free DOPA in the latex of this plant by an isolation procedure, but made no attempt to study the variation throughout the growth cycle.

In the present work, samples of the latex were removed from the plant at regular intervals and examined on paper chromatograms, location being with diazotized p-nitroaniline/NaOH. The DOPA concentration was found to be consistently high throughout the whole growing cycle.

It was also found to be fairly high in the outer tissue of the fruit, from which extraneous latex had been carefully removed by washing. Other compounds having the same colour reaction as DOPA were also present in the latex, but were not examined further.

Despite the high concentration of such compounds, blackening or discolouration of any tissue was never observed. The role of DOPA, and the reason why it appears to be limited to only one Euphorbia species is as yet unknown.

Baptisia australis (Leguminosae).

This species (also known as B. minor), was the first in the present survey to constitute a new source of DOPA. It seems to resemble Vicia faba in the fact that DOPA is found in virtually all parts of the plant throughout its life, but it differs in not forming any glucosyl derivative in the pod.

The presence of DOPA in this plant was confirmed by extensive chromatographic and electrophoretic studies and by isolation using lead acetate precipitation.

Unfortunately only one species of Baptisia was available for study, but Alston et al (137) report that DOPA occurs in about half the Baptisias they have examined, wherein it seems to be responsible for the subsequent blackening upon drying.

Mucuna species (Leguminosae).

It was possible to confirm the original findings (25, 26 27) for both Mucuna deeringianum and Mucuna pruriens. The black pigment present in the hairs covering the pods, formed during the actively growing phase might turn out to be a true phytomelanin. Unfortunately, insufficient material was available for chemical studies.

Vicia angustifolia, V.sativa, V.sepieum, Astralgus cicer and Lupinus polyphyllus (Leguminosae).

These are discussed further in section IV 2.

Vicia faba (Leguminosae).

The occurrence of free DOPA in the broad bean has been recognised for over half a century, but little work has been done on its distribution in the various tissues throughout the growth cycle. A more comprehensive survey was therefore undertaken with this plant, and the results are reported in the following sections.

III 2. Qualitative studies on the distribution of L-tyrosine, L-DOPA and DOPA 3-O-β- D-glucoside in broad bean tissues.

The broad bean, (Vicia faba L.) is perhaps the commonest and horticultually most valuable of the 120 or so species of the genus Vicia. The origins of this particular species are lost in antiquity, but it has been suggested that it was derived from one of the southern Mediterranean species such as Vicia narbonensis, which morphologically it resembles closely.

Three sub-species are recognised. These are minor, with seeds about 1.5 cm.long; major with seeds of about 2.5 cm.; and equina, the horse or field bean, with seeds of about 1.25 cm. Major is the usual culinary type, whilst equina is a valuable fodder crop.

Since DOPA was first isolated from the green pod of this plant by Torquati (1) and Guggenheim (2) in 1913, the distribution of DOPA in the broad bean had received little attention except for the work of Nagasawa and his co-workers (5,138), who reported the occurrence of both tyrosine and DOPA in various tissues. They were also the first to appreciate the occurrence of large amounts of a DOPA-O-β- D-glucoside, principally in the seed coat. The structure of this compound has already been discussed.

The observations of these workers have been re-examined and extended in the present study, in which a fairly comprehensive, qualitative

and later quantitative examination has been made of broad bean tissues throughout their growth cycle. Qualitative assessments were made after concentrated tissue extracts had been fractionated on paper chromatograms (Solvents B,C,E,F) and electrophoretograms (0.1 M-formic acid), location being with diazotized p-nitroaniline/NaOH.

Observations were made with seeds and etiolated seedlings( ) grown on damp cotton wool (var. Johnson's Longpod), and with field-grown plants (var. Seville Giant Longpod); the results were as follows:-

The cotyledons, testa, and radicle of dormant seeds were found to contain only traces of tyrosine and DOPA. Soaking the seeds for 24 hours caused little change, except for an increase in the tyrosine content of the cotyledons. After about 36 hours, the radicle appeared through the seed coat. Extracts prepared from this latter tissue over the next few days revealed that a rapid build up in the DOPA concentration takes place at this stage and at the later emergence of the plumule, so that after 7 days growth DOPA was by far the most abundant phenolic compound in both radicles and shoots, being particularly evident from the plumules. During the first week's growth tyrosine was not very evident in the radicles, possibly because it is actively converted to DOPA.

As growth continued, the DOPA content of leaves remained at a consistently high level, whereas it diminished slowly in the stem and roots,

the tyrosine concentration still remaining at a fairly low level.

In plants at the flowering stage, the DOPA content was found to be unchanged in the leaves, but much lower in the stems, where the concentration fell from the upper to the lower portions. Tyrosine followed a similar pattern. The roots and root-nodules had little DOPA present and the other phenols differed noticeably from the above-ground parts of the plants.

At first DOPA content of the young flowers was similar to that of the leaves, although there appeared to be much more tyrosine present, but as they reached maturity there was strong evidence for a rapid increase resulting in a very high DOPA concentration which was maintained in the young pods. However, as the pods increased in length, the DOPA concentration diminished gradually, falling to approximately the same level as the leaves by the time the pods had reached a length of about 15 cm.

As the pods and their seeds reached maturity, DOPA formation in the plant was no longer evident, the DOPA content of all tissues slowly diminished. It is perhaps interesting to note, however, that even old, yellowing leaves had a relatively high DOPA content although blackening was rarely observed.

The metabolism of DOPA in the pods was of particular concern to us. Previously, Nagasawa *et al* (5) had studied the distribution of

DOPA glucoside in the green pods of a late-ripening (Japanese) variety, and had found that it was particularly located in the testa. It was present to a lesser extent in the radicle, hilum and inner tissue of the green pod, but was absent from the outer tissue of the pod and the cotyledons. Our own observations on pods at approximately the same stage confirmed these findings, although we were unable to detect any glucoside in the radicle.

The distribution of the glucoside appears to depend very much on the state of growth. For example, it was present in relatively large amounts in the funicles of very young seeds (5 mm.) slightly less evident at the stage normally used for culinary purposes (seeds 2.5 cm.), and completely absent from the funicles of fully matured seeds.

It was mentioned previously that the DOPA 3-O-β-D-glucoside was originally detected by us in the cotyledons from young seedlings. It was thought that it must have been formed during the germinating stage, but it was later shown that the dormant seeds also contained this compound. To detect whether in fact any further glucoside was formed during soaking, a semi quantitative comparison was made of the amount of glucoside in dry cotyledons, and in the cotyledons from seedlings germinated with and without their testas. The result of this experiment together with the results obtained on the pods are summarised in Table 10.

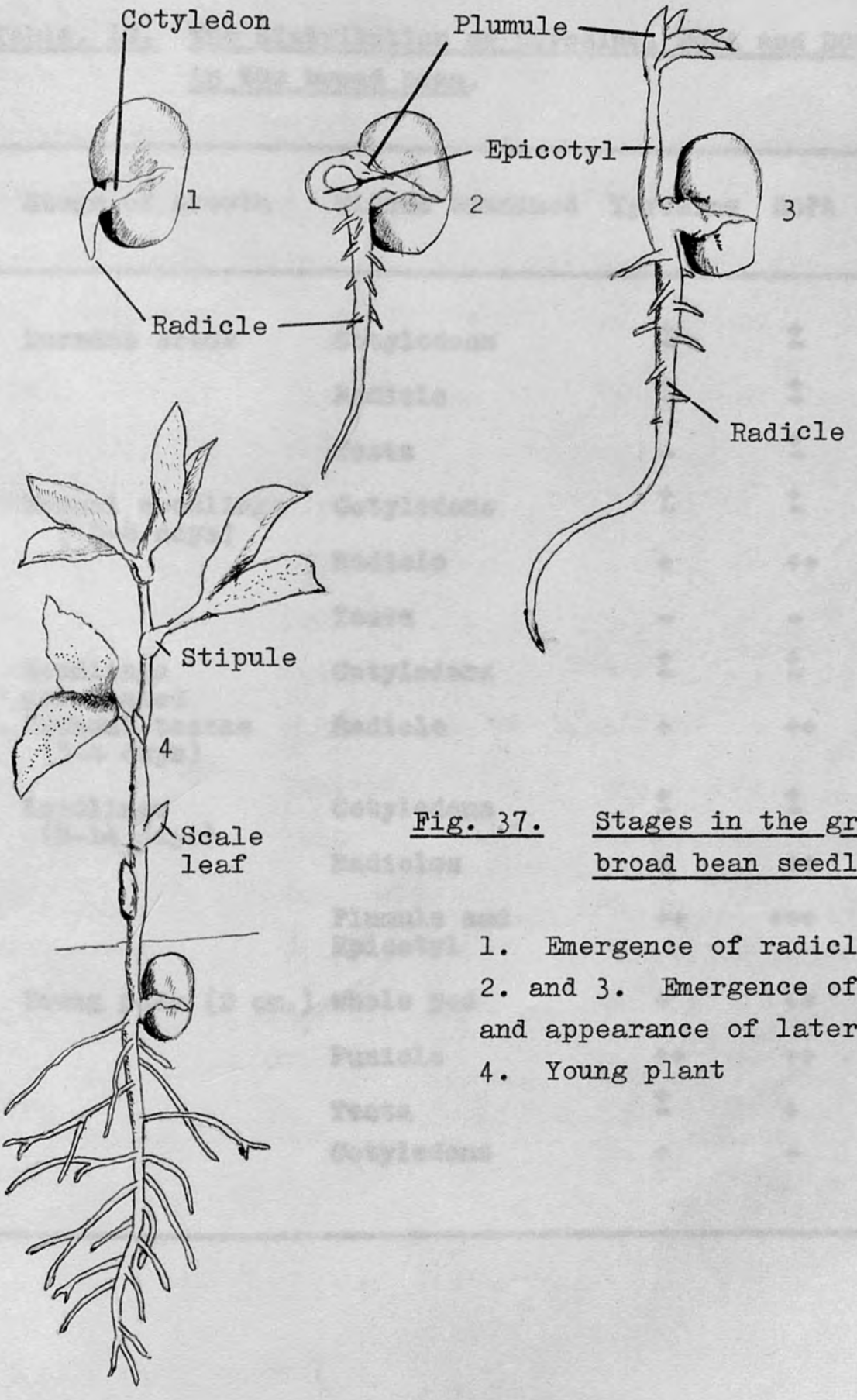


Fig. 37. Stages in the growth of broad bean seedlings.

1. Emergence of radicle
2. and 3. Emergence of plumule and appearance of lateral roots
4. Young plant

Table. 10. The distribution of tyrosine, DOPA and DOPA glucoside in the broad bean.

Stage of growth	Tissue examined	Tyrosine	DOPA	DOPA glu.
Dormant seeds	Cotyledons	±	±	+
	Radicle	±	±	±
	Testa	-	±	+
Normal seedlings (3-4 days)	Cotyledons	±	±	+
	Radicle	+	++	-
	Testa	-	-	±
Seedlings germinated without testas (3-4 days)	Cotyledons	±	±	+
	Radicle	+	++	-
Seedlings (8-14 days)	Cotyledons	±	±	+
	Radicles	+	++	-
	Plumule and Epicotyl	++	+++	-
Young pods (2 cm.)	Whole pod	+	++	-
	Funicle	++	++	+
	Testa	±	+	+++
	Cotyledons	+	-	-

Table 10 (continued)

Stage of growth	Tissue examined	Tyrosine	DOPA	DOPA glu.
Fully grown pods	Outer tissue of pod	+	++	-
	Inner tissue	+	++	+
	Funicle	++	++	+
	Testa	±	±	+++
	Cotyledons	+	±	±
	Radicle	+	+	-
As above, 3 weeks later	Outer tissue	+	++	-
	Inner tissue	+	++	+
	Funicle	++	++	-
	Testa	-	-	+++
	Cotyledons	-	-	±
Mature seeds	Cotyledons	-	+	+
	Radicle	-	+	-
	Testa	-	+	++

The key to the quantities in Table 10 is as follows:-

***	large amounts present
**	medium amounts
*	small amounts
*-	possible trace
-	none detected

It must be emphasised that these values have no strict quantitative significance and are only intended as a rough guide. Some conclusions, however, may be drawn from them.

(1) Glucoside formation takes place even in the earliest stages of pod development, the cotyledons, for example, never appear to contain DOPA in any quantity. It would seem that the plant ensures that free DOPA is excluded from this tissue, possibly because of an injurious effect.

(2) The glucoside disappears from the funicle as the seeds reach maturity.

(3) The glucoside diminishes considerably from the fully grown to the dormant seed. Some apparently is transferred into the cotyledons, but the fate of the remainder is unknown.

Nagasawa's hypothesis (5) was that the glucoside is hydrolysed in the seed coat, and the liberated DOPA converted into the brown pigments characteristic of that tissue. There seems to be little precedent for the formation of these pale brown pigments from DOPA, however.

The scheme which Nagasawa suggested is shown in Fig.38.

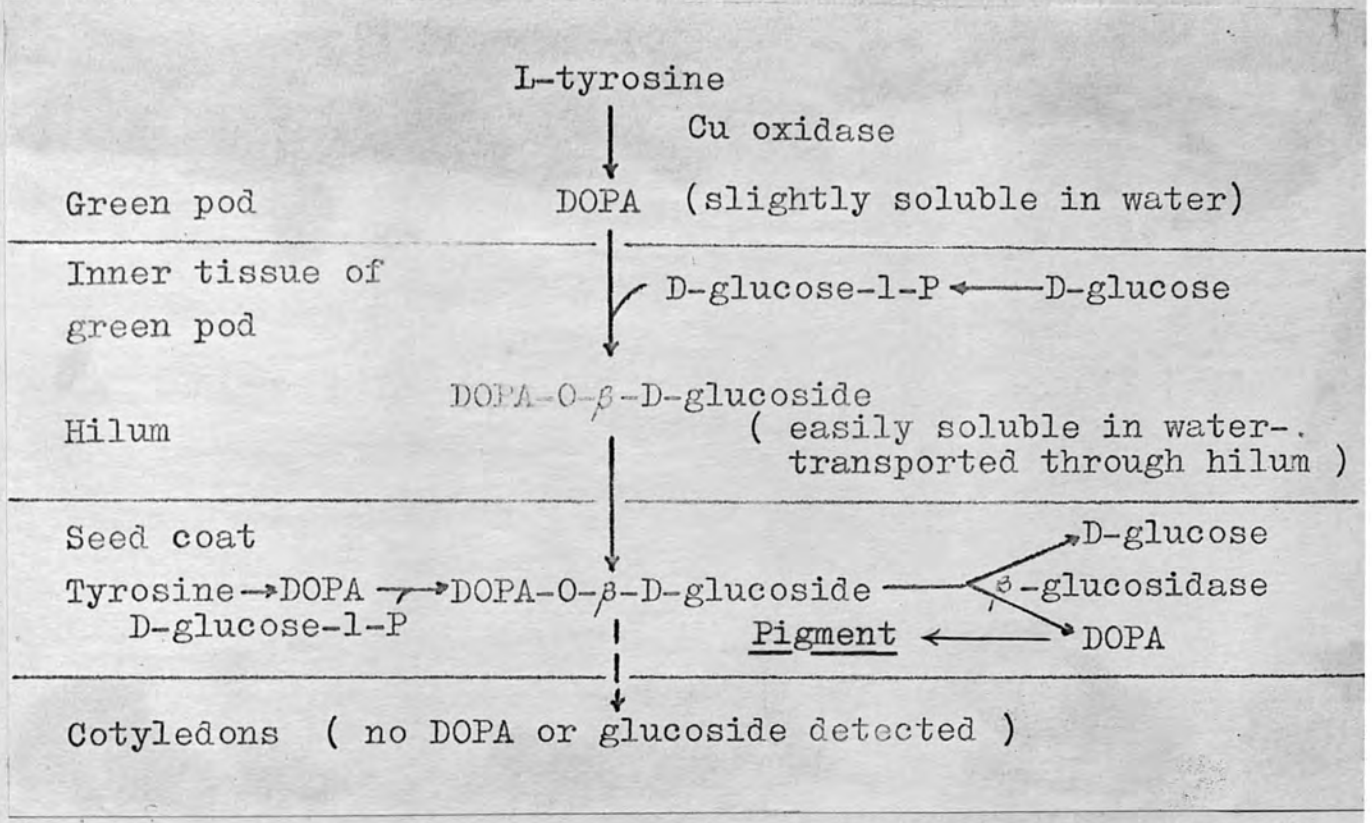


Fig. 38. Scheme to show the possible mechanism of browning in broad bean tissue (5).

The experimental evidence on which Nagasawa based this hypothesis seemed to be highly inadequate and was in any case founded upon work with mature seeds which contain large amounts of glucoside. Any appreciable hydrolysis which takes place must occur after this stage, because browning is not obvious until after then. The browning cycle in the Japanese variety, however, may be different from that observed with the 'Seville Giant Longpod'.

III 3. Quantitative studies on the distribution of DOPA in the broad bean.

Although the survey reported in the previous section gave an indication of the distribution of DOPA in the broad bean, it was obviously desirable to record the actual concentrations of this compound in each tissue throughout the growth cycle. This necessitated devising suitable quantitative procedures.

(a) Colorimetric methods.

A variety of colour reactions have been described for the detection of compounds like DOPA and a few have been found which can be applied quantitavely. The merits of such methods have been reviewed by Barker et al (139)

Two of the methods investigated by Evans and Raper (140), specifically for the determination of DOPA were based on the observations that o-dihydroxy compounds such as DOPA, produce a yellow-brown complex with ammonium molybate, or a red oxidation product when treated with Lugol (iodine) solution.

Methods based on either of these procedures were found to give good proportionality over a wide range of DOPA concentrations, but the molybdate method was found to be unreliable in the presence of ascorbic acid. As this compound is thought to co-occur with DOPA in plant tissues, the iodine method was chosen for further study.

Determination of DOPA by the iodine method.

The development of a red colour when adrenaline is oxidised with iodine solution is often referred to as the Vulpian reaction, after the original discoverer (141).

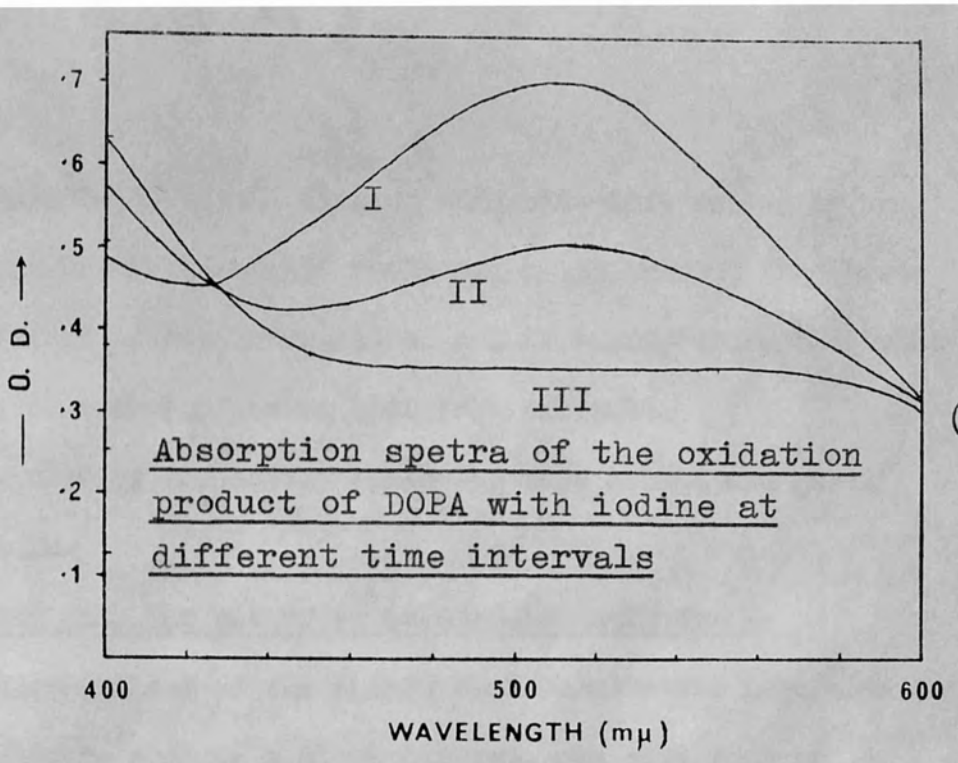
Schild (142) and von Euler (143) used it for the quantitative estimation of adrenaline itself, and later Evans and Raper applied it to the determination of DOPA. Kendal (144) improved the method by employing a Spekker absorptiometer instead of visual colour matching. Kendal's method, however, required 8 ml. of sample for each determination, which seemed rather excessive for the present investigation, so his method was scaled down by a factor of ten, and a spectrophotometer used in preference to an absorptiometer. This latter change necessitated determining the absorption maximum of the red oxidation product. Oxidation of a sample of DOPA carried out and the spectrum of the product followed at various time intervals. The three spectra shown in Fig. 39 (a) were obtained over a period of about 6 mins. and show the rapid diminution of the red product. They also show that the absorption maximum occurs at about 515 mμ. This value was, therefore, used in all determinations.

The modified method was found to give strict proportionality over the entire range of DOPA concentrations studied. The standard graph is shown in Fig. 39 (b).

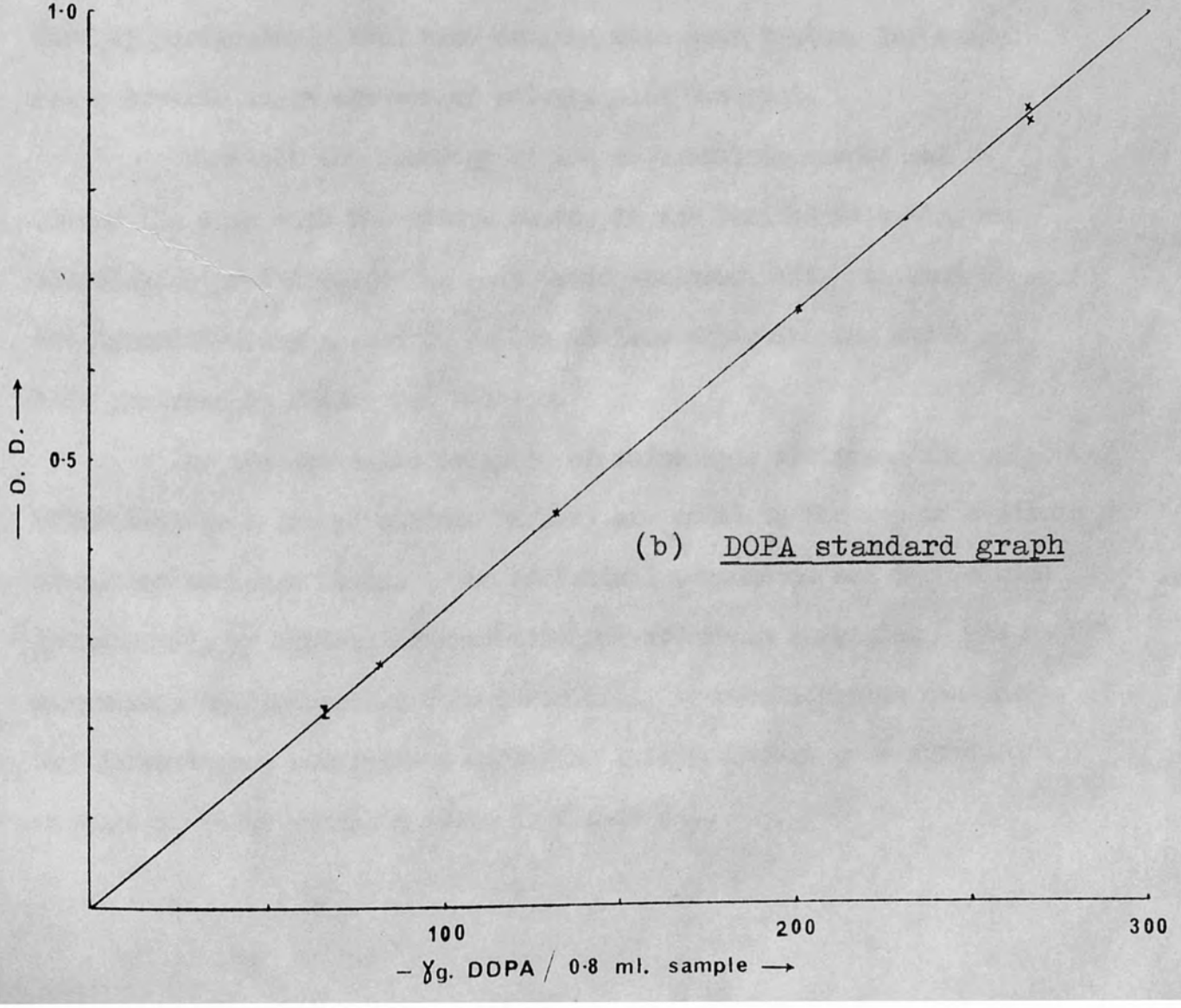
A number of compounds are known which interfere with this

Fig. 39

Determination of DOPA by the iodine method



(a) Absorption spetra of the oxidation product of DOPA with iodine at different time intervals



(b) DOPA standard graph

method, but comparison of determinations made with this method by an independent method using amino-acid analyser, on an extract from broad bean tissue, gave very close correlation, and it seems, therefore, that it can be used with some confidence with such extracts.

The results of the survey employing this method are given in Tables 11 and 12.

(b) Determinations with the aid of an amino-acid analyser.

It was recognised at the outset that coloimetric determinations on any complex mixture such as a plant extract, can give rise to misleading results due to (generally unknown) interfering substances. This is particularly true when dealing with bean testas, for example, which contain large amounts of polyphenolic material.

To check the accuracy of the colorimetric method and to extend the work with the mature seeds, it was decided to employ an alternative procedure. The amino-acid analyser, which is both accurate and discriminating seemed to be the obvious choice. The model was that produced by Bender and Holbein.

In the automatic analysis of amino acid mictures, the samples (dissolved in a low pH citrate buffer) are added to the top of a column of cation-exchange resin. The individual components are then eluted successively by buffers of increasing pH and ionic strength. The eluted components are determined colorimetrically by reaction with ninhydrin. The chromatogram obtained by employing this procedure on a standard mixture of amino acids is shown in Fig.40 (a).

Table 11. DOPA content of etiolated V. faba tissues.

Days growth	Tissue examined	No.	Total wt.(g.)	Total vol. (ml.)	Aliquot (ml.)	O.D. zero time	TOTAL DOPA(mg.)	DOPA/g. fresh wt.	DOPA/tissue (mg.)
2	Radicle(½ cm.) and plumule	120	5.28	205	0.8	0.073	5.61	1.06	0.05
3	Radicle(1 cm.) and plumule	55	5.99	205	0.8	0.224	17.22	2.87	0.31
4	Radicle(2 cm.) and plumule	39	9.00	208	0.8	0.569	44.38	4.93	1.14
5	As above,plumule and root hairs emerging	26	8.83	208	0.8	0.661	51.56	5.84	1.98
7	Cotyledons	-	17.75	300	0.8	0.153	1.72	0.10	-
	Radicle	23	8.40	207	0.8	0.513	39.82	4.74	1.73
	Plumule and (a) epicotyl	23	8.91	208	0.8	0.900	70.20	7.89	3.05
	(5 cm.) (b)	25	9.21	308	0.8	0.641	74.03	8.05	2.96

Table 11 (continued)

Days growth	Tissue examined	No.	Total wt.(g.)	Total vol. (ml.)	Aliquot (ml.)	O.Dx zero time	Total DOPA(mg)	DOPA/ g fresh wt.	DOPA/tissue (mg.)
8	Whole root	24	10.00	209	0.8	0.573	44.91	4.49	1.87
	Whole shoot	24	9.75	257	0.8	0.841	81.05	8.31	3.34
9	Epicotyl	18	8.81	258	0.8	0.581	56.21	6.38	3.12
10	Plumule	22	5.25	204	0.8	0.830	63.49	12.09	2.89
	Epicotyl	16	10.32	209	0.8	0.668	52.35	5.07	3.27
	Radicle	14	9.50	208	0.8	0.556	43.36	4.56	3.10
12	Cotyledons	-	24.81	130	0.8	0.074	3.61	0.14	-
	Epicotyl	-	15.82	90	0.4	0.934	63.07	3.98	-
	Whole root	-	13.75	120	0.4	0.626	56.38	4.10	-

Table 12. DOPA content of field-grown broad bean tissues.

Stage of growth	Tissue examined	Total wt. (g)	Total vol. (ml.)	Aliquot (ml.)	O.D. (zero time)	Total DOPA (mg)	DOPA/ g fresh wt
Plants 15 cm.	Leaves	16.14	214	0.8	1.380	110.74	6.86
	Stem	7.01	110	0.8	0.531	21.90	3.12
	Roots	4.20	153	0.8	0.126	7.23	1.72
Plants 25 cm.	Upper leaves	17.58	215	0.8	0.725	58.45	3.32
	Lower leaves	14.41	213	0.8	0.711	56.79	3.94
	Upper stem	11.75	210	0.8	0.226	17.80	1.51
	Lower stem	21.05	219	0.8	0.203	16.67	0.79
Plants 50 cm. (at flowering stage)	Upper leaves	6.07	205	0.8	0.226	17.37	2.86
	Lower leaves	9.92	209	0.8	0.271	21.23	2.14
	Flowers (a)	2.51	202	0.8	0.347	26.28	10.47
	(b)	5.24	204	0.8	0.813	62.19	11.86

Table 12 (continued)

Stage of growth	Tissue examined	Total wt.(g)	Total vol. (ml.)	Aliquot (ml.)	O.D. (zero time)	Total DOPA (mg)	DOPA/ g fresh wt.
Mature plant (during pod formation)	Pods 4-8 cm.	15.83	200	0.4	0.881	132.15	8.35
	Pods 10-14 cm.	24.40	200	0.4	0.813	121.95	5.00
	Pods 20 cm.	26.25	223	0.8	0.515	43.06	1.64
Pods fully grown	Pods 25 cm. (a)	24.16	221	0.8	0.530	44.12	1.83
	(b)	9.14	208	0.8	0.219	17.08	1.86
	Funicle	2.03	122	0.8	0.135	5.67	2.79
	Upper stem	26.57	223	0.8	0.212	17.73	0.65
	Middle stem	28.82	225	0.8	0.107	9.03	0.32
	Lower stem	32.54	229	0.8	0.062	5.24	0.16
Old plants (pods blackening)	Old leaves	3.53	100	0.8	0.253	9.77	2.76
	Very old leaves	4.68	104	0.8	0.322	12.56	2.68
	Cotyledons	13.41	112	0.8	0.010	0.39	0.03

The sequence in which the amino acids are eluted from the column is dependent upon the elution procedure, but under standard conditions the retention time on the column is constant and a valuable diagnostic property of any particular amino acid.

Before undertaking the analysis of broad bean extracts it was first necessary to establish the retention time of both DOPA and the glucoside by applying known mixtures to the column. The results of this procedure are shown in Fig. 40 (b).

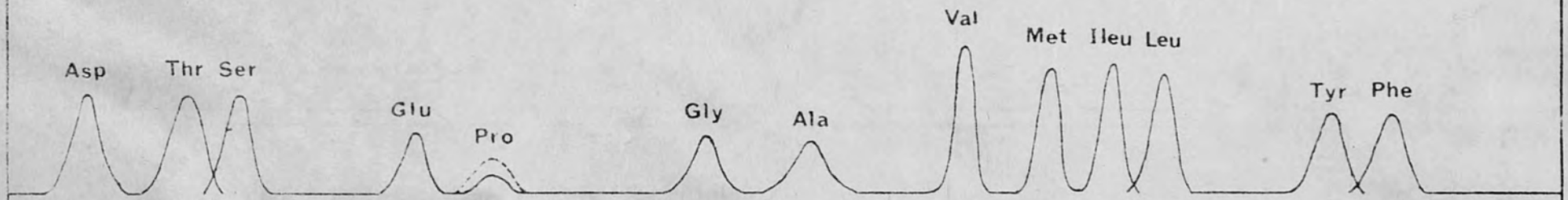
Unfortunately, there was only limited access to the analyser and so there had to be some discrimination in the tissues chosen for analysis. Finally, the extracts from 9-day epicotyls, mature funicles and mature testas were chosen. The first chosen was an example of a tissue relatively free of interfering substances, whilst the latter two were chosen, partially because they contained interfering substances which made the colorimetric procedure unusual, but also because of the high concentrations of tyrosine and DOPA glucoside, which could not be determined by other methods.

The results of the analyses are shown in Fig. 41. Their validity was checked by examining the extracts on paper chromatograms and electrophoretograms, after spraying with ninhydrin and diazotized p-nitroaniline/NaOH. It was thus possible to establish, for example, that the glucoside was absent from funicles and DOPA was completly absent from the seed coat.

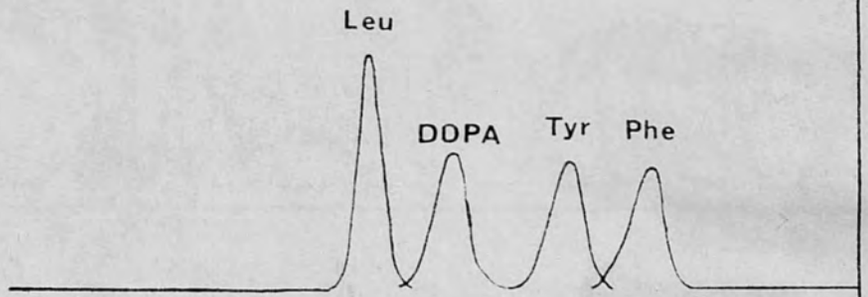
The quantities of tyrosine, DOPA and DOPA glucoside found in each of the tissues are given in Table 13.

Fig. 40. The separation of standard amino-acid mixtures by ion-exchange column chromatography

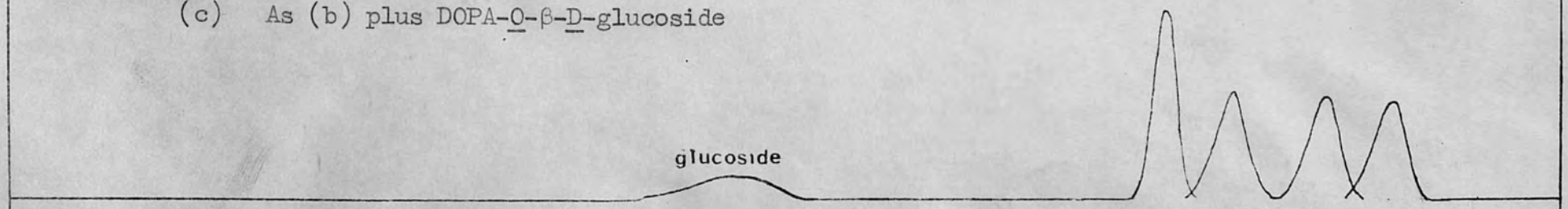
(a) 'EEE' standard amino acid mixture



(b) Mixture of leucine, DOPA, tyrosine and phenylalanine



(c) As (b) plus DOPA-O-β-D-glucoside



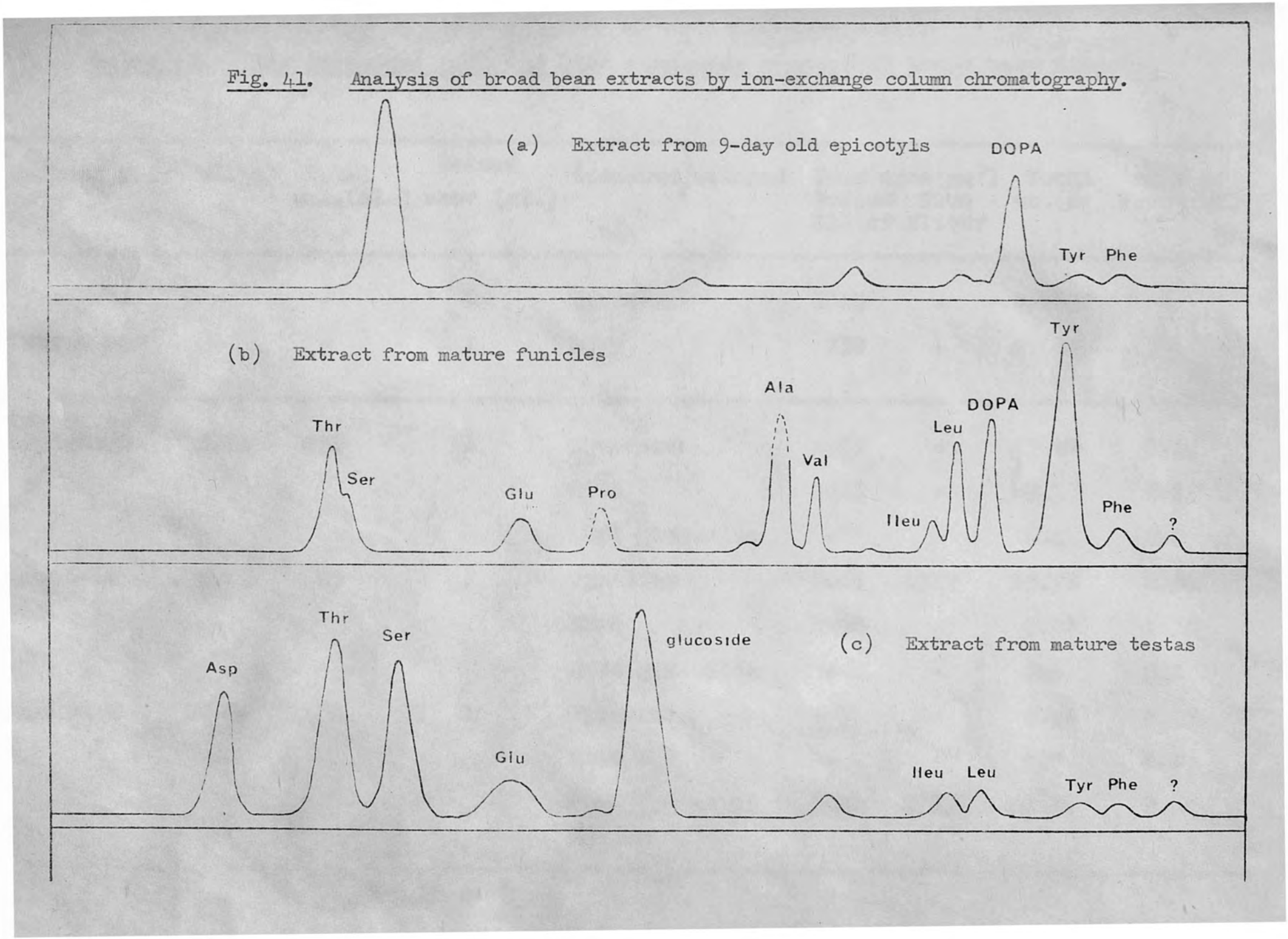


Fig. 41. Analysis of broad bean extracts by ion-exchange column chromatography.
(a) Extract from 9-day old epicotyls
DOPA
Tyr
Phe
(b) Extract from mature funicles
Thr
Ser
Glu
Pro
Ala
Val
Ileu
Leu
DOPA
Tyr
Phe
?
Asp
Thr
Ser
Glu
glucoside
(c) Extract from mature testas
Ileu
Leu
Tyr
Phe
?

Table 13. The tyrosine, DOPA and DOPA glucoside content of broad bean tissues.

Extract from	Wt.(g)	Total vol.(ml.)	Volume used (ml.)	Compound studied	Peak area(mm^2) Yellow filter	Peak area(mm^2) Blue filter	Total wt.(mg)	mg / g fresh wt.
Standard	-	-	1	Tyrosine	1005	-	0.1822	-
Standard	-	-	1	DOPA	932	-	0.1926	-
Epicotyl	8.81	258	1	Tyrosine	73	-	3.43	0.39
F				DOPA	1012	-	53.96	6.12
				DOPA glucoside	-	-	0.0	0.0
Funicle	5.75	25	1	Tyrosine	3029	1317	13.73	2.38
				DOPA	1556	-	7.94	1.38
				DOPA glucoside	-	-	0.0	0.0
Testa	20.00	72	1	Tyrosine	91	-	0.59	0.03
				DOPA	-	-	0.0	0.0
				DOPA glucoside	3526	1533	50.18	2.51

PART IV

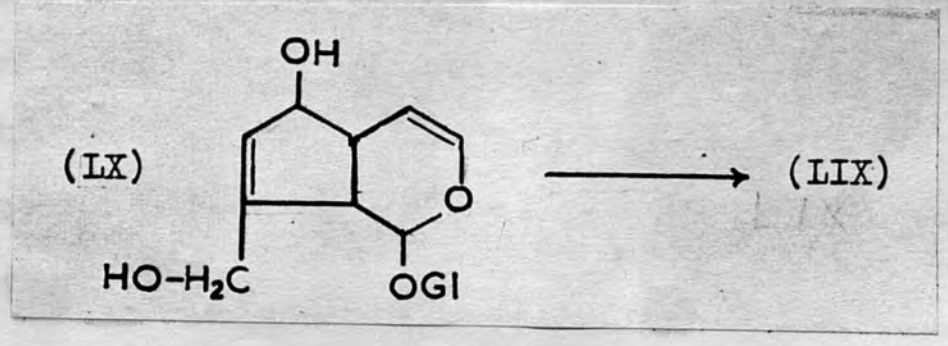
The formation and nature of dark pigments in plants with particular reference to those found in the Papillionaceae

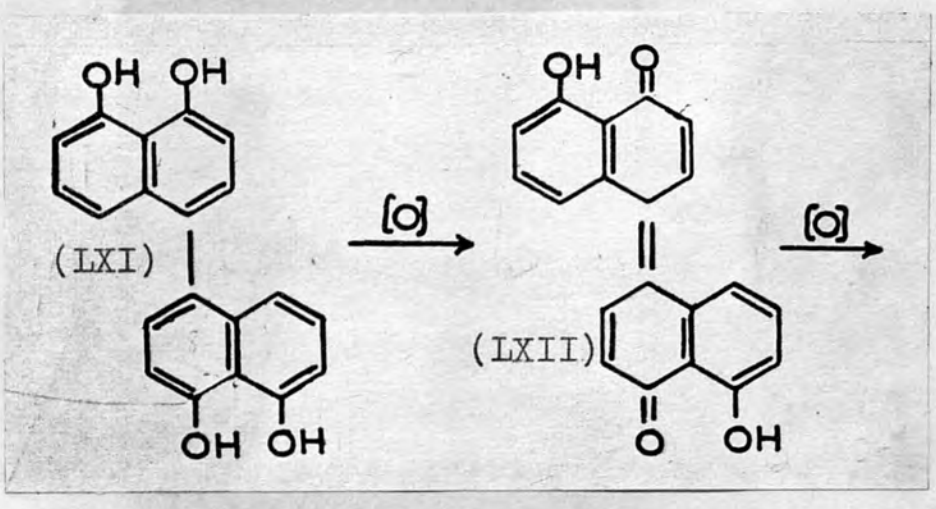
IV 1. Dark coloured plant pigments.

Although dark brown and black pigments are common in animal tissues, they are far less obvious in higher plants and fungi. They are, nevertheless, a characteristic feature of species such as Lathyrus niger and Aspergillus niger, the names obviously being derived from this fact.

The pigments present in the seeds of melons, sunflowers etc. were shown by Nicolaus and Piatetelli to be derived from catechol (Table 1), and are thus true phytomelanins within our definition, but many of the black pigments which have been investigated do not appear to be similar in type . Thus the black pigment present in the leaves and fruit of the Japanese variegated laurel, Aucuba japonica, has been shown to be derived from aucubigenin (LIX). In the actual tissues of the plant this compund exists as the glucosoide aucubin (LX) which is hydrolised by β- glucosidase just prior to oxidation (145).

The black pigment present in the fungus Daldinia concentrica is thought to be derived from LXI by oxidation, 4,9-dihydroxyperylene-3,10-quinone (LXII) being an intermediate in the reaction.(120).





There have been no reports of phytomelanins corresponding to the animal eumelanins, which are derived from tyrosine or DOPA even though several plant species such as Vicia faba, Baptisia australis, Cytisus scoparius and Musa spp. are known to contain high concentrations of DOPA or dopamine in tissues which subsequently blacken.

As DOPA and related compounds and tissue blackening are most commonly observed in species belonging to the Papilionaceae, the present study was mainly limited to this family. The features which have been investigated included the biogenesis, histological appearance, and chemical and physical properties of the pigments.

IV. 2. The biogenesis of dark pigments in Papillionaceous species.

Very little work appears to have been done on the sequence of reactions leading to the observed blackening in plant tissues. Indeed, without radioactive tracer compounds, the approach is limited to recording the fluctuations in the concentrations of likely precursors and attempts to draw conclusions from this. Nevertheless, valuable information can be gained by such a method.

(a)

(b)

Fig. 42. Examples of blackened pods

(a) Vicia faba equina

(b) Astragalus cicer

Many species belonging to the Papillionaceae exhibit marked pod and/or seed blackening, and the following were selected for study. Vicia angustifolia, V. faba, V.hirsuita, V. sativa, V. sepium, Astralgus cicer, Lupinus polyphyllus, Baptisia, Lathyrus montanus and Laburnum vulgare.

(a) Blackening of pods

In several of the above species, blackening is limited to pod tissue, as shown in Fig 42. DOPA had been detected in high concentration in V. faba and B. australis and it seemed possible that it might be responsible for the blackening in the other species. Attempts to detect DOPA in the green pods of other species, however, were mainly unsuccessful, although in many cases tyrosine was shown to be present. This posed the question whether DOPA formation took place in the mature pods just before the observed blackening.

In order to investigate this, extracts were prepared from pods which were partially blackened or had just turned black, and examined on paper chromatograms and electrophoretograms.

The results for many of the species such as Vicia angustifolia, V. sepium, V. sativa, Astragalus cicer and Lupinus polyphyllus were remarkably consistent. In each case large quantities of tyrosine and lesser quantities of DOPA were detected in the extracts. This can be seen from the colour photograph of a chromatogram of two of the species shown in Fig. 43.

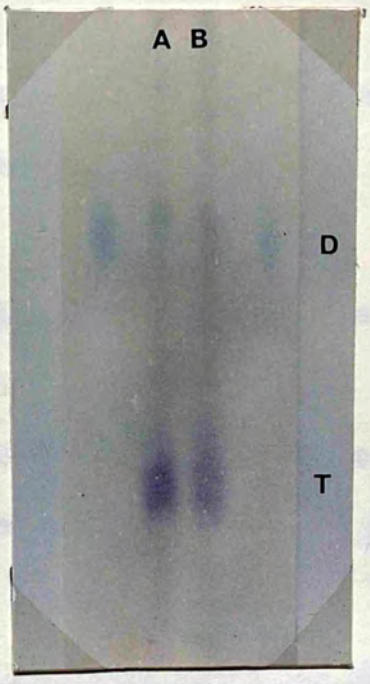


Fig. 43. Colour photograph of a chromatogram showing the phenolic compounds present in extracts (70 % aqu. ethanolic) of Vicia sepium (A) and Lupinus polyphyllus (B) pods during the blackening process. The presence of relatively large amounts of tyrosine (T) and of DOPA (D) is apparent.
Development by the descending technique in Solvent C. Location by diazotised p-nitroaniline / NaOH.

This information seemed to lend considerable support to the idea that the pigments formed by these species are true tyrosine or DOPA based phytomelanins.

Although minute amounts of tyrosine and DOPA were also detected in blackening pods of Vicia hirsuita, much greater quantities of tyramine and dopamine were found to be present. In this respect V. hirsuita appears to resemble Cytisus scoparius more closely than the other Vicia species.

Repeated attempts to detect compounds which might be responsible for the pod blackening in Lathyrus montanus were without success.

IV 3. Eumelanins and plant pigments under the microscope.

Laboratory produced melanins are amorphous in structure, but the numerous studies which have been made on the histological appearance of eumelanins(have shown that in mammalian tissues) the pigment is produced and deposited in specialised cells known as melanocytes. In these cells the pigment is usually combined with protein to form discrete granules.

In pigmented tissue the granules are formed in the melanocytes and some are transported into adjoining cells. Fig. 44 shows eumelanin granules in human epidermal cells adjacent to melanocytes.

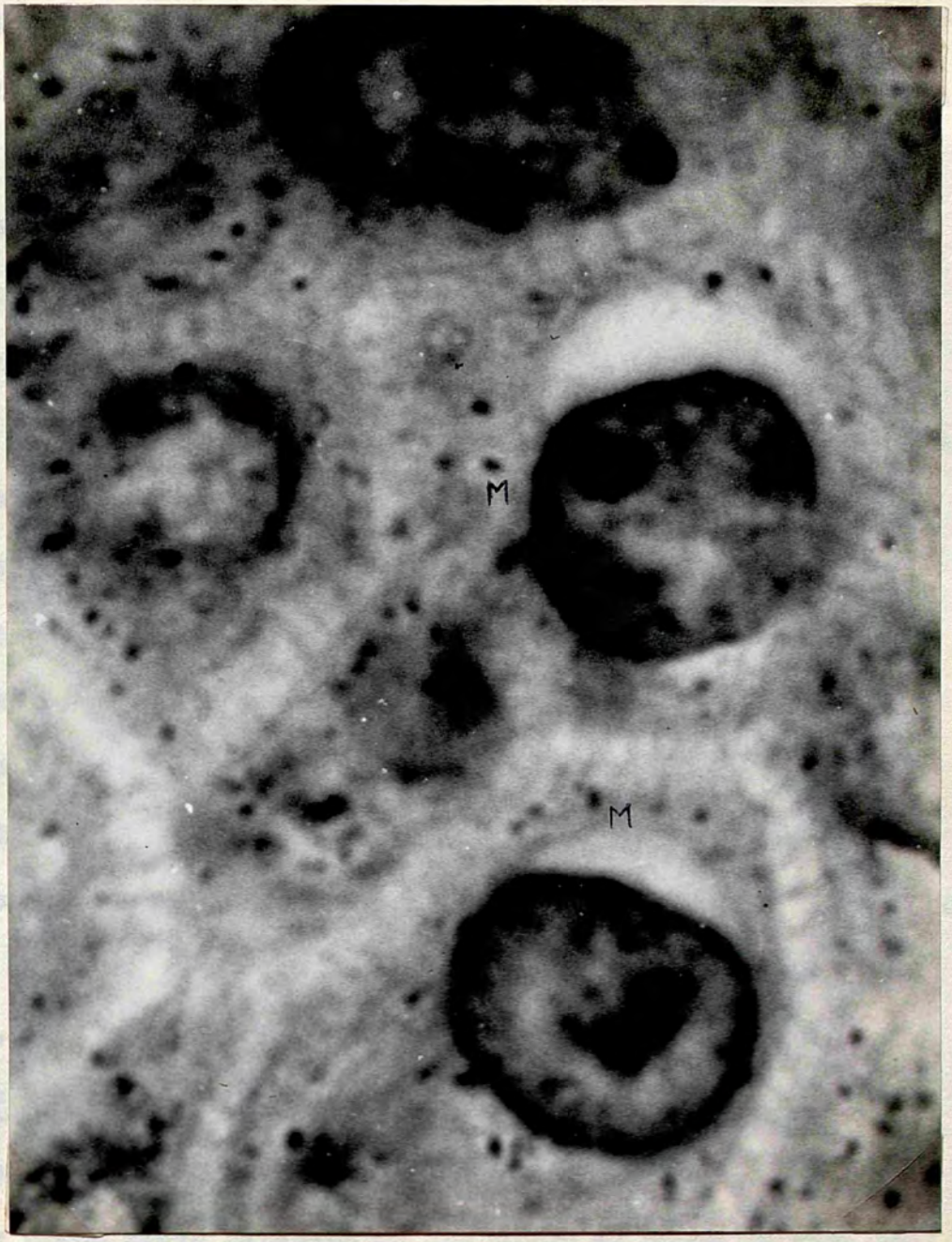


Fig. 44. Eumelanin granules (M) in human epidermal cells

(X 8000)

Mason (146) found that granules from human skin, beef choroid and Harding-Passey melanomas appear, under the electron microscope, as formed elements with a limited number of rod-like and spherical forms. The dimensions of such elements of the order of 0.1-2 microns.

In the pigmented hair of humans, sheep, dogs, horses etc. Laxer (117) has shown that the granules have a compound structure in which a sheath of eumelanin is deposited on a colourless matrix. He found that the pigment could be removed with hydrogen peroxide to leave a colourless granule.

There have been similar studies on the form of plant pigments and so this aspect was included in the present work. Investigations were limited to species in which tyrosine or DOPA had been demonstrated. Sections of blackened tissues were sectioned by hand and mounted in water or 'Euparol' mounting medium for examination.

Broad bean flowers.

The flowers of the broad bean are white in colour except for two dark brown spots situated on the wings as shown in Fig. 45. The spots are present throughout the life of the flower, and it is thought that they serve as insect guides to help in cross-pollination.

Histological examination of the flower wings revealed that they have a three-layered, sandwich like structure with the pigmented cells only occupying the centre layer.

Fig. 45. Pigment formation in broad bean flowers

If the pigment is a true phytomelanin then one might class these cells as melanocytes. It is interesting to note that cells adjacent to the melanocytes and those above and below were completely pigment free.

The pigment in the cells had no structural organisation, being rather in solution or in colloidal suspension. In this respect, therefore, the broad bean melanocytes differ from their animal counterparts. Another important difference is the colour of the pigment itself, which is dark brown in the flowers but black in animals.

Pigmentation in leguminous pods.

Pigmentation in the pods of leguminous species is quite common, but unlike the flower pigmentation just discussed, it does not occur until the pods have ripened, and appears to coincide with the death of that tissue. It is difficult to say, in fact, whether the onset of pigment formation results from a breakdown in cellular organisation, or whether melanin formation plays an integral part in the death of the cells, perhaps by combining with enzyme protein. As most of the species which show pod blackening disperse their seeds by the explosive opening of the dead, dry pods, this could be an important function of the pigment.

The dry pods were found to be difficult to section and the assessment of results was correspondingly more arduous. It was generally found, however, that pigmentation was far less ordered than in animal tissues. The melanin was mainly located in a seemingly amorphous form in dead cells.

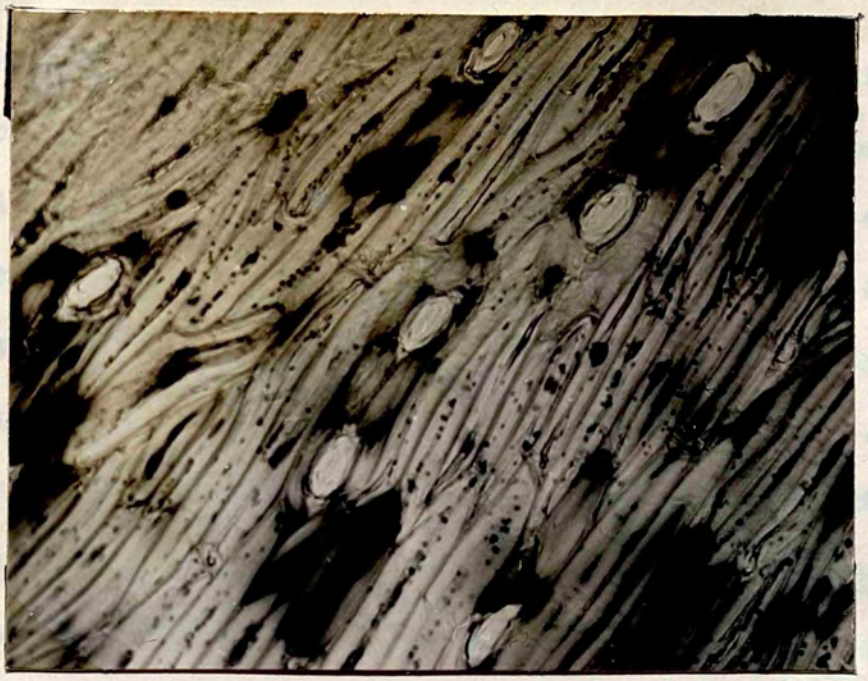
In Lupinus polyphyllus pods the pigment existed as large dense-black aggregates; in Cytisus scoparius as much paler parallel bands of no general structure, but in most species the dark brown or black pigment was located in collapsed cells and had little structural organisation. A diagramatic representation of this is shown in Fig.47 (a). Species of this type included Vicia angustifolia, V. sepium, V. hirsuita, Astragalus cicer, Baptisia australis, and Lathyrus montanus. In certain cases, however, some structural organisation of pigment 'granules' was observed, and two examples are shown in Fig. 46.

In most of the species examined, other pigments were found which contributed to the observed colour of the pods. Vicia sepium pods,for example, contained a red, presumably anthocyanin-type pigment in addition to melanin, and nearly all contained additional brown polyphenolic material.

Seed-coat pigmentation.

Many leguminous species produce seeds which appear black. Sometimes this occurs in addition to pod blackening, but it also occurs in species in which the pods do not turn black.

Chromatographic examination of seed-coat extracts failed to show the production of tyrosine and DOPA as had been found with pod tissues, and so the source of the pigment remained a mystery. The reason for this failure soon became apparent when sections of the seed-coats from apparently black seeds were examined under the microscope.



(a)

(b)

Fig. 46. Surface sections of blackened pods

(a) Vicia sepium

(b) Vicia faba equina

The dark areas of the seeds of a large number of Vicia species were found to be due to dark blue or violet anthocyanins, melanin-like pigments being completely absent. The jet-black colour of the seeds of Vicia dumetorum, for example, was found to be due to a combination of a violet anthocyanin, chlorophyl and polyphenolic material. This is shown diagramatically in Fig.47(b). Melanin-containing structures, however, were tentatively indentified in the seed-coats of Lupinus polyphyllus and various Laburnum species. Tyrosine was also detected in the former. The black seeds of Laburnum vulgare result from a combination of melanin and a violet anthocyanin in the seed-coat. A photomicrograph of the seed-coat is shown in Fig. 48. Certain other Laburnum species which lack anthocyanins in the seed-coat have much paler coloured seeds.

As tyrosine and DOPA were not identified in the seeds of Laburnum species the source of the pigment is unknown. However, a compund tentatively identified as P_1 was isolated from the seed-coat of L. vulgare, and if this is the case then one might visualise the formation of melanin taking place after β- glucosidase action on this compound.

The only other species showing black seed-coat pigmentation to be examined was the runner bean (Phoseolus vulgars) . Sections taken from the seed-coat of a dormant seed have the appearance shown in Fig.47(c), in which black areas are found together with russet anthocyanins.

(a) The distribution of black pigment and brown 'polyphenolic material' commonly observed in blackened pods of leguminous species

(b) The distribution of anthocyanins and plastid pigments in the testas of Vicia dumetorum seeds

(c) Section of the dark area of Phaseolus vulgaris seeds

Fig. 47 Histological appearance of black tissues

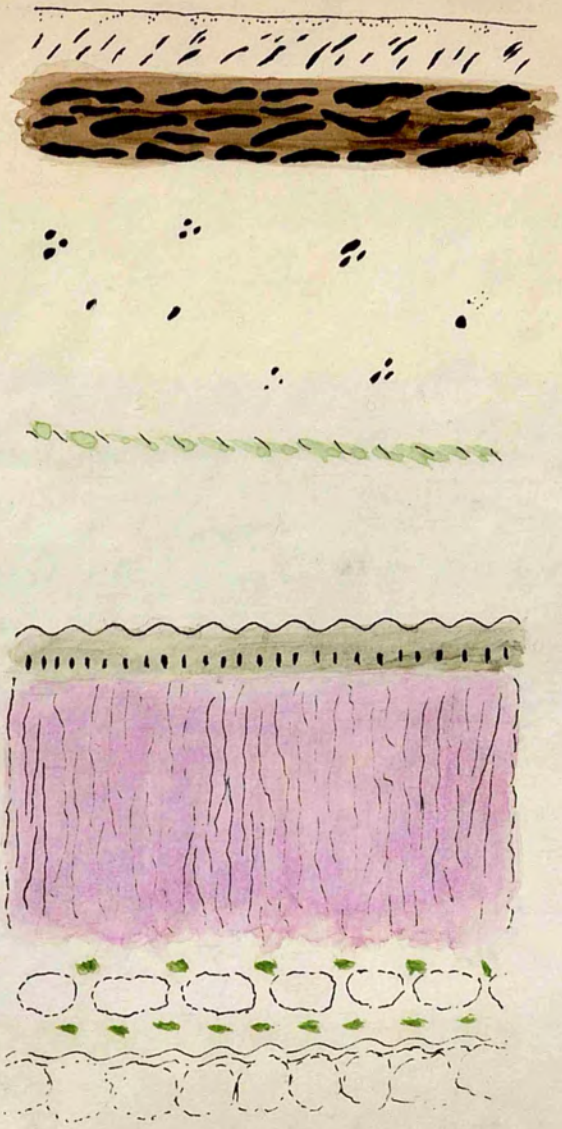


Fig. 47

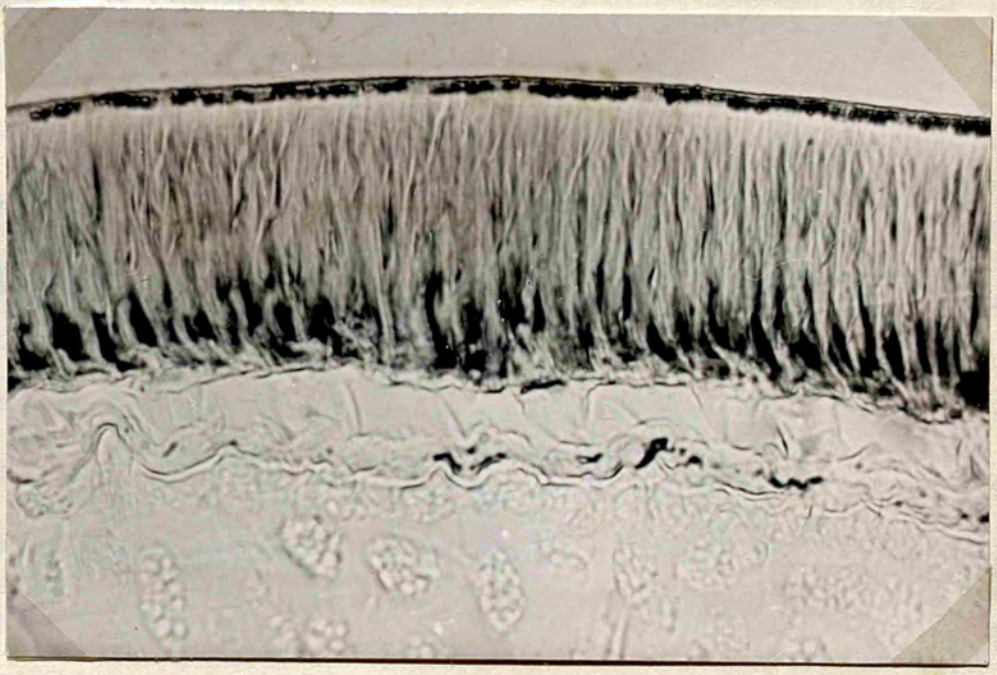


Fig. 48. Blackening in Laburnum vulgare testa

Examination of sections taken throughout the ripening process of the seeds, reveals that the black pigmentation follows blue pigmentation in the same regions, and it is suggested therefore that it is not melanic in nature.

IV 4. Isolation of pigments.

As large quantities of pigment were required for degradative studies, only a few readily available species were examined. These were the pods from V. angustifolia, V. faba equina, Baptisia australis, Lupinus polyphyllus and Astragalus cicer; the flowers of V. faba major, and banana peel. The latter was included a a possible source of a dopamine-based pigment.

The isolation of pigments from leguminous pods was found to be extremely difficult as they occur in association with large quantities of unwanted material such as lignin, polyphenolic compounds and other cell debris.

After trying a number of methods for purifying the pigments, the following method, which involved both chemical and physical treatment, was found to be the most satisfactory:

(i) Dry, pigmented pods were soaked in distilled water containing a little detergent for about 3 days. This softened the dead outer tissue, and facilitated the removal of extraneous materials.

(ii) The pigment-containing outer layers were carefully scraped off from the hard inner tissue with a knife, and any fibrous elements discarded.

(iii) An acetone 'powder' of the pooled outer tissue was prepared. The acetone powders were grey in colour, so that the white fibrous tissue was easily seen and could be removed with forceps. This simple procedure proved to be extremely valuable and was employed at the end of each chemical step.

(iv) The acetone powder from above was transferred to a porous thimble which was placed in a Soxhlet apparatus and extracted successively with methanol, pyridine, ethanol, acetone and ether. Each extraction was continued until no further coloured material could be removed.

(v) The air-dried solid from (iv) was heated under reflux with 6N-HCl for 5 days, after which the suspension was filtered under reduced pressure and the residue washed with several changes of water, methanol, ethanol, acetone and ether and then air-dried.

In the case of Astragalus cicer pods and the flowers of the broad bean which had sofer and more readily extractable tissues, steps (i) and (ii) were omitted.

For comparative purposes, biosynthetic melanins were also prepared by the oxidation of L-tyrosine and catechol with a potato phenolase preparation.

IV. 5. Characterisation of pigments.

The characterisation of a particular pigment as a melanin is a somewhat difficult operation as no simple tests are available for this purpose. In general, if a dark pigment is insoluble in ordinary solvents, is decolourised by oxidising agents such as hydrogen peroxide, and reduces ammoniacal silver nitrate it is deemed to be a melanin. These properties, however, are also exhibited by other polyphenolic materials. Eumelanins and biosynthetic melanins derived from tyrosine, DOPA or dopamine have nitrogen contents between 6 - 10%, whereas those derived from catechol have a much lower value (ca. 1%), and this again is a useful distinguishing feature.

The nirtogen content of the plant pigments and biosynthetic melanins are recorded in Table 14 . The low values of the plant pigments are rather surprising, being of the same order as those reported for the catechol derived melanins.

The most satisfactory means for characterising pigments is to subject them to degradative procedures such as oxidation or fusion and study the products. As the alkali fusion technique had proved to be extremely valuable in the hands of other workers, it was chosen for the present study.

Alkali fusion of pigments.

Samples of each of the pigments were degraded by the alkali fusion technique of Piattelli and Nicolaus (85). After cooling, the fused mass was taken up in $Na_2S_2O_4$ solution and acidified with acetic acid.

Table 14. General properties of melanin preparations.

Specimen	Appearance	N (%)
Plant pigments		
Astragalus cicer (pod)	black-brown powder	1.17
Baptisia australis (pod)	black powder	1.55
Lupinus polyphyllus (pod)	black powder	1.33
Musa sp. (peel)	black powder	1.47
Vicia faba		
(i) flowers	dark brown powder	2.12
(ii) pod	dark brown powder	1.31
(iii) seed-coat polymer	dark brown powder	1.05
Vicia angustifolia (pod)	dark brown powder	1.39
Biosynthetic melanins		
Tyrosine (potato tyrosinase)	black powder	7.09
As above, after fusion	black powder	6.73
Tyrosine (mushroom)	black powder	5.88
Dopamine (potato)	black powder	6.75
Catechol (potato)	black powder	1.11

Insoluble material was removed by centrifugation and the centrifugate extracted with peroxide-free ether. The ether extract was then evaporated to dryness under reduced pressure and the residue dissolved in a few drops of water.

Phenolic products were examined on paper chromatograms (Solvents A and D) and on paper electrophoretograms (using 0.2 M sodium acetate or sodium molybdate (pH 5.2) as electrolytes). Compounds were located on the papers with U.V. light, diazotised p-nitroaniline / NaOH and $AgNO_3$ / NaOH spray reagents.

Standards of catechol, protocatechuic acid, salicylic acid, 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid were applied to each of the papers. The latter two compounds were prepared from a sample of 5,6 dibenzyloxyindole-2-carboxylic acid (generously donated by Professor Nicolaus) by hydrogenolysis in the presence of a palladium catalyst. To produce the 5,6 dihydroxyindole it was first necessary to decarboxylate the dibenzyl derivative by heating to 200°.

In trial experiments it was discovered that the plant pigments when subjected to this procedure gave rise to a large number of unidentified products. For purposes of comparison therefore, they were compared with the products obtained from a biosynthetic tyrosine melanin. The low nitrogen content might suggest that the pigments are of the catechol type, and catechol and protocatechuic acid could be detected amongst the fusion products.

However, these could equally well have been formed indole units, and in fact, 5,6-dihydroxyindole was identified in the products from the pigments of V. faba flowers and V. angustifolia and L.polyphyllus pods.

In many respects, the fusion products from the plant pigments were similar to those from the biosynthetic melanin, although there were some differences in the range of compounds produced and the relative amounts. The plant pigments invariably produced quantities of soluble 'polyphenolic material' on fusion which partially obscured chromatograms and electrophoetograms and made interpretation difficult, and nearly thewhole was solubilised by the procedure. The biosynthetic melanin, by contrast, produced little material of this type and most of the pigment could be recovered and resubjected to the same procedure.

These findings show quite conclusively that the pigments produced by Papillionaceous species differ from the true indole-type melanins found in animals. This brings one back to the suggestion of Nicolaus and Piattelli that most phytomelanins are formed by the in vivo polymerisation of catechol.

Some of the reported findings are consistent with this view, but catechol was not detected in the tissues studied, and it appears to be relatively uncommon in the plant kingdom (147, 148). Nevertheless, one cannot rule out the possibility that the pigments are largely composed of catechol or similar residues co-polymerised with some indole units.

Alternatively, the pigments might be derived from polyphenolic material formed from leucoanthocyanidins or other flavonoid compunds. This kind of material is common in pod tissue and often occurs in association with the darker coloured pigments under study.

Nagasawa et al (5) suggested that the browning of broad bean testa arose by the hydrolysis and oxidation of the DOPA glucoside. In an attempt to isolate the pigment, mature testas were digested with strong HCl. This resulted in the formation of anthocyanidins, followed by rapid darkening, even when the reaction was carried out under nitrogen. The final product, which had a nirtogen content of 1.1 %, was dark brown in colour, and was almost identical in appearance to the pigments isolated from the flowers and pods of the same plant. Unlike these, however, it yielded phoroglucinol, as a major product upon alkaline fusion together with other unidentified phenolic compounds.

At this stage, no conclusions can be drawn with any certainty. It appears that these leguminous pigments are formed mainly be the polymerisation of catechol-type or flavonoid-type units with some co-polymerisation with indole units. The possibility that the non-nitrogenous precursor is derived from DOPA cannot be discounted, bearing in mind that pigment formation often occurs in DOPA-rich tissue, but the metabolic pathway followed remains unknown.

A further possibility also suggests itself; it is well known that in melanogenesis free-radicles are formed (149). One might explain the fact that DOPA or dopamine was detected in the majority of tissues studied, by postulating that the initial stages of pigment formation involves the production of an indole melanin. The free radicles so produced could then initiate a chain reaction involving nitrogen-free polymers already present in the tissue, the whole resulting in the production of a pigment with a small nitrogen content.

Spectroscopic studies.

During the work described above, it became apparent that there is a considerable problem in establishing the identity of the range of products formed during degradation.

As the appropriate standard compounds are not readily available in most laboratories, a comprehensive picture of the structure of a pigment cannot be obtained.

This difficulty has led other investigators to search for alternative characterising procedures such as spectroscopy, which has the great advantage of requiring only minimal amounts of pigment.

Unfortunately, ultraviolet and visible spectra are of little value, as most pigments show only generalised absorption in this region.

The infrared spectra, by contrast, provide much more information, and they have been used in studies on gallstone (149a) and other animal pigments (150) with apparent success.

This technique was therefore investigated in an attempt to establish or disprove its validity. In order to make measurements, the pigments (generally in the acid form) were incorporated in potassium bromide discs.

The results of this study represented in Figs. 49 - 53, revealed several interesting features. The spectra of the natural plant pigments (Fig.49 (a) - (f)) had many features in common with one another, and with the biosynthetic melanins (Fig.51). They all showed a strong band at 1600 cm^{-1} indicating their quinonoid nature. Simple quinonoid structures normally give bands in the region 1645 - 1780 cm^{-1} (151), but a lowering of the frequency to 1600 cm^{-1} could conceivably occur in structures where there are conjugation effects.

Absorptions were also given at 2850 - 2900 cm^{-1}, due to stretching vibrations of alkane -CH-. Two bands were particularly evident in the case of the Baptisia australis pod pigment (Fig.49 (f)).

With one exception, the plant pigments also exhibited fairly strong absorption at 1700 cm^{-1}, characteristic of C = O (as in COOH), the exception being the banana pigment (Fig.49 (f)). As banana peel was the only tissue in which dopamine (rather than DOPA) had been detected, this observation seemed to be significant, particularly when it was found that the biosynthetic dopamine melanin exhibited this same feature (Fig.51 (a)).

When treated with alkali the spectra of the pigments showed a marked reduction in the band at 1700 cm^{-1} and an increase in the band at 1380 cm^{-1} ascribed to carboxylate formation.

Reacidification led to a change in the spectrum back to the original form, thus showing the reversibility of the changes (Fig.50).

The spectra of both plant pigments and biosynthetic melanins showed a complex series of absorptions between 1000-1550 cm^{-1}, much of which is difficult to ascribe to specific groupings in the macromolecules apart from aromatic nuclei. All the pigments showed little absorption below 1000 cm^{-1}.

In general, the spectra of the plant pigments were closely similar to those of the biosynthetic melanins.

The features revealed by the spectra, however, whilst suggesting the presence of a quinonoid structure, and providing avidence for the presence (or absence) of other groups, do not provide sufficient evidence for critical diagnostic purposes.

For spectra to be used for this purpose, it was necessary to demonstrate that the spectra of true melanins and closely related pigments were sufficiently different from those of other dark non-melanin pigments. Did flavonoid polymers, for example, exhibit different spectra ?.

Spectra of pigments believed not to be melanins.

Two dark, presumed non-melanic pigments were included in the present study. The first was obtained by heating unpigmented broad bean testa in the presence of strong acid, whilst the other was isolated from black gallstones.

Chemical examination of the testa pigment, the results of which have already been described, suggested that it was a flavonoid polymer. The infrared spectral characteristics (Fig.52), however, were similar to the natural plant pigments. They were, in fact, almost identical to those of the bean flower pigment, showing the same changes on treatment with alkali (Fig.52 (b)) and on reacidification (Fig.52 (c)).

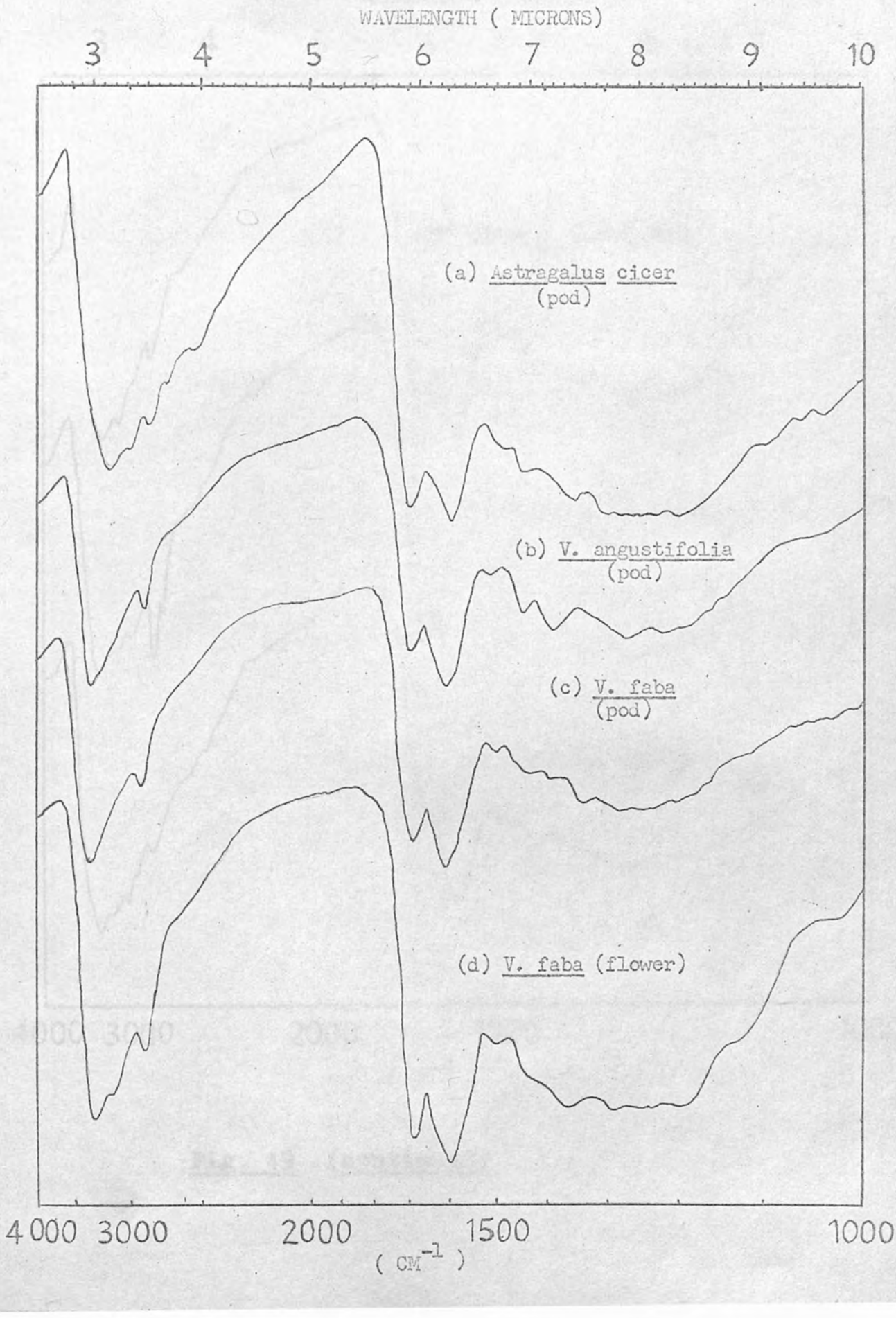
Black gallstones (Fig.53 (a)) are occasionally encountered in haemolytic jaundice and other conditions, and often contain relatively high concentrations of pigment.

Mirjake and his collaborators (152) claimed that the pigment was a melanin, but Suzuki (149) thought that melanin in quantity was unlikely to be formed in the hepato-biliary system which does not normally produce such pigments. He therefore compared the spectra of gallstone pigments and biosynthetic melanins in much the same way as in the present work. He came to the conclusion that the pigment was not a melanin, but possibly belonged to the class of bile pigments.

The spectra reported by Suzuki, however,lacked detail. His work was therefore repeated.

The spectra shown in Fig. 53 (b), although having the same general properties as biochemical melanins and the plant pigments, suggest a different chemical structure for the pigment. The gallstone pigment appears to contain more carboxylate groups, as shown by the pronounced absorption at 1380 cm^{-1}. The spectra also shows other differences.

Fig. 49 Spectra of plant pigments



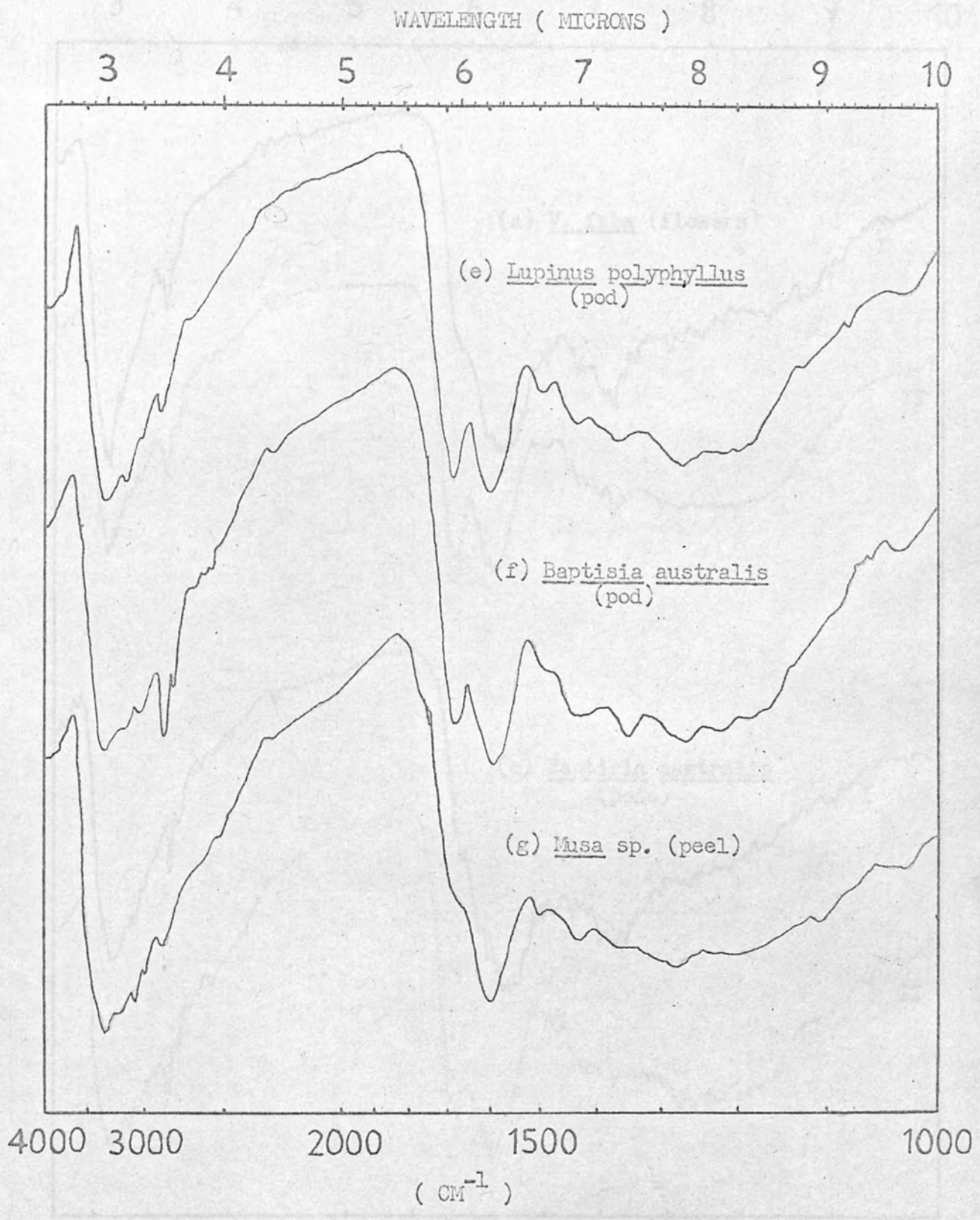


Fig. 49 (continued)

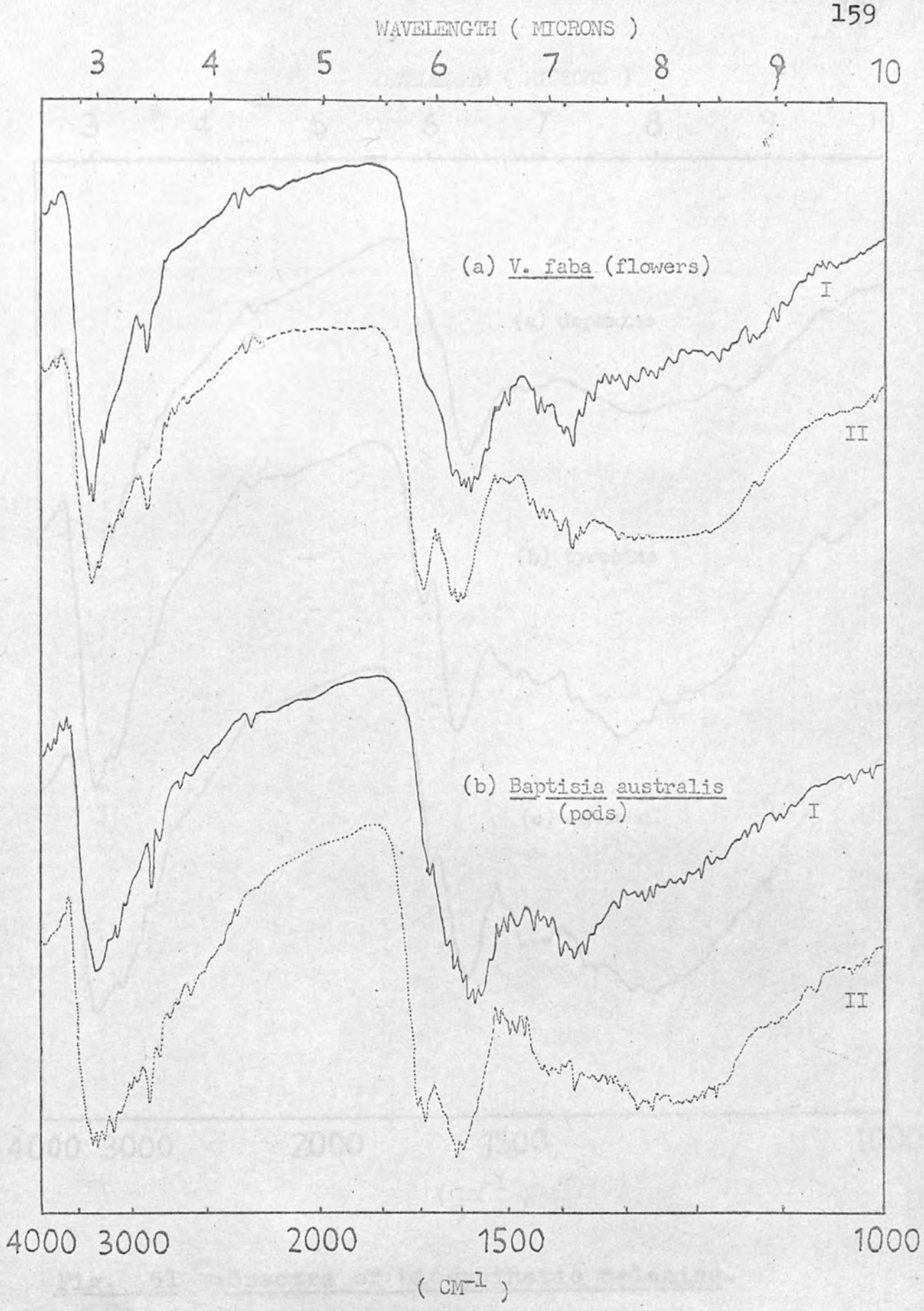

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
(a) V. faba (flowers)
I
II
(b) Baptisia australis
(pods)
I
II
4000
3000
2000
1500
1000
(CM-1)

Fig. 50

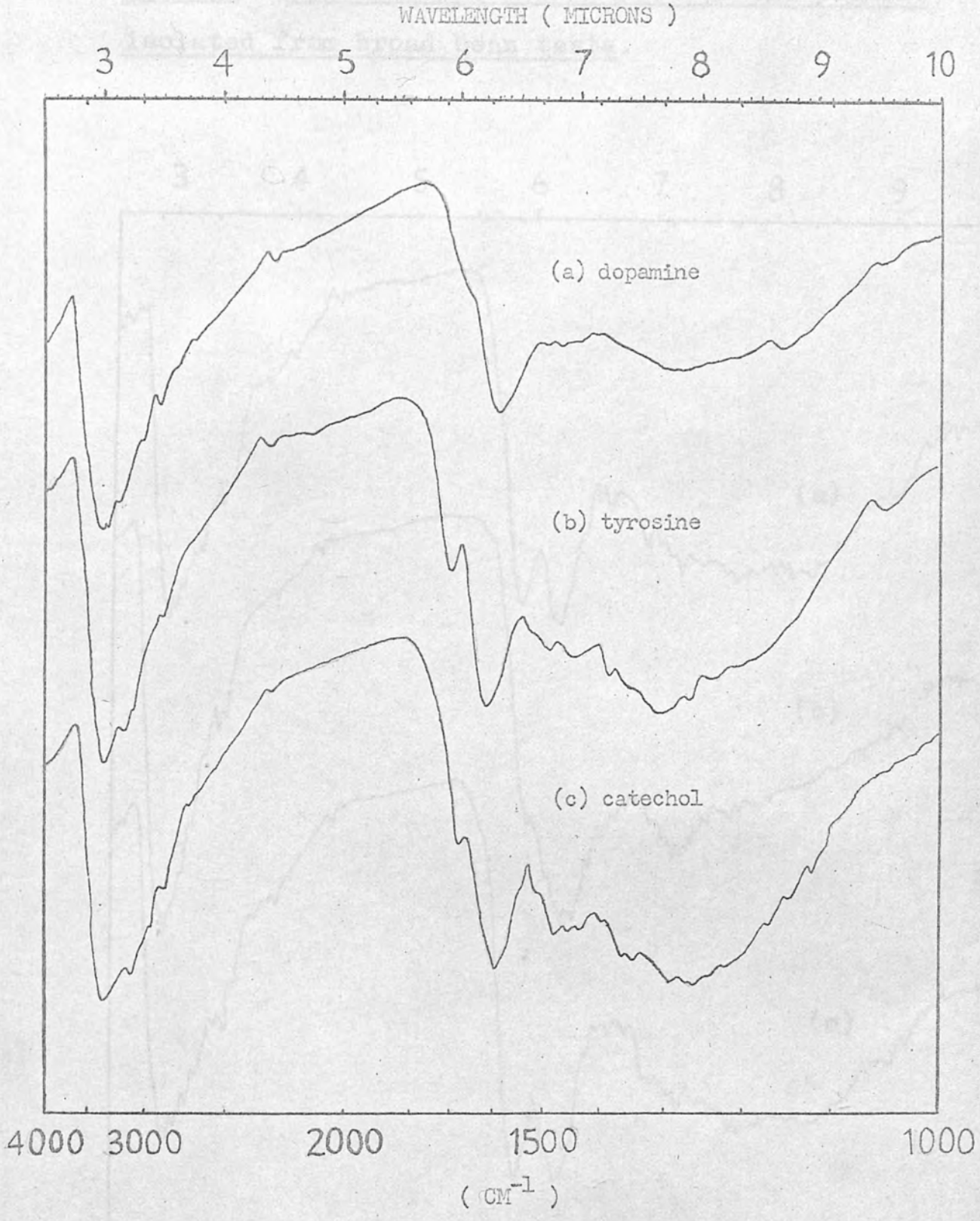


Fig. 51 Spectra of biosynthetic melanins.

Fig. 52 Spectral characteristics of the pigment isolated from broad bean testa.

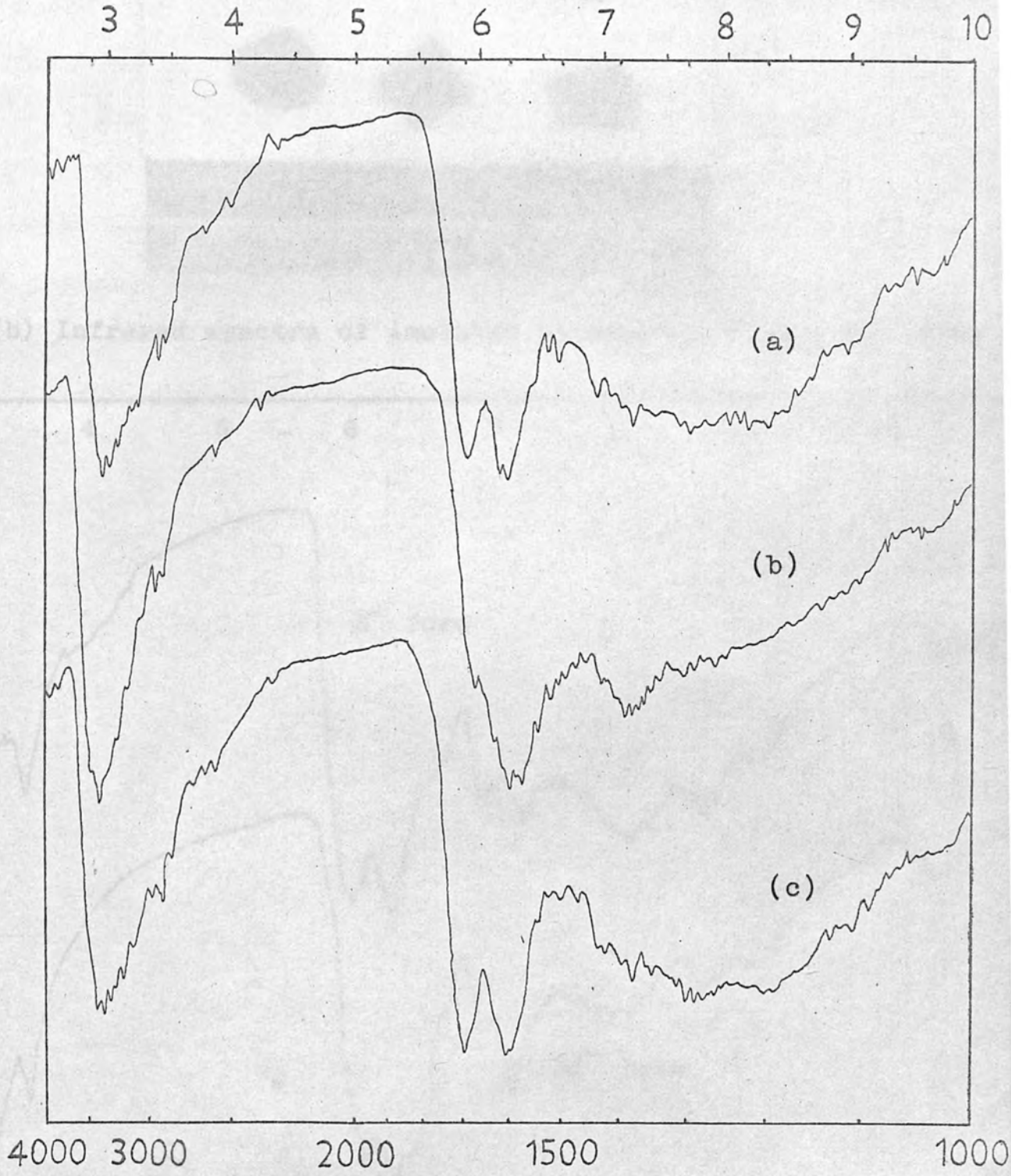
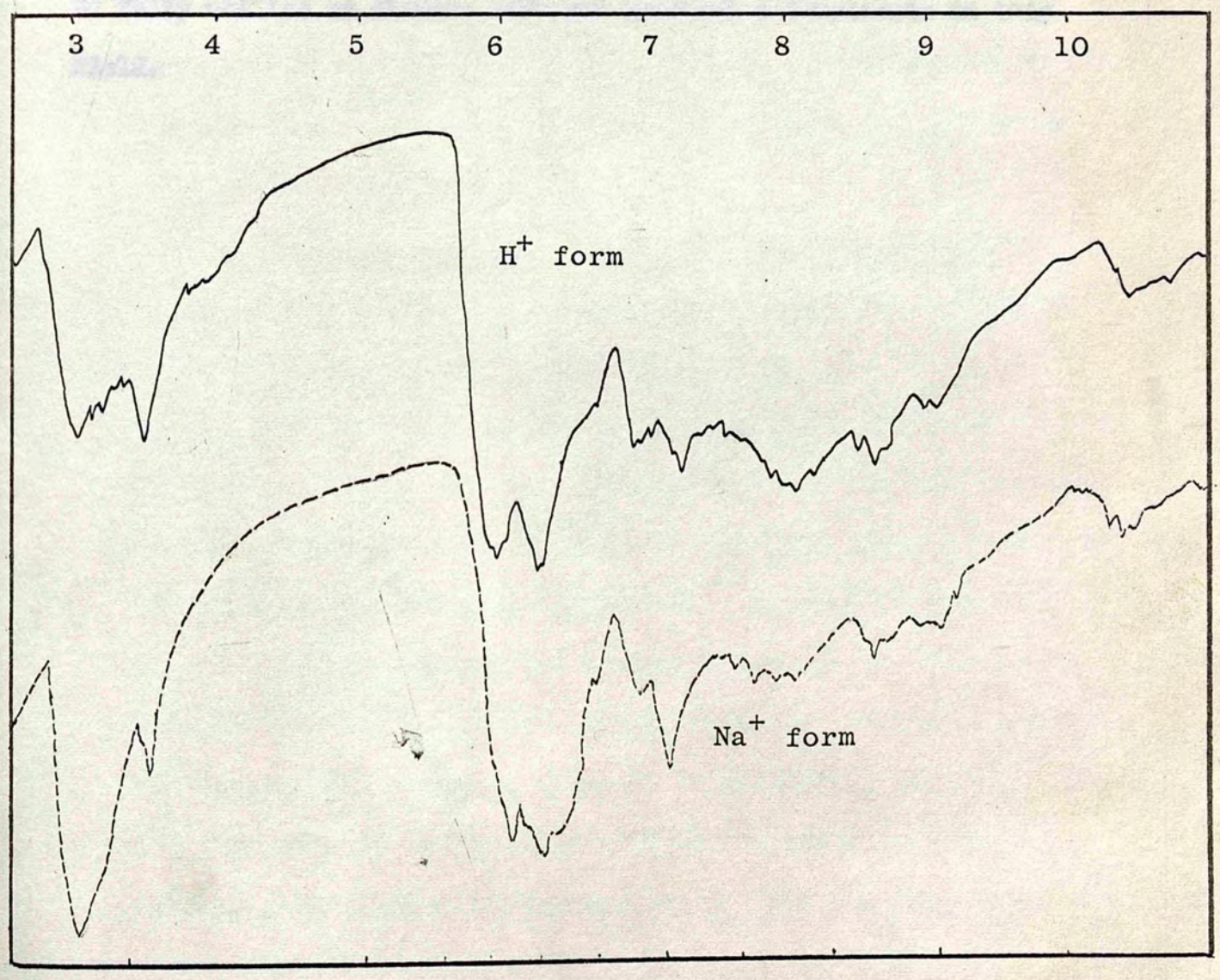


Fig. 53 Pigmented gallstones (a)

(a)

(b) Infrared spectra of isolated pigment in H^+ and Na^+ forms



Nicolaus and Picinelli have stated (85) that infrared spectra are of little value for the characterisation of melanins. These workers have unrivalled experience in this fields, and there is little doubt that their general conclusion is a valid one.

Nevertheless, our results suggested that the spectra may have been of some value for 'finger-printing' individual pigments. They might be useful, for example, in distinguishing between pigments from dopamine-containing tissues and those from DOPA-containing tissues. Much more work will have to be conducted before it will be possible to fully utilise or dismiss infrared spectral measurements in this field.

PART V

A study of enzymic reactions involving DOPA

V. 1. A DOPA ammonia-lyase from dandelion leaves.

Since the first reports of Koukol and Conn (121) that phenylalanine ammonia-lyase activity could be demonstrated in barley, there have been many reports of this enzyme. It appears to be widely distributed in plants and occupy an important metabolism role. Tyrosine ammonia-lyase activity, by contrast, seems to be limited to members of the Graminae.

MacLeod and Pridham (124) demonstrated that acetone powders prepared from Taraxacum officinale (dandelion) leaves possessed DOPA ammonia-lyase activity. The trans-caffeic acid produced from DOPA by this enzyme was characterised by means of its colour reactions, chromatographic and electrophoretic properties and by spectrophotometry.

The optimum pH for the reaction was shown to be 8-8, and the ammonia liberated in digests as a result of the reaction was detected by means of Nessler's reagent.

As this was the first demonstration of DOPA ammonia-lyase activity in a species not belonging to the Graminae, the findings of McLeod and Pridham were reinvestigated and their findings confirmed.

The work was then extended by a study of the specificity of the preparation whereby the ability of digests to deaminate a number of aromatic amino acids was considered. Ethereal extracts from digests were separated on paper chromatograms, and the ability of the preparation to deaminate a particular amino acid was established by a visual assessment of the amount of the appropriate

cinnamic acid derivative formed. The results are given in Table 15. This part of the study was purely qualitative.

The product obtained after incubating L-phenylalanine with a digest was shown to be trans-cinnamic acid by comparison of its chromatographic and electrophoretic behaviour with an authentic sample of that compound. The ultraviolet spectrum of the product was also shown to be identical to that of trans-cinnamic acid (Fig.54)

Table 15.+

Amino acid	Expected product	Activity.
L-phenylalanine	cinnamic acid	strong
D-phenylalanine	" "	none
L-tyrosine	p-coumaric acid	very weak or absent
L-DOPA	caffeic acid	medium
3-O-methyl DOPA	ferulic acid	none
4-O-methyl DOPA	isoferulic acid	none

The preparation was shown to be specific for L-phenylalanine, as no cinnamic acid could be detected in extracts from digests incubated with the D-isomer.

Several attempts to show the formation of p-coumaric in digests containing L-tyrosine were unsuccessful and it is therefore concluded that tyrase activity in T.officinale is either weak or absent.

+ See ref. (49)

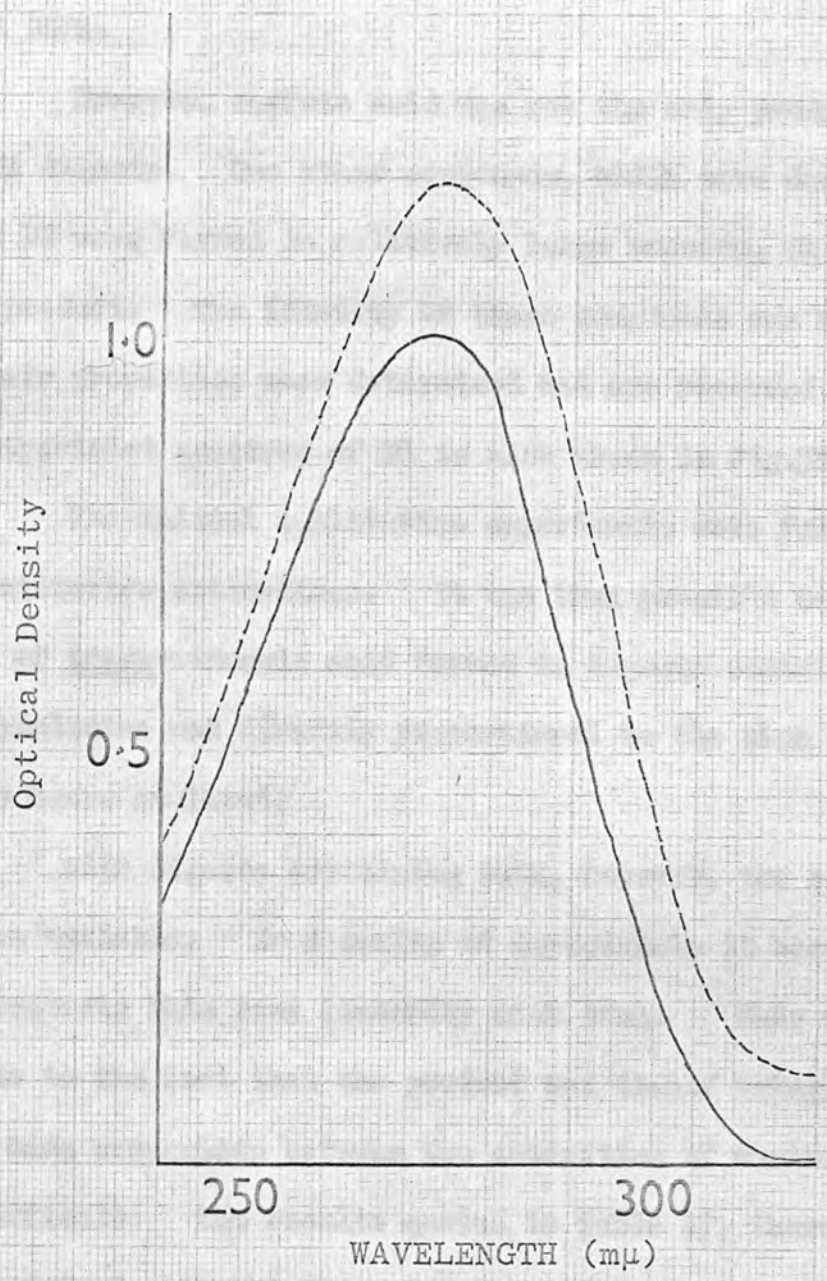


Fig. 54. A comparison of the spectra of trans cinnamic acid and the compound isolated from L-phenylalanine digests, measured in ethanol. The spectrum of cinnamic acid (dotted lines) has been displaced by 0.1 O.D. units

The crude preparation showed deaminase activity towards both the D and L forms of DOPA but no activity to either 3-O or 4-O-methyl DOPA.

However, caffeic acid was not the only product formed in DOPA digests. Two other compounds, which were designated D1 and D2 were formed in relatively large amounts, D1 being the major product. The identity of these compounds was not established but their properties were determined and are recorded in Table 16. The ultraviolet spectrum of D1 is also shown in Fig.55.

The initial qualitative experiments were followed by quantitative estimations. It was thus possible to show that the amount of trans-cinnamic acid formed in digests containing L-Phenylalanine was directly proportional to the time of reaction up to 3 hours at least.

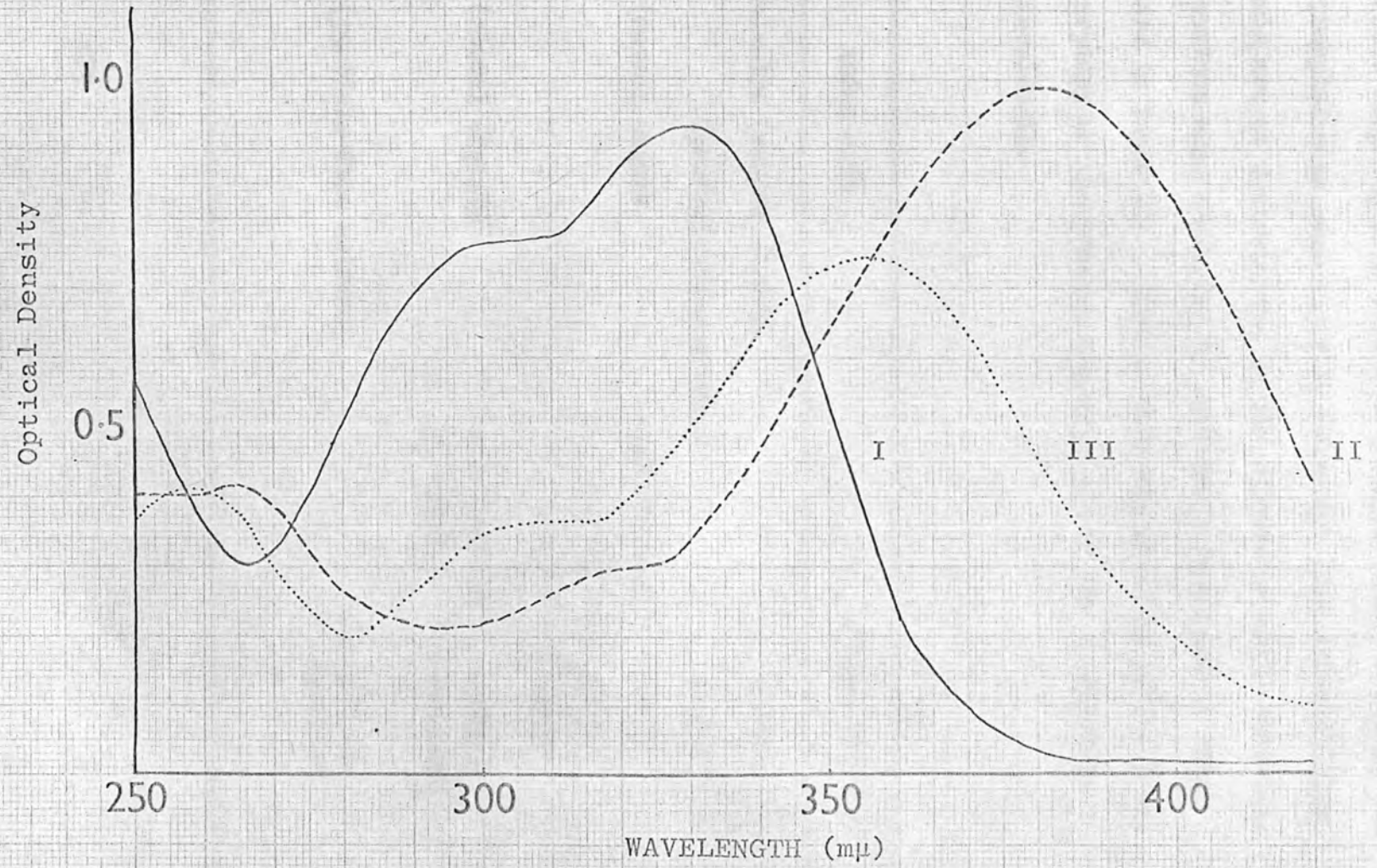
With digests containing DOPA, however, the results were far more variable. In a series of experiments it was never possible to demonstrate this same linearity with time. This may well have been due to the fact that the product was itself being acted upon, but it made comparison between the activities of various preparations very difficult. The results quoted in Table 17, therefore, were all determined with the same acetone powder.

Table 16. Properties of Compounds D1 and D2.

		D_1	D_2
Colour in ultraviolet	alone	pale blue	pale blue
	with NH_3	green	green
Chromatographic behaviour			
(R_f values)			
Solvent B		0.4	0.3
C			
E		1.0	1.0
F			
Electrophoretic behaviour.			
(M_{SA} corrected values)			
Borate (pH 9.8)		1.19 (chlorogenic acid 0.90)	1.29
Tris (0.05 M pH 8.8)		1.13 (chlorogenic acid 0.88)	1.35
($M_{\text{caffeic acid}}$ value)			
Molybdate (pH 5.0)		1.52	

Fig. 55. The ultraviolet absorption spectrum of compound D_1 from DOPA digests.

I in ethanol II with NaOEt III with boric acid



Further qualitative experiments also showed that whereas soluble extracts prepared from dandelion acetone powder retained much of their L-phenylalanine ammonia-lyase activity, DOPA ammonia-lyase activity could not be demonstrated in the absence of solid material. These observations were confirmed by quantative measurements which are summarised in Table 17. Digests were conducted at 37°for 3 hr. and the absorption of the product was measured at the appropriate maximinium wavelength.

Table 17.

Amino acid			Absorption
L-phenylalanine	(normal digest)		0.692
	(particle-free extract)		0.558
L-DOPA	(normal digest	caffeic acid	0.077
		D_1	1.34
		D_2	0.330
D-DOPA	(normal digest)	caffeic acid	0.078
		D_1 and D_2	not measured
L-DOPA	(particle-free extract)		No products detected
D-DOPA			

These results confirmed the earlier finding that both the D and L forms of DOPA were deaminated with equal ease. This is extremely unusual, and ought to be reinvestigated with a guaranteed completely pure sample of the D isomer.

The DOPA ammonia-lyase activity would appear to be intimately bound to the particulate fraction of the powder, unlike the phenylalanine ammonia-lyase activity. Particle-free extracts prepared directly from fresh dandelion leaves were also free of activity, thus establishing that the enzyme had not been rendered insoluble during the preparation of the acetone powder.

All these factors suggest that the DOPA ammonia-lyase in dandelion leaves is quite distinct from the phenylalanine ammonia-lyase present in that tissue.

V.2. A DOPA ammonia-lyase from barley stem.

Neish established the ability of barley stem preparations to deaminate DOPA by showing that ammonia was produced in DOPA-containing digests (119).

Macleod and Pridham (124) confirmed this finding, and further showed that trans-caffeic acid was the other product of the reaction. This was achieved by means of chemical and chromatographic procedures.

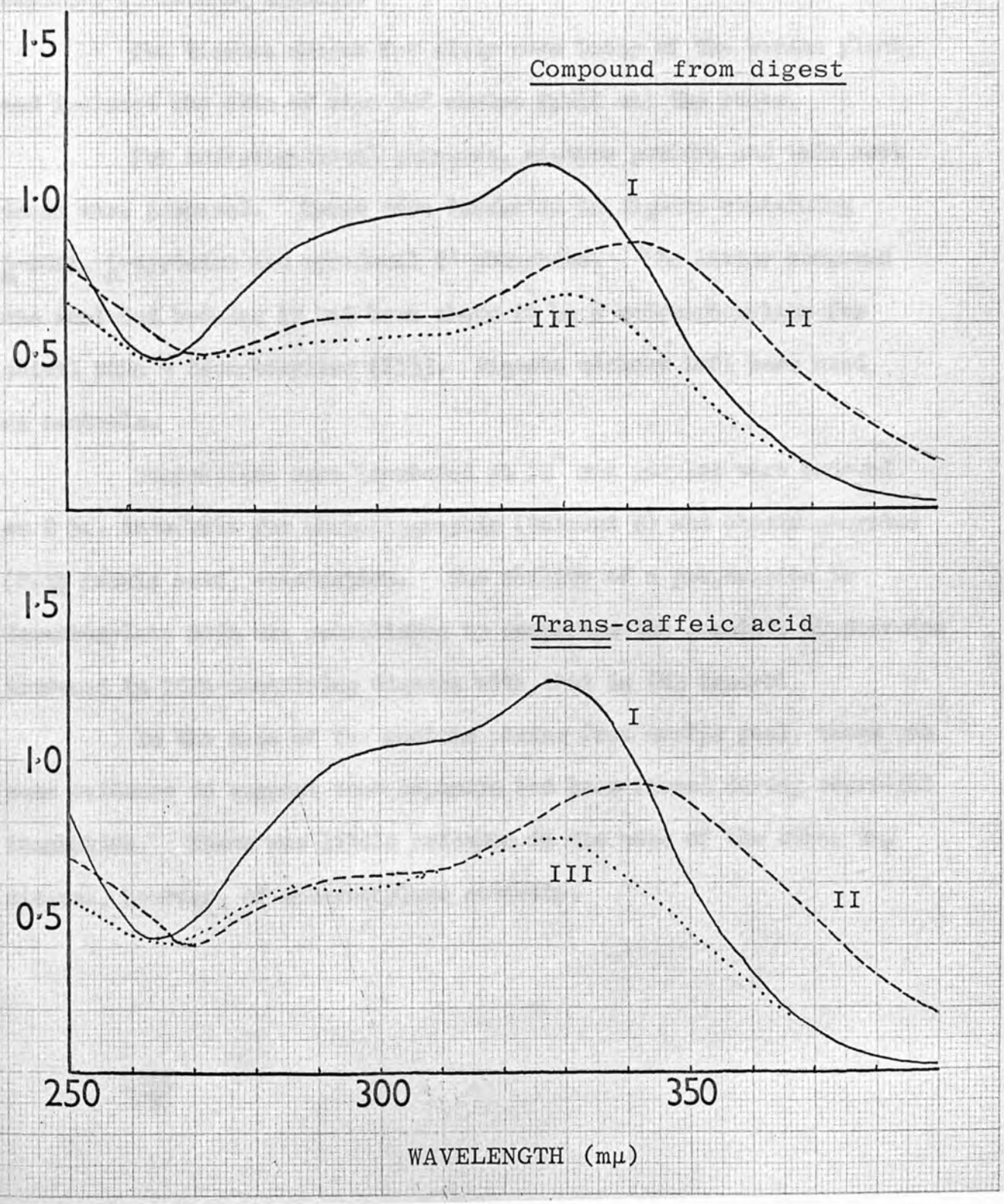
They also established that the pH optimum for this enzyme was the same as that of the phenylalanine and tyrosine ammonia-lyases, namely 8.8.

In the present work, acetone powders prepared from the stems of etiolated seedlings grown from a small quantity of seed supplied by MacLeod were shown to actively deaminate DOPA in digests. The reaction product was recovered from paper chromatograms (Solvent B), and its identity confirmed as trans-caffeic acid by comparison of its U.V. spectra in alcohol, with added sodium ethoxide, and after addition of boric acid, with an authentic sample of that compound (Fig.56).

Preparations prepared from the etiolated seedlings of other varieties of barley, however, showed little activity, making a detailed study difficult. Attention was therefore directed to preparations prepared from mature, field grown plants, but these also showed very little deaminase activity. The work was therefore discontinued.

Fig. 56. Comparison of the spectra of trans-caffeic acid and the product isolated from DOPA digests

I in ethanol
II with NaOEt
III as II with boric acid



V.3. DOPA - decarboxylase activity in banana peel.

As DOPA is a possible precursor of dopamine in plants, attempts were made to demonstrate decarboxylase activity in dopamine-containing tissues.

The tissues chosen for study were those of the banana plant, and included the skin of ripe and unripe fruit and the roots.

For investigational purposes, acetone powders and thin root discs were prepared. These were incubated in digests containing L-DOPA, L-cysteine and pyridoxal 5'-phosphate. The latter compound was included because it had been shown to be a codecarboxylase for animal DOPA - decarboxylase (153). Digests without DOPA were used as controls.

Suspensions were incubated at 30° and samples were removed at 2 hr. intervals for chromatographic (Solvent B) and electrophoretic (0.1M formic acid) examination. The ability of a preparation to decarboxylate DOPA was established by comparing the quantity of dopamine produced in DOPA-containing digests with that in the control.

In the case of the acetone powder from unripe peel, there was some evidence to suggest that dopamine had been formed during overnight incubation. There was little evidence in the case of the other two tissues, however, of decarboxylase activity.

PART VI

Discussion

Discussion.

The large number of reports on various aspects of DOPA metabolism in higher plants which have appeared in the literature during the past few years provide some guide to the increasing interest which is being shown in this field.

DOPA is probably formed in plants, as it is in animals, by the phenolase-catalysed oxidation of tyrosine. Despite the considerable amount of information which is available on these oxidative enzymes, much more is required before a full understanding of their mode of action can be reached.

As tyrosine can be readily oxidised to DOPA in test tube experiments, it seems, at first sight surprising that DOPA is not of more widespread distribution. Free DOPA, however, appears to be almost exclusively limited to a relatively small number of species in the Leguminosae or Centrospermae.

The uncontrolled oxidation of tyrosine in the majority of species would undoubtedly prove fatal by removing tyrosine from its role in protein formation. Evolutionary changes have probably equipped most species with the ability to stop this reaction, by separating the enzyme and its substrate or by careful control of the redox potential of the cell.

In mammals the presence of eumelanins confer protection against ultraviolet light, whilst in marine animals, fish and some insects it provides camouflage. Neither of these functions appears to be of importance in plants.

The dark spots on broad bean flowers presumably act as insect attractants, and the production of quinone-induced pigments by leguminous pods could aid in seed dispersal.

The production of DOPA in a small minority of species could have enabled further metabolic changes with survival value to take place. The production of brightly coloured betacyanin pigments, for example, by members of the Centrospermae would have provided them with similar advantages to those species producing anthocyanin and related pigments. Although alkaloids have a less obvious function, their production could also have an important survival value.

The widespread involvement of DOPA in the biogenesis of alkaloids remains to be confirmed, possibly by a continuation of radioactive feeding experiments or by a continuation of enzyme experiments such as those recently reported by Jindra, Kovacs and their co-workers (162). Studying the biogenesis of the opium alkaloids in Papaver somniferum, these workers were able to demonstrate the activity of a number of enzymes involved in the metabolism of DOPA by both *in vivo* and *in vitro* experiments. They detected enzyme systems in the poppy plant which could bring about the oxidation of tyrosine to DOPA, and also the oxidative deamination, transamination and decarboxylation of DOPA. The approach adopted by this team could obviously be adopted with advantage with other alkaloid-producing plants.

The deamination of aromatic amino acids, particularly phenylalanine, by plants is now firmly established. Phenylalanine ammonia-lyase activity is widely distributed, so that it appears to occupy an important role in plant biochemistry. The tyrosine ammonia-lyases found in grasses and in one species of Eucalyptus (163), and the DOPA ammonia-lyase found in dandelion leaves both seem to be individual enzymes, but at this stage it is difficult to say of what importance they are in the metabolism of these plants.

In conclusion, despite the considerable progress which has been made in the field of secondary [illegible] substances such as DOPA, little is known of their functions nor of their metabolism at the cellular level. This is a field, therefore, which offers considerable scope and the prospects of many interesting discoveries.

PART VII

General methods

GENERAL METHODS.

1. Paper chromatography.

Paper chromatography was carried out using Whatman No.1 paper for carbohydare compounds and Whatman No.3 and 3 MM papers for phenolic compounds and for preparative work. Separartions were performed by the descending technique in the following solvent systems. All proportions are given by volume.

A Butan-1-ol/acetic acid/water (6:1:2)

B Butan-1-ol/ethanol/water (40:11:19)

C Ethyl acetate/acetic acid/water (9:2:2)

D 2% hydrochloric acid

E Ethyl acetate/acetic acid/formic acid/water (18:3:1:4)

F Ethyl acetate/pyridine/water (10:4:3)

G Butan-1-ol saturated with water.

$$R_{DOPA\ values} = \frac{\text{distance travelled by substance}}{\text{distance travelled by DOPA}}$$

2. Paper electrophoresis.

Paper electrophoresis was carried out on Whatman No.3 and 3 MM papers using a Shandon high-voltage apparatus.

Buffers used included : sodium molybdate (pH 5.0,1.5% w/v)
phosphate (pH 7.0, 0.1M)
formic acid (0.1 M)

3. Spectrophotometric measurements.

Ultraviolet and visible absorption measurements were made with a Unicam S.P. 500 spectrophotometer, whilst spectra were obtained using a Perkin-Elmer 137 UV recording spectrophotometer.

Infrared spectra were measured with a Perkin-Elmer Infracord spectrophotometer.

4. Melting point determinations.

Uncorrected melting points are recorded.

Location reagents employed.

p-Anisidine hydrochloride.

1 % in butan-1-ol

Papers were sprayed with the reagent, and then heated at 100° for 5 min. Monosacharrides and reducing oligosacharrides gave yellow, brown or red products with a characteristic fluorescence.

Diazotized p-nitroaniline / NaOH.

A stock solution was prepared by dissolving 1.5 of p-nitroaniline in 45 ml. concentrated HCl and 950 ml. water. 20 ml. of this solution was diazotized by the dropwise addition of 5 per cent aqueous sodium nitrite until the colour changed to a faint straw colour, and diluted with 2 volumes of distilled water.

Papers were first sprayed with this reagent and then oversprayed with 2N NaOH. Most phenols gave intense stable colours, although catechol derivatives such as DOPA generally give weaker colours which fade rapidly.

Silver nitrate.

(a) Silver nitrate, saturated solution in water 1 vol.
Acetone 20 vol.

(b) Sodium hydroxide, 0.5 per cent in 80 % ethanol.

Papers were dipped in reagent (a) and the acetone allowed to evaporate off. They were then dipped through the alkali. Some of the more reactive dihydroxyphenolic compounds reacted without the application of alkali, but other phenolic compounds and reducing sugars required this step.

The products in either case were grey or grey-brown. This reagent was particularly useful in the present work as it made it possible to detect all the products in some of the hydrolysis studies on carbohydrate derivatives.

Where it was necessary to preserve the chromatograms, they were washed in dilute (0.1 M) sodium thiosulphate solution.

Perric chloride-ferricyanide reagent.

0.3 % aqueous solution of ferric chloride 1 vol.

0.3% aqueous solution of potassium ferricyanide 1 vol.

Papers were dipped into the reagent. Phenolic compounds gave a green or blue colour which slowly developed in the cold. Excess reagent was removed with dilute hydrochloric acid.

Ninhydrin.

Amino acids and related compounds were located by dipping papers in an acetone solution of ninhydrin (0.2 per cent) containing a little pyridine. Blue and violet reaction products were formed by heating at 110 for 5 mins. or for longer periods at lower temperatures.

Dinitrophenylhydrazine reagent.

Aldehydes produced the yellow 2, 4 dinitrophenylhydrazones on treatment with a saturated solution of 2,4 dinitrophenylhydrazine in 2N HCl. If the papers were oversprayed with alkali, the colour changed to purple.

Method 1. Preparation of a plant tissue extract for chromatographic examination.

A suitable quantity of tissue was macerated in an excess of aqueous ethanol (70%) and insoluble materal removed by filtration and/or centrifugation. Plastid pigment was removed with petroleum ether (40-60°).

Extracts were then taken down to dryness under reduced pressure and a small quantity of water added to the residue. After shaking for a few minutes the aqueous suspensions were again centrifuged. The centrifugates were usually suitable for chromatographic examination, but with unknown tissues it was sometimes necessary to run trial chromatograms to check the concentration of substance in the extract.

Some tissues, such as the seed coat of V. faba were found to contain considerable amounts of polyphenolic material which completely obscured chromatograms sprayed with diazotized p-nitroaniline/NaOH. In such cases, the concentrated extract was applied to the top of a small column filled with nylon powder and washed through with distilled water. The combined effluent from the column was reconcentrated under reduced pressure before being applied to paper.

In cases where plants contained abundant sap or latex, this was spplied without further treatment.

Method 2. Determination of DOPA in solution.

The method is a modified version of that outlined by Kendal (144) which in turn was derived from the earlier method of Evans and Raper (140).

Solutions required.

(a) Standard DOPA or sample solution(s) containing between 0.1 and 0.4 mg DOPA/ml. Preliminary determinations were performed on unknown solutions and the concentration adjusted to within these limits where necessary.

(b) Phosphate buffer (0.5 M, pH 6.0)

(c) 0.192 N-NaOH solution.

(d) 0.1 N-iodine.

(e) 0.1 N-sodium thiosulphate.

Procedure.

All the following steps were carried out in a 1 cm. glass cell. The reagents were added in the following order and the mixture was agitated by means of a plastic stirrer after each addition.

(i) 0.80 ml. DOPA or standard solution.

(ii) 1.40 ml. phosphate buffer.

(iii) 1.00 ml. NaOH solution.

(iv) 0.38 ml. iodine.

Exactly 90 secs. after the iodine addition, 0.42 ml. of the sodium thiosulphate solution was added and the time accurately noted by means of a stop-watch. A fresh control, containing 0.8 ml. distilled water instead of the iodine and thiosulphate was prepared for each determination. The cells were transferred to a spectrophotometer and readings of optical density at 515 mu made at approximately half minute intervals over a period of 3 mins. A plot of log (O.D.) against time after thiosulphate addition gave a straight line, from which log (O.D.) at zero time could be found by extrapolation.

The results of such a procedure with a number of broad bean extracts is shown in Fig.57. The actual plots are for extracts from.-

(a) 4-8 cm. pods.

(b) plumules and epicotyls of 7-day old seedlings.

(c) epicotyls of 9-day old seedlings.

(d) upper mature leaves.

(e) lower mature leaves.

The accuracy and the reproducibility of the method can be seen from the close agreement obtained between duplicate determinations on the same extract.

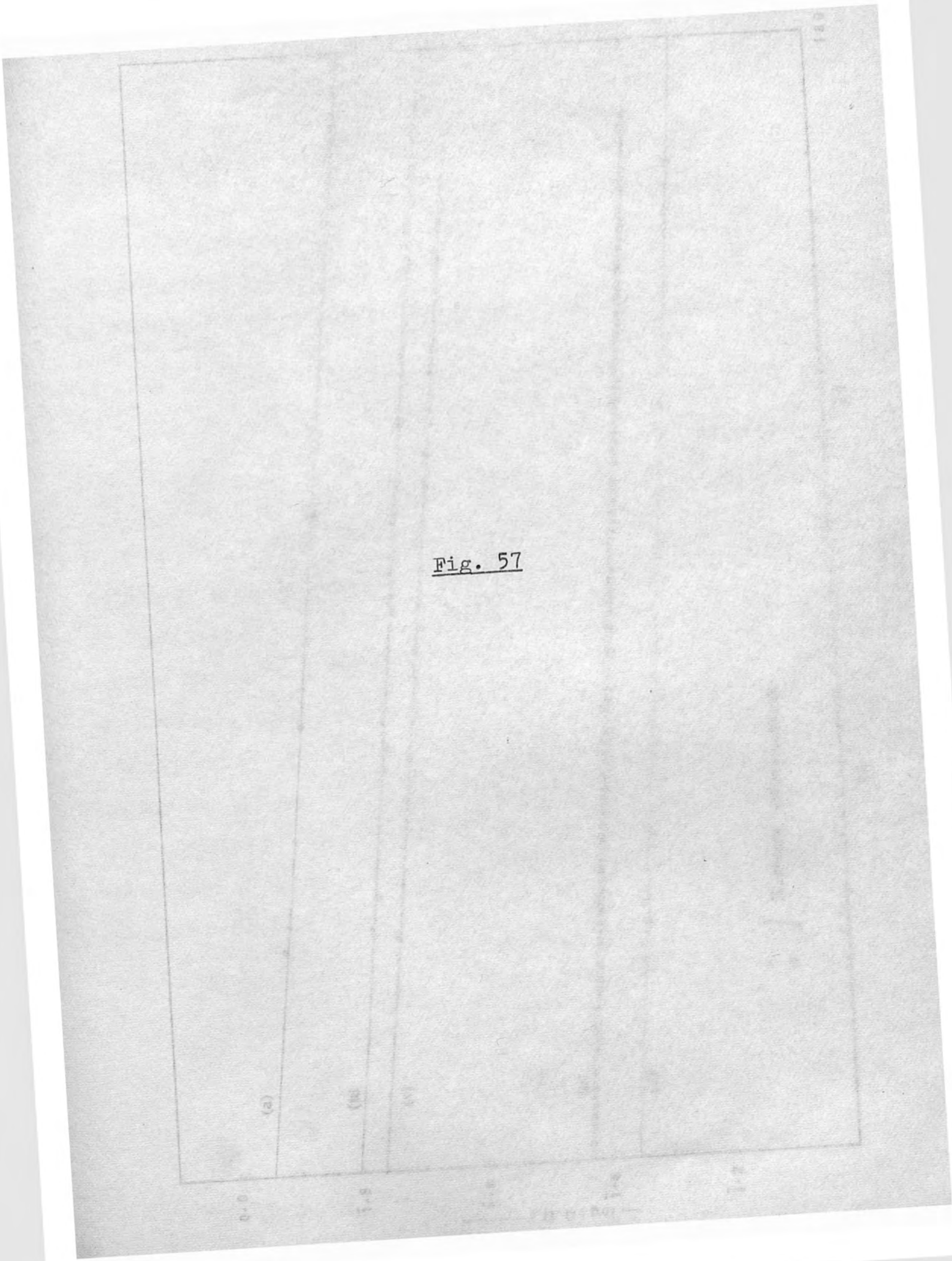
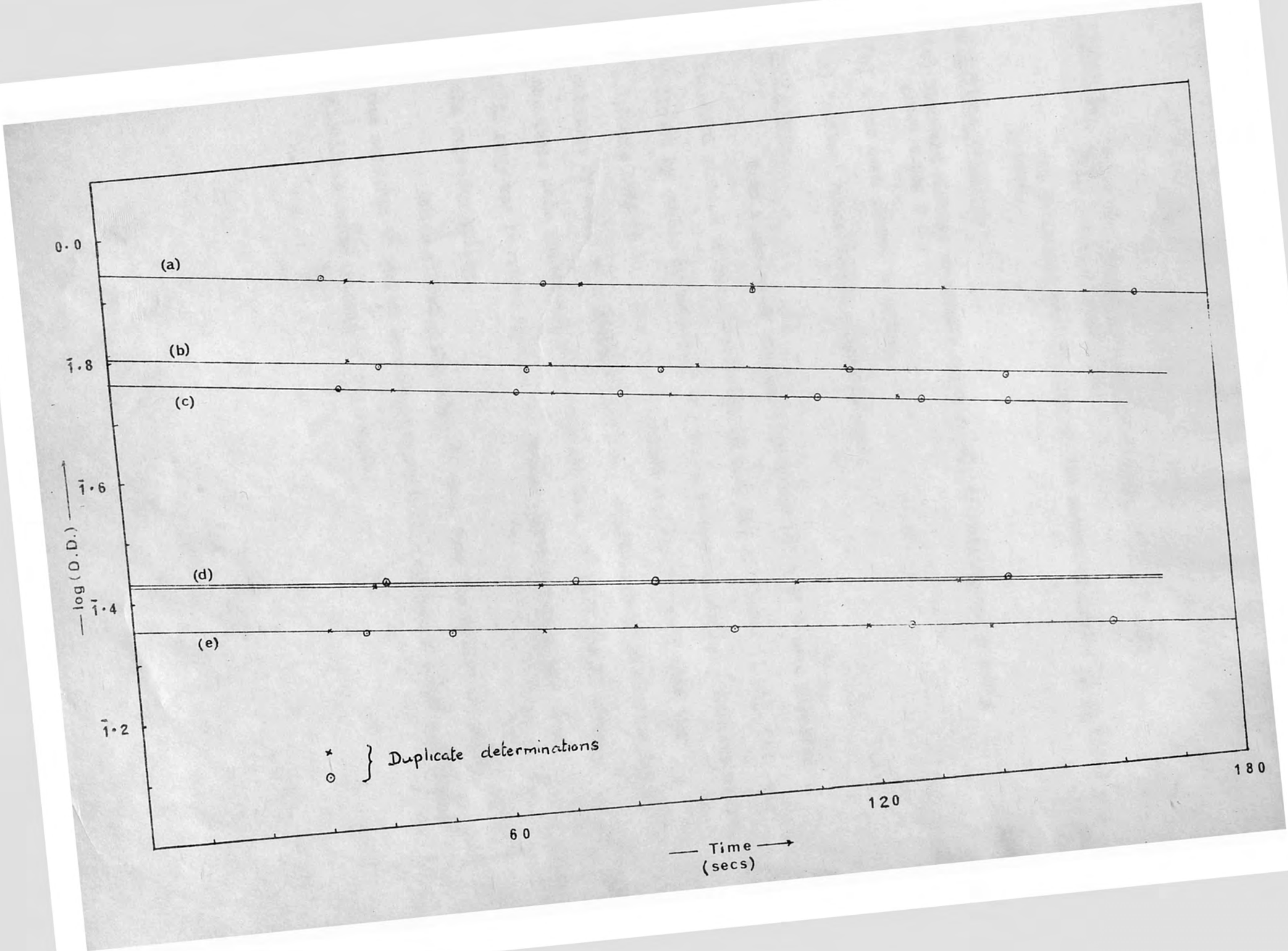


Fig. 57


0·0
(a)
(b)
1̄·8
(c)
1̄·6
(d)
1̄·4
(e)
1̄·2
—log (O.D.)—
Duplicate determinations
60
120
180
— Time —
(secs)

Method 3. Phenol-sulphuric acid colourimetric method for the determination of glucose.

The following modification of the method of Dubois et al (154) was used.

Solutions required.

(a) Standard glucose or sample solution(s), containing up to 150γg glucose per 2 ml.

(b) 4 per cent phenol in water.

(c) 'Analar' concentrated sulphuric acid.

Procedure.

Into a series of similar test tubes ($\frac{3}{4}$" x 6") were pipetted standard glucose or sample solution (2 ml.) and 4% phenol (1 ml). This was followed by gentle agitation of the tubes to ensure mixing. Concentrated sulphuric acid (5 ml.) was then pipetted rapidly, directly into the solution by means of a special pipette. Approximately 30 minutes later, the tubes were shaken and then immersed in a 40° bath for 15 mins. This step was found to improve the colour yield slightly and also the reproducibility.

After allowing the tubes to cool down the colour intensity was measured at 490 mμ spectrophotometrically against a blank containing distilled water instead of the sample.

Method 4. The preparation of diazomethane (155).

Potassium hydroxide (8 g) was dissolved in water (5 ml.) and then ethanol (5 ml.) and ether (5 ml.) added. This mixture was then poured into a flask fitted with a dropping-funnel and a side arm with a downward condenser leading to a collecting vessel cooled in ice-salt mixture.

The contents of the flask were warmed until the ether just began to distil over and a solution of N-methyl-N-nitroso toluene p-sulphonamide (1 g) in ether (10 ml.) added dropwise so as to keep the ether distilling gently over. The distillation was continued until the distillate was no longer yellow. The ethereal solution of diazomethane thus produced was usually used immediately but where necessary it was stored at 0°.

Method 5. Methylation with diazomethane (156)

A small quantity of the compound to be methylated (5 mg) was dissolved in water (0.1 ml.) and methanol (0.5 ml.). The solution was cooled to 0° and treated with an ethereal solution of diazomethane (1 ml.), prepared as described in Method 4.

The initial colour of the reaction mixture was straw-yellow, but as the reaction proceded, nitrogen was evolved and the colour faded. Further additions of diazomethane were made until the colour remained stable for several hours. The solution became decolorized on being allowed to regain room temperature overnight.

The residue obtained by evaporating the solution under reduced pressure was then remethylated by the above procedure.

Method 6. Methylation with methyl iodide and silver oxide.

(after Kuhn (134))

5 mg of the compound was suspended in dimethylformamide (2 ml.) cooled to 3°. Methyl iodide (0.5 ml.) and silver oxide (50 mg) were added to the reaction vessel which was shaken in the dark for 1 hour and for a further 7 hours at room temperature. Further quantities of methyl iodide and silver oxide were then added and the suspension shaken for another 18 hours.

At the end of this period the contents of the flask were centrifuged. The residue was washed with several portions of chloroform and the washings were combined with the centrifugate, the whole then being under reduced pressure.

Method 7. Oxidation with nitrobenzene/NaOH. (131).

A small amount of compound (5 mg.) was dissolved in 2N NaOH (0.4 ml.) in a small tube, and nitrobenzene (0.05 ml.) added. The tube was then sealed and heated in an autoclave at 160°for 3 hours.

When cool the tubes were opened and ether (1 ml.) added and the tube gently agitated. The ether was removed with a pipette and the aqueous layer extracted twice more with fresh ether to remove the last traces of nitrobenzene. The solution was then acidified with N-HCl and the solution extracted with ether (3 x 1 ml.). The ether extracts were pooled and taken down to dryness, any residue taken up in a few drops of ethanol ready for electrophoretic examination.

Method 8. Alkaline fusion of plant pigments (85).

An intimate mixture of the pigment (200 mg) sodium, hydroxide (600 mg.), sodium dithionite (100 mg.) moistened with a few drops of water were heated in a platinum crucible for 10 mins. at 300°.

After cooling under a stream of nitrogen, the fused mass was suspended in an ice-cold solution of sodium dithionite (10%, 100 ml.) agitated by means of a magnetic stirrer. The solution was acidified by the addition of acetic acid (2 ml.) and any insoluble material removed by centrifugation.

The centrifugate was extracted with peroxide-free ether (5 portions of 40 ml.). The pooled ether extract was dried over anhydrous sodium sulphate with a little dithionite, before being taken down to dryness under reduced pressure.

The residue was taken up in water (0.1 ml.) and the products examined on paper chromatograms (Solvents A and C) and electrophoretograms (formic acid and molybdate buffer).

Method 9. Amino acid analyses by means of an automatic analyser.

A Bender and Holbein automatic analyser was generously put at our disposal by the Director, and Dr. J.Bowes of the British Leather Manufacturers R.A.

The apparatus employed a glass column (135 x 0.9 cm) packed with an 8% cross-linked sulphonated polystyrene/resin (30-40 μ beads) in a pH 2.2 citrate buffer. The mixture to be analysed was applied to the top of the column and the individual components were separated by eluting in a stepwise manner with citrate buffers of increasing pH and ionic strength.

The effluent from the column was continuously mixed with a solution of ninhydrin reagent and the colour developed by passing the mixture through a long Teflon coil immersed in a boiling water bath. The optical density of the resulting solution was measured continuously at 570 mμ (for amino acids) and 440 mμ (for imino acids), and the results printed on a moving roll of paper.

The amount of any amino acid in the mixture was then determined by integrating the area under the appropriate peak by means of a planimeter, and comparing this area with that produced by a known quantity of the compound.

The chromatogram shown in figs. 40 and 41 were those given at 570 mμ.

Method 10. The preparation of plant tissue acetone powders.

(All operations were carried out in a cold room at 0°.)

A suitable quantity of freshly gathered plant material was washed in cold tap water and surplus water removed by blotting between sheets of absorbent paper. The tissue was then placed to a refrigerator at -20° for about 30 mins.

The cooled tissue was transferred to a Waring blendor and sufficient cold (-20°) acetone to just cover the tissue added. Homogenisation was carried out for 2 mins. and the insoluble material removed by filtration under reduced pressure. The residue was placed in the blendor and the homogenisation repeated with fresh cold acetone.

After removing most of the acetone by filtration, the remainder was removed by placing the resulting 'powder' under a fume hood for 15 mins. at room temperature.

This procedure resulted in the preparation of acetone-free, free-flowing powders, which were stored at 3° until required.

PART VIII

Experimental

Experiment 1. Attempts to prepare glucosyl derivatives of DOPA by synthetic means.

The method attempted was based on those described by Irvine and Gilmour (126) and Irvine and Hynd (127).

DL-DOPA and anhydrous D- glucose were intimately mixed and heated under reflux in a number of solvents such as water, methanol and 83% aqu. ethanol. The latter procedure will be taken as an example.

DL-DOPA (1g) and anhydrous D glucose were suspended in 83% aqu. ethanol (100 ml.) and heated under reflux for 1 hr. and then the solution was rapidly filtered. A small quantity of the filtrate was applied to chromatograms and electrophoretograms for examination.

This revealed that about 4 compounds in trace amounts had been formed by this procedure. On electrophoretograms (0.1 M formic acid) they all gave the characteristic blue colouration with diazotised p-nitroaniline/NaOH of o-dihydric phenols. They were not, however, detected on chromatograms developed in Solvent C, which suggested that these derivatives are unstable to prolonged contact with acid.

Experiment 2. Attempts to produce DOPA glucosides with an A. niger enzyme.

A. niger mycelia were macerated in 0.1 M sodium acetate buffer (pH 5.1) and the insoluble material removed by centrifugation.

To 40 ml. of this crude preparation, maltose (0.6 M, 14 ml.), saturated L-DOPA solution (4 ml.) and L-cysteine solution (1.2 M, 2 ml.) were added. The reaction mixture was incubated for 24 hrs. at 30° and then the reaction was stopped by the addition of trichloracetic acid (1.2 M, 4 ml.).

The solution was concentrated under reduced pressure and aliquots taken for examination on paper chromatograms (Solvents A,B,C and E). No products having the expected properties of DOPA-glucose derivatives could be detected amongst the products, although tyrosine was tentatively identified.

Experiment 3. Attempts to prepare derivatives with an E.coli enzyme.

Experiments were kindly performed by Dr. M.Walter (R.H.C.) but no products of interest were found.

Experiment 4. Feeding experiments with Pisum sativum seeds.

Seeds of P. sativum var. 'Early Onward' were soaked overnight in a solution (0.2%) of L-DOPA. They were then washed and placed on damp cotton wool in the dark. Seeds soaked in distilled water were used as controls.

In a preliminary experiment, the seeds were taken after 0, 2, 3 and 4 days and macerated in aqueous ethanol. The roots and shoots of the seedlings after 4 days growth were also examined. The added DOPA in the seeds appeared to have no adverse effect on either the germination or growth.

The extracts were shaken with light petroleum and the aqueous layer concentrated under reduced pressure. Paper chromatographic examination of the extract revealed five compounds not present in the controls (P_1 - P_5). These were isolated as follows.-

The extract was streaked onto Whatman No.3 papers which were developed in solvent C for 24 hr. Strips were cut from the ends of the papers and sprayed with diazotized p-nitroaniline/NaOH. Three sections corresponding to (1) a crude mixture of compounds P_1 and P_2, (a) a mixture of P_3 and P_4, and (3) crude P_5, were cut out and the compounds eluted off with water. (1) and (2) were then reapplied to separate sets of papers which were developed for up to 72 hr. in solvent C. Compound P_5 decomposed under such treatment and was therefore not isolated in this manner.

Experiment 5. Fractionation of a crude mixture of compounds P_1 and P_2.

After the second development, the strips containing these compounds were cut out and eluted with water. The eluted material was concentrated under reduced pressure and then reapplied to the baseline of prewashed No.3 paper. Electrophoretograms were run in 0.1 M aqueous formic acid (pH 2.4) (4000 v./1 hr.). This led to a clear separation of P_1 and P_2 from each other and from traces of impurities. The compounds were eluted from the electrophoretograms with water and the eluates either freeze-dried or concentrated under reduced pressure and stored at 5 C. Both compounds yielded hygroscopic powders, very pale yellow in colour.

Experiment 6. Fractionation of a crude mixture of compounds P_3 and P_4.

P_3 and P_4 were first separated from one another on No.3 paper using Solvent B. They were finally purified on electrophoretograms as described above, and isolated as freeze-dried powders.

Experiment 7. The isolation of compound P_5.

Compound P_5 was found to be extremely labile and it resisted complete purification. It was found to be fairly stable to chromatographic and electrophoretic separations in acid and neutral solvents and could be washed off paper strips with water containing SO_2, but concentration of these solutions, even by freeze-frying caused considerable reddening and blackening. By contrast, it was found to be stable to prolonged acid hydrolosis. No attempts were made to isolate the compound directly, but for purposes of measuring the ultraviolet spectrum, the stable glucoside P_1, was isolated. This was then hydrolised in dilute hydrochloric acid, and the spectrum measured directly on this solution.

Experiment 8. Experiments with compound P_2 Acid hydrolysis.

A small sample of P_2 was dissolved in N-sulphuric acid (2 ml.) and the solution heated on a water bath under reflux for 4 hr. Excess acid was removed by adding barium carbonate and centrifuging off the insoluble residue. The centrifugate was concentrated under reduced pressure and divided into two portions. One portion was shaken with a small quantity of Amberlite IR-120 (H^+ form) to remove any remaining barium ions whilst the other was spotted directly onto paper without treatment.

Chromatograms to isolate the carbohydrate component(s) were developed in Solvents C, E, F and G (Whatman No.1), whilst the phenolic component was identified on chromatograms run in 0.1M formic acid. Carbohydrates were located with p-anisidine and alkaline silver nitrate and phenolic compounds with diazotized p-nitroaniline/NaOH.

The only products identified in this way were glucose and DOPA. Some of the DOPA produced in the hydrolysis appeared to be absorbed onto the barium sulphate making identification difficult. Better results could be obtained by carrying out the hydrolysis with N-hydrochloric acid or 50% formic acid. Again in each case the only detectable products were glucose and DOPA. All remaining acid hydrolysis were conducted with these two acids.

Experiment 9. Hydrolysis with β-D-glucoside glucohydrolase.

β-D-Glucoside glucohydrolase (B.D.H.preparation, 2 mg) was added to a solution of P_2 (2 mg in 0.1 ml. water) and the solution incubated for up to 24 hr. at 25°. The products were indentified as glucose and DOPA. This procedure gave similar results to earlier experiments in which the digest was buffered with sodium acetate buffer (0.05M pH 5.1). The presence of the acetate, however, made electrophoresis in formic acid difficult.

Experiment 10. Rate of hydrolysis studies.

A sample of P_2 was hydrolised in 50% formic acid on a boiling water bath and samples of the solution were withdrawn at 15 minute intervals and applied to Whatman No.1 and 3 papers. These were developed in Solvent C and the disappearance of P_2 and the appearance of glucose and DOPA was followed by spraying the chromatograms with alkaline silver nitrate or diazotized p-nitroaniline/NaOH as previously. This showed that hydrlysis was relatively slow, 2 - 3 hours being necessary for complete hydrolysis. It was therefore concluded that the glucoside is in the pyranose form.

Experiment 11. Conformation of the glucosyl residue in P_2.

A sample of P_2 was hydrolised with N-HCl for 90 min. and then the acid was removed under reduced pressure. The residue was dissolved in a little water and streaked onto Whatman No.3 paper which was developed in Solvent C. DOPA and glucose were located on edge strips

with alkaline silver nitrate and the glucose containing strips cut out and eluted with water. The eluate was concentrated and divided into two equal portions, one to act as control, the other being treated with D-glucose oxido-reductase.

After overnight incubation, samples of the control and enzyme treated solution were spotted onto papaer together with standards of D-gluconic acid. Papers were developed in Solvents B, C, E and F, compounds being located with alkaline silver nitrate. This revealed that complete oxidation of glucose had resulted in the enzyme treated sample, and the products, D-gluconic acid and its lactone were present. Thus the glucosyl residue in P_2 has the D-configuration.

Experiment 12. The glucose/DOPA ratio in compound P_2.

Both glucose and DOPA are water soluble, organic solvent insoluble. They are therefore difficult to separate from one another in a quantative manner. Attempts were made to find methods of determining each of them in the presence of the other. Preliminary investigations showed that the enzymic method of Hugget and Nixon (157) for glucose was very much influenced by the presence of DOPA. The phenol-sulphuric acid colourimetric method of Dubois et al (154), however, was not affected at the concentrations studied and was chosen for this work.

Although the iodine colourimetric method for DOPA was not ingluenced by the presence of glucose, it proved to be far less sensitive than the phenol-sulphuric acid method (i.e. far more of the glucoside was needed to yield enough DOPA to give a measurable colour) and a simpler and more sensitive method was to measure the absorbance of the

hydrolysates at 280 mμ and compare this with suitable standards.

P_2 was dissolved in N-HCl to produce a solution with an optical density of approximately 0.5 at 277 mμ. Standard solutions containing known amounts of DOPA and glucose were also prepared. 10 ml. of the P_2 solution and each of the standards was heated under reflux on a boiling water bath for 3 hours and allowed to cool. The absorbance at 280 mu was then measured and samples were taken for glucose determinations by the phenol-sulphuric acid method (Method 3.).

By comparing the P_2 hydrolysate with the standards, the molar ratio of glucose to DOPA was found to be 1.00 : 1.01.

Experiment 13. Position of the glucosyl residue on the aromatic nucleus of P_2.

A small quantity of P_2 (5 mg) was methylated with diazomethane. At the completion of the reaction excess diazomethane was removed with acetic acid. The solution was taken down to dryness under reduced pressure and the residue oxidised by the nitrobenzene/NaOH procedure (Method 7). Isovanillin was indentified as the only aldehydic product on electrophoretograms run in borate buffers at pH 8.7 and 10.0.

Experiment 14. The isolation of a compound resembling P_2 from the cotyledons of V, faba seeds.

The cotyledons from young (1 week) broad bean plants were removed and an aqueous ethanolic (70%) extract prepared. After filtering off insoluble material the solution was extracted with pet. ether (40-60°) and then concentrated under reduced pressure.

Streaks of the extract were applied to Whatman No.3 paper together with standards of the DOPA 3-O-β-D-glucoside (P_2) isolated from DOPA-fed P. sativum seeds.

Chromatography in Solvents B, C, E and F followed by location with diazotized p-nitroaniline/NaOH revealed the presence of a compound having the same chromatographic behaviour and colour reactions as the standard.

The compound was isolated by preparative paper chromatography using Solvent C and then Solvent F. It was finally purified on paper electrophoretograms using 0.1M formic acid as electrolyte. After eluting from the paper with water and freeze-drying a small quantity of the compound was obtained as a cream coloured powder.

Experiment 15. Hydrolysis of the methylated product of the bean compound.

A sample of the DOPA glucoside isolated from broad bean testa was methylated with diazomethane as described previously. The product was hydrolysed by heating for 90 mins. with N-HCl on a boiling water bath.

After cooling, the solution was taken down to dryness under reduced pressure and the residue dissolved in a little water for chromatographic and electrophoretic examination.

Electrophoretograms (formic acid pH 2.4) after location with diazotized p-nitroaniline/NaOH, showed three products, each giving the same colour with the reagent. One of the produced was identified as 4-O-methyl DOPA, after extensive comparison of its properties with an authentic sample of that compound. The identity of the other products was not established. It was shown, however, that neither of these compounds was affected by further prolonged hydrolysis in N-HCl or the concentrated acid.

Experiment 16. Studies with compound P_1.

Preliminary experiments, on the lines described above for P_2, showed that P_1 yielded only glucose and compound P_5 on hydrolysis. In order to determine the glucose /P_5 ratio, the assumption was made that the phenolic compound had the same extinction coefficient as DOPA. With this assumption the value obtained was 0.91/1.0.

A small quantity of P_1 (10 mg) was methylated with diazomethane. The product was dissolved in a small quantity of water, and the solution divided into two equal portions.

One portion was subjected to alkaline nitrobenzene oxidation, the products being isolated and examined in the usual manner. Unlike the case of compound P_2, however, no aldehydic products could be detected.

The other portion was hydrolysed in N-HCl under reflux for 3 hr. Excess acid was removed under reduced pressure, and the residue was examined on paper electrophoretograms (formic acid). Six compounds were located with diazotised p-nitroaniline/NaOH, each giving similar colour reactions to P_1. The nature of these compunds was not determined, but their colour reactions add further weight to the suggestion that P_1 is not a simple DOPA derivative.

Experiment 17. Attempted synthesis of ethyl 2, 3 dihydro-5,6 dihydroxy-3-iodoindole-2-carboxylate.

Attempts were made to synthesise this compound according to the method of Bu'Lock and Harley-Mason (158).

(i) Preparation of L-DOPA ethyl ester.

L-DOPA (2g) was suspended in ethanol (50 ml.) and dry HCl gas passed through. The DOPA slowly went into solution and when the solution was saturated with the gas the flask was stoppered and left overnight.

Removal of the solvent under reduced pressure and drying the residue over P_2O_5 and KOH in vacuuo left a pale amber glass.

This was dissolved in water (10 ml.) and 2.3 g potassium iodate in 20 ml. water added, quickly followed by n-butanol (20 ml.). The reaction mixture was shaken mechanically for 45 mins. and then filtered on a Buchner. The solid was washed carefully with water, methanol, and ether to yield the product, 2, 3-dihydro-3-iodoindole 5,6 quinone-2-carboxylate as long, bright red needles (2.06 g; m.p.129°). This compares with a yield of 1.0 g; m.p.127° obtained by the original authors, who used slightly more starting material. The product was characterised by its U.V. spectrum and by converting it to known derivatives. It is intersting to note that a compound with the same U.V. spectral characteristics as the P_4 aglucone was formed simply by diluting a sample in water. Under similar conditions, however, the quinone could also be isomerised to the dihydroxyindole derivative.

When a solution of the quinone in aqueous ethanol in an atmosphere of nitrogen was treated with solid sodium dithionite, rapid decolourisation occurred. The decolourised solution was extracted with several portions of ether, which were pooled and dried over sodium sulphate. Evaporation of the dried solution yielded a pale yellow gum, which was assumed to be impure 2:3-dihydro-5:6 dihydroxy-3-iodoindole-2-carboxylate. Repeated attempts to crystallise this compound from ethyl acetate light petroleum were all unsuccessful.

Experiment 18. Feeding DOPA to V.faba seeds.

Broad bean seeds were soaked for 2 days in a sturated solution of L-DOPA, and then washed and allowed to germinate for a further 5 days in the dark. Similarly treated seeds soaked in distilled water acted as controls.

Extracts of roots and shoots (combined) and cotyledons were prepared and quantitative determinations carried out, using the iodine colourmetric method. The results for the roots and shoots were similar in both treated and untreated tissues. The results for the cotyledons are given below.

Table 18

	Wt.of tissue (g)	Total DOPA (mg)	DOPA mg/g fresh wt.
Cotyledons (DOPA fed)	18.50	4.12	0.22
Cotyledons (control)	17.75	1.73	0.10

Concentrated extracts were also examined chromatographically. This showed that relatively large amounts of P_1 and P_5 had been formed in the DOPA fed plants, but there was no evidence to suggest that P_2, the DOPA glucoside, was formed in anything but trace amounts.

In a later experiment, the period of growth was extended to 14 days. Extracts of the cotyledons then showed that most of the P_5 had disappeared, presumably by glucosylation to P_1, as this was the only other compound detected in any quantity.

Experiment 19. Feeding experiments.

The feeding experiments were performed with actively growing plants, generally at the flowering stage, during the early part of the Summer.

Suitable lengths of the stem (9" - 12") from each plant were cut by means of a pair of scissors. The lower leaves and shoots were quickly removed and the cut ends dipped into a saturated solution of the compound being fed (50 ml.) contained in a boiling tube. Similar sections were dipped into distilled water to act as controls.

The boiling tubes were set in soil, out of doors, as shown in Fig. 58, and a few days allowed for the tissues to absorb and metabolise the compound. During this time the tubes were inspected daily, and where necessary distilled water was added to the tubes to maintain the level of liquid at a satisfactory level.

At the end of the experiment, leaves were removed from each of the cuttings and extracts prepared in the usual manner ready for chromatographic examination.

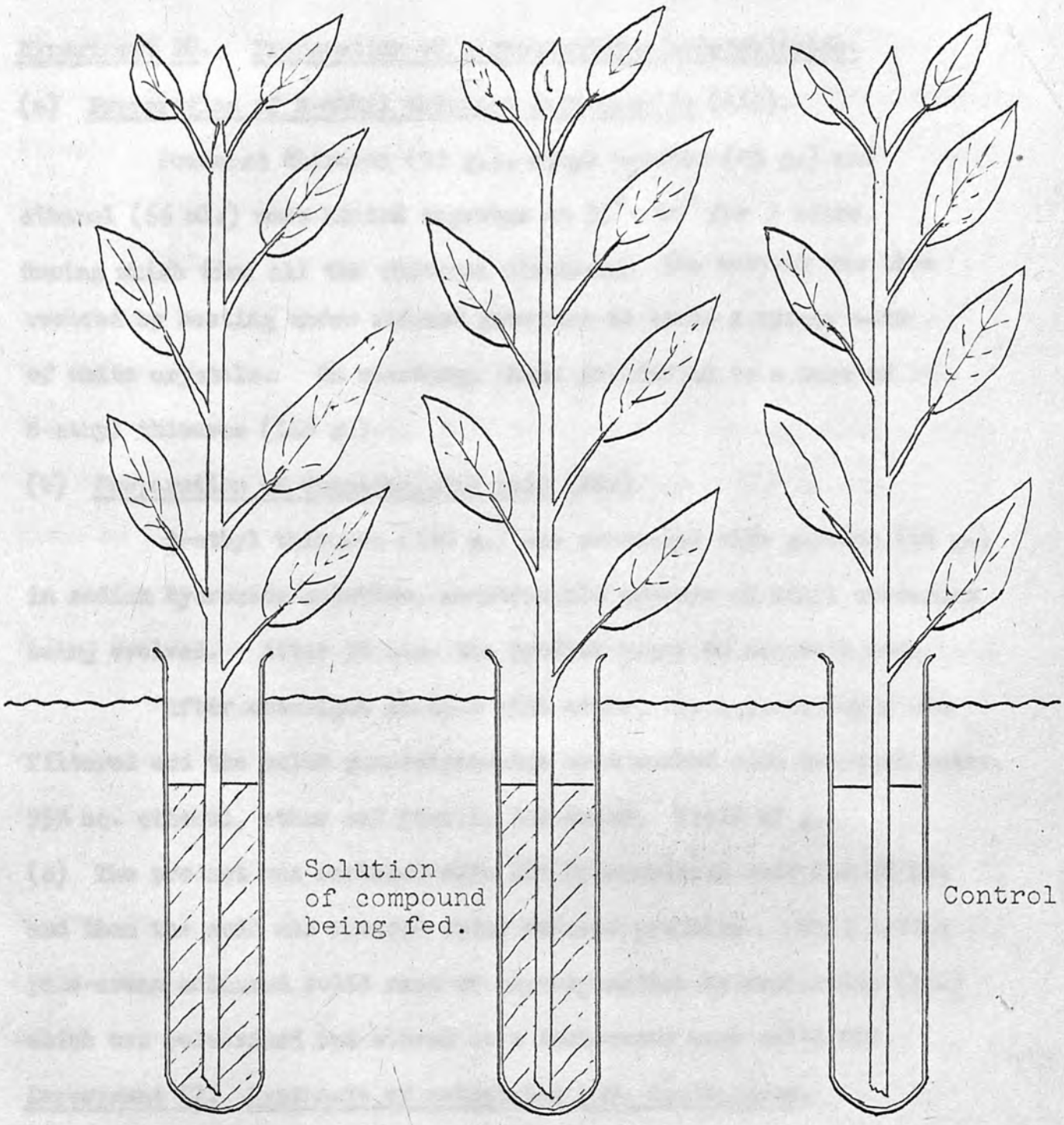


Fig. 58. Feeding Experiments with Plant Tissues.

Experiment 20. Preparation of glycocyanidine hydrochloride.

(a) Preparation of S-ethyl thiourea hydrobromide (159).

Powdered thiourea (50 g.), ethyl bromide (83 g.) and ethanol (66 ml.) were heated together at 55°- 60° for 3 hours, during which time all the thiourea dissolved. The solvent was then removed by heating under reduced pressure to leave a syrupy mass of white crystals. On standing, these solidified to a mass of S-ethyl thiourea (118 g.).

(b) Preparation of Guanidoacetic acid (160)

S-ethyl thiourea (110 g.) was condensed with glycine (48 g.) in sodium hydroxide solution, considerable amounts of ethyl mercaptan being evolved. After 30 min. the product began to separate out.

After overnight contact with ether, the aqqeous layer was filtered and the solid guanidinoacetic acid washed with ice-cold water, 95% aq. ethanol, ether and finally air-dried. Yield 61 g.

(c) The product was refluxed with 18% hydrochloric acid for 18 hr. and then the acid was removed under reduced pressure. This left a pale-cream coloured solid mass of glycocyanidine hydrochloride (161) which was pulverised and stored in a desiccator over solid KOH.

Experiment 21. Synthesis of methylated DOPA derivatives.

As the purpose of these syntheses was to prepare small samples for comparison, no attempt was made to record yields.

(a) N-Methyl-(3-methoxy-4-hydroxyphenyl) alanine - (by the method of Deulofeu and Guerrero (135).

The condensation of creatinine with vanillin was carried out

by heating an intimate mixture of these two compounds at 170° for about 15 mins.,after which a solid mass of impure 5-(3-methoxy-4-hydroxy-benzal) creatinine was deposited. The mass was extracted with ethanol and then washed with water.

The crude product thus obtained was suspended in water and sodium amalgam (3%) added in small amounts (with continuous shaking) over about 30 mins. The suspension first turned brilliant red, but the colour gradually changed, until at the end of the reduction a clear straw-coloured solution was obtained. The whole reduction took about an hour.

The solution was decanted from the mercury and filtered. Dilute hydrochloric acid was added to the filtrate until the pH reached 6.6, and the mixture was stored for 4 hours at 0°. The precipitated material was filtered and washed with a little cold water, finally being air-dried.

The 5-(3-methoxy-4-hydroxybenzyl) creatinine thus produced was finally hydrolised by refluxing with an aqueous suspension of barium hydroxide for 12 hr. The barium was removed by the addition of a slight excess of 6N sulphuric acid. After removing the precipitated barium sulphate and concentrating the solution and adjusting the pH to 8.0 with ammonium hydroxide solution, the product, N-methyl-(3-methoxy-4-hydroxyphenyl) alanine (N-methyl 3-O-methyl DOPA) was obtained.

It gave colourless rhombic crystals (m.p. 232-234o lit m.p. 276-278°) after recrystallising three times from water.

Calc for $C_{11}H_{15}O_4N$; C; 58.65; H, 6.71; N, 6.22%

Found ; C; 58.69; H, 6.75; N, 6.29%

(b) N-Methyl (3-hydroxy-4-methoxyphenyl) alanine.

5- (3-Hydroxy-4-methoxybenzal) creatinine prepared by the condensation of isovanillin with creatinine, as described above, was suspended in water and reduced with sodium amalgam (3%) over a 1 hr. period.

Unlike its 3-methoxy analogue, however, the solution did not eventually change to a pale straw colour, but darkened considerably and on acidification a large quantity of tarry material was deposited. Attempts to purify this were unsuccessful.

The mother liquor was concentrated slightly and left for 14 days, during which time an almost colourless mass of crystals separated out (m.p. 238°). These were filtered off and hydrolised with barium hydroxide. The excess barium was removed with sulphuric acid, and after filtration, concentration and adjustment of the solution to pH 8.0 with ammonium hydroxide, the produce precipitated out.

After storing the mixture for 6 hr. at 0°, the precipitate was removed, dissolved in hot water and allowed to crystallise at room temperature. Recrystallisation was repeated twice more, the N-methyl (3-hydroxy-4-methoxyphenyl) alanine being obtained as rosettes of long colourless needles (m.p. 250-252°).

Calc for $C_{11}H_{15}O_4N$: C, 58.65; H, 6.71; N, 6.22%

Found: C, 58.99; H, 6.74; N, 6.23%

(c) 3-Methoxy-4-hydroxyphenyl alanine.

Vanillin was condensed with glycocyanidine hydrochloride, and the product reduced with sodium amalgam and then hydrolysed with a suspension of barium hydroxide. The resulting 3-methoxy-4-hydroxyphenyl alanine was recrystallised five times from water as long square colourless needles which lost water of crystallisation at 100° and melted at 233-235°.

Calc for $C_{10}H_{13}O_4N.2H_2O$; C, 48.57; H, 6.93; N, 5.67%

Found ; C, 48.47; H, 7.14; N, 5.71%

Johnson and Bengis (136) prepared this compound by an alternative method and also found that it crystallised from hot water with 2 molecules of water of crystallisation. Their compound, which had the same crystal structure as the one prepared by the above method (a photomicrograph was included in their paper), had a m.p. of 255-256°.

(d) 3-Hydroxy-4-methoxyphenyl alanine.

This compound was prepared by the condensation of isovanillin with glycocyanidine hydrochloride fllowed, as before, by reduction and hydrolysis. Attempts to crystallise the product from water and aqueous ethanol were unsuccessful, and it was finally crystallised by dissolution in a minimum quantity of boiling water and addition of ethanol (6 vol.). After storing overnight at 0°, the 3-hydroxy-4-methoxyphenyl alanine separated out as small clusters of long colourless microcrystalline needles (m.p. 240-242°).

Calc for $C_{10}H_{13}O_4N$: C, 56.86; H, 6.20; N, 6.63;

Found: C, 56.76; H, 6.25; N,6.72%

(e) N-Methyl 3,4-dihydroxyphenyl alanine.

N-methyl (3-methoxy-4-hydroxyphenyl) alanine prepared in 'A' was O-demethylated with phosphorus and hydriodic acid. The resulting material, N methyl DOPA, after three recrystallisations from water containing sulphur dioxide, was obtained as colourless rhombic crystals (m.p. 275-277° lit m.p. 282-283°

Cal cor $C_{10}H_{13}O_4N$: C, 56.86; H, 6.20; N, 6.63%

Found: C, 57.06; H, 6.31; N, 6.55%

Experiment 22. Preparation of standard solutions and extracts used with the amino-acid analyser.

Amino-acid solution (Fig.41 (a))

An aliquot (1 ml.) of an 'EEL' mixture of amino-acids supplied for calibration purposes.

B. DOPA, tyrosine, phenylalanine and leucine mixture (Fig.41 (b))

DL-DOPA (192.6 mg.), L-tyrosine (182.2 mg.), L-phenylalanine (172.3 mg.) and L-leucine (134.9 mg.) were dissolved in water (1 l.), and an aliquot (1 ml.) applied to the top of the column.

C. DOPA glucoside (Fig.41 (c)).

A freeze-dried sample of the glucoside (approximately 2 moles), isolated from broad-bean testa, was dissolved in Solution B (2 ml.) and 1 ml. applied to the column.

D. Extract from epicotyls (Fig.41 (d)).

See Experiment 23.

E. Extract from funicles (Fig.41 (e)).

The funicles (5.75 g.) were removed from fully grown pods,

and homogenised in aqueous ehtanol (70%, 250 ml.). The extract was filtered and evaporated almost to dryness under reduced pressure, and then made up to 25.0 ml. with 0.01 N HCl. This solution was again filtered, 1 ml. being applied to the column.

F. Extracts from seed coats (Fig.41 (f)).

The seed coats (20.0 g.) were obtained from fully grown seeds. They were homogenised in aqueous ethanol (70%, 250 ml.), the extract filtered and evaporated down to a volume of about 50 ml. under reduced pressure.

The solution was applied to the top of a small nylon column (12 x 5 cm.) and washed through with a little distilled water. The column was washed with a further 200 ml. water, and the combined eluates evaporated down to 72 ml. under reduced pressure. 2 ml. of this solution was applied to the top of the analyser column.

Experiment 23. Comparison of DOPA determination by the iodine colourmetric and column chromatographic methods.

An extract containing approximately 1 umole of DOPA per ml., was prepared by homogenising the epicotyls (8.81 g.) from 9-day old, etiolated broad bean seedlings in dilute hydrochloric acid (2.01 N.250 ml.) and then filtering.

A known volume of the extract (1 ml.) was applied to the top of the column of the amino-acid analyser and analysed, the remainder being analysed using the iodine colourmetric procedure. In the case of the analyser, the DOPA content of the extract was found by comparing the area under the curve with that produced by a known quantity of DOPA on a previous run. The results are quoted below.

Table 19.

Procedure.	Sample	Vol. (ml.)	O.D. (zero time)	Area under curve(mm^2)	DOPA (umoles)
Analyser	Standard	0.995	-	932	0.977
	Extract	0.995	-	1012	1.066
Iodine colourmetric	Extract	0.80	0.581	-	1.105

These results show a good correlation between the methods.

Experiment 24. Incubation of an amino-acid with dandelion acetone powder.

Dandelion acetone powder (0.4 g) was suspended in Tris-HCl buffer (40 ml., 0.05 M, pH 8.8) containing cysteine (0.1 %) and the appropriate amino acid (0.1 %). The suspension was incubated for 3 hr. at 25° and was then filtered. An aliquot of the filtrate (20 ml.) was taken, diluted with water (30 ml.) and after acidifying with 2N HCl (1 ml.) it was extracted with two portions of ether (100 ml.)

The combined ether extracts were taken down to dryness under reduced pressure. The residue was suspended in ether (5 ml.) and the insoluble material removed by centrifugation. This final step was found to be necessary if strictly comparable and reproducible results were required. The centrifugate was then taken for chromatographic examination.

Experiment 25. Quantitative estimation of cinnamic acid produced from L phenylalanine.

Ethereal extracts from digests containing L phenylalanine were applied quantitatively to a chromatogram together with extracts from control digests. The chromatograms were developed in Solvent B for 16 hr. and then allowed to dry at room temperature before being examined under ultraviolet light. The 'strips' of paper containing the cinnamic acid and control 'strips were cut out and eluted with ethanol (approximately 5 ml.) The solution was then made up to 10 ml. with ethanol and the absorption of each solution measured at 260 mμ.

Experiment 26. Quantitative estimation of the products produced from DOPA.

Ethereal extracts from DOPA digests were separated and the products detected and eluted as described above. Caffeic acid was determined by measuring the absorbance at 320 mμ, and D_1 and D_2 by measuring the absorbance at 330 mμ.

Experiment 27. DOPA decarboxylase activity in the banana.

Attempts were made to demonstrate the presence of DOPA-decarboxylase activity in a number of banana (Musa sp.) tissues such as the skin of ripe and unripe (green) fruit and roots.

Enzymic decarboxylation of amino acids usually involves pyridoxal 5' -phosphate as the codecarboxylase (153). This compound was therefore included in the digests.

Procedure.

Acetone powders or thin root discs (20 mg) were added to a solution of L-DOPA (0.1 % w/v), L -cysteine (1 % w/v) and pyridoxal 5' -phosphate (1 % w/v), in 0.05 M phosphate buffer (pH 7.0) (0.2 ml.) Boiled acetone powders were used as controls.

The reaction mixtures were incubated at 30° and samples were removed at 2 hr. intervals for chromatographic (Solvent B) and paper electrophoretic (0.1 M formic acid) examination.

The banana tissues were shown to be rich in dopamine, and this obscured there general picture. In the case of root tissue and ripe peel, there was no detectable difference in the concentration of dopamine in digests after overnight incubation. In these therefore, the DOPA- decarboxylase activity is either absent or very weak.

In digests containing acetone powder from unripe peel, there was some evidence, particularly from electrophoretograms, that dopamine had been formed after overnight incubation.

PART IX

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Structure of a Dopa Glucoside from *Vicia faba*

β-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) has only been detected in a limited number of plants, and it appears to be confined mainly to leguminous species. Unlike most other phenols of low molecular weight, however, it exists in relatively high concentrations in the free state. This is particularly the case, for example, with the broad-bean, *Vicia faba*[1], but Nagasawa and his associates[2] have also isolated an *O*-β-D-glucoside of DOPA from the testa of the bean seed. The Japanese workers did not, however, determine whether it was a 3- or 4-*O* substituted derivative and in view of our interest in the formation and metabolism of DOPA in plants we have reinvestigated the structure and distribution of this compound.

As *V. faba* contains only small amounts of this DOPA glucoside a preliminary examination of the glucoside obtained in high yield by feeding *Pisum sativum* seeds with DOPA was undertaken and a comparison then made of this compound with the corresponding derivative obtained from the bean.

Seeds of *P. sativum* var. 'Early Onward' were soaked overnight in a saturated solution of L-DOPA. They were then washed and left on moist cotton wool for 4 days in the dark (*cf.* Pridham and Saltmarsh[3]). The resulting seedlings were extracted with aqueous ethanol (80 per cent) and the concentrated extract was examined on paper chromatograms. This showed the presence of at least five compounds which are not normally present in the tissues. By careful fractionation of the extract on paper, using ethyl acetate/acetic acid/water (9 : 2 : 2 v/v) solvent followed by electrophoresis (0·1-*M* formic acid, *p*H 2·4, 4,000 V/1 h), a small quantity of a compound, P_2, was isolated as a syrup which was freeze-dried to a hygroscopic powder. Hydrolysis of P_2 with N H_2SO_4, N HCl, 50 per cent formic acid or β-D-glucoside glucohydrolase yielded only DOPA and D-glucose in the molar ratio of 1 : 1. The configuration of the glucose was determined by reaction with D-glucose oxido-reductase, and the rate of acid hydrolysis of P_2 suggested that the glucosyl residue was in the pyranoid form.

β - (3 - Hydroxy - 4 - methoxyphenyl) - DL - alanine and β-(3-methoxy-4-hydroxyphenyl)-DL-alanine were synthesized as model compounds and their colour reactions with diazotized *p*-nitroaniline/NaOH and ultra-violet spectra under neutral and alkaline conditions determined. This revealed several significant differences between the 3-*O*- and 4-*O*-substituted compounds and in particular a variation in the relative absorbance at ~ 240 and ~ 295 mμ

on addition of alkali. P_2 had all the characteristics of a 3-*O*-substituted DOPA. The absence of a free *O*-dihydroxyl grouping was further indicated by the fact that it gave no colour reaction with aqueous sodium molybdate and did not exhibit a characteristic hypsochromic shift in spectrum under alkaline conditions when treated with borate.

Confirmation of the position of the D-glucopyranosyl residue on the aromatic ring was obtained after methylation of P_2 by the Kuhn procedure with methyl iodide and silver oxide in dimethylformamide[4]. Alkaline nitrobenzene oxidation[5] of the methylated derivative gave rise to isovanillin as the only aldehydic product. This was characterized by paper electrophoresis using phosphate buffers at *p*H 8·7 and 10·0, with 2,4-dinitrophenylhydrazine/NaOH as the locating reagent (isovanillin is readily distinguished from vanillin by this method as the latter possesses a more strongly dissociated phenolic hydroxyl group).

The cotyledons from 4-day-old broad-bean (var. 'Johnson's Longpod') seedlings were extracted and examined by the same procedure, and a compound identical to P_2 in all respects was isolated. The conclusion is, therefore, that both P_2 and the derivative from *V. faba* are β-[3-(β-D-glucopyranosyloxy)-4-hydroxyphenyl]-L-alanine. The colour reactions and chromatographic and electrophoretic behaviour of this compound are summarized in Table 1.

Table 1. PROPERTIES OF DOPA GLUCOSIDE

	P_2 and *V. faba* glucoside
Colour reaction with:	
Diazotized *p*-nitroaniline/NaOH	Plum red—grey green
Ninhydrin	Blue-violet
Sodium molybdate	No reaction
R_{DOPA} *values*	
Butan-1-ol/acetic acid/water (6 : 1 : 2, v/v)	0·47
Butan-1-ol/ethanol/water (40 : 11 : 19, v/v)	0·60
Ethyl acetate/acetic acid/water (9 : 2 : 2, v/v)	0·35
Ethyl acetate/acetic acid/formic acid/water (18 : 3 : 1 : 4, v/v)	0·37
Ethyl acetate/pyridine/water (10 : 4 : 3, v/v)	0·56
Butan-1-ol saturated with water	0·30
M_{DOPA} *values**	
0·1 *M* formic acid (*p*H 2·4)	0·85

* Electrophoretic mobility relative to L-DOPA, corrected for electroendosmosis.

Nagasawa and his colleagues[2] reported that DOPA glucoside occurred mainly in the green testa of a late ripening variety of *V. faba* and to a lesser extent in the hilum and the inner tissue of the pod. Our own chromatographic investigations with the 'Johnson's Longpod' variety confirm their findings but also suggest that this compound is present in both the dormant and germinated cotyledons. There is no evidence, however, for its occur-

rence in either the roots or shoots of young plants or in other issues of the mature plant other than the pods.

Investigations are continuing on the DOPA–DOPA glucoside equilibrium in *V. faba*. The apparent lack of glucoside in the tissues could be due to a weakly active specific glucosylating enzyme (although other phenols are readily glucosylated[3]) or to a physical barrier between the enzyme and its substrate, L-DOPA.

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