# ENZYMES OF CINNAMIC ACID METABOLISM IN PLANTS

R.E.H. Walton, B.A.

Lady Margaret Hall.

Thesis submitted for the degree of Doctor of Philosophy in the University of Oxford.

Long Vacation, 1969.

BODLEIAN LIBRARY OXFORD
DEPOSITED THESIS
15.1.70.

## ACKNOWLEDGEMENTS

The research described in this thesis was carried out in the Botany School, University of Oxford, between 1966 and 1969, with the kind permission of Professor C.D. Darlington.

The supervision by Dr.V.S.Butt of this research, and his help in the preparation of this thesis, is gratefully acknowledged.

I am grateful to Dr.K.W.Fuller for use of growth chambers, to Dr.E.A.Newsholme of the M.R.C. unit in the Department of Zoology for his permission to use the scintillation counter in that unit, and to Mr.P.G.Turner for preparing the photographs.

I acknowledge the constant support of my husband throughout this work.

I am indebted to the Science Research Council for the award of a Research Studentship during this work.

## ABSTRACT

The wide variety of phenolic compounds found in higher plants originate from cinnamic acid. Derivatives of this molecule are formed by two types of conversion: hydroxylation of the benzene ring, and activation of the side-chain. The first hydroxylation is *para* to the side-chain and gives rise to *p*-coumaric acid. The necessity of a carboxylic activation step prior to side-chain conversions which lead to complex phenolic compounds by reduction, esterification, $\beta$-oxidation or condensation, is suggested by thermodynamic considerations, and by analogy with similar reactions which occur in higher plants.

In this thesis the extraction of two enzymes from higher plants is described: one catalysed the *para*-hydroxylation of cinnamic acid, and the other the activation of cinnamic acid. Damage to the enzymes by the high concentrations of phenols and oxidising enzymes released during extraction was reduced by the use of acetone powders, the inclusion of antioxidants such as ascorbate in the extraction buffers, and the use of insoluble polyvinyl-pyrrolidone as a phenolic adsorbent.

*The activating enzyme*

The work on the activating enzyme was concerned with the extraction, measurement, and characterization of an enzyme

capable of catalysing the activation of cinnamic acid, distinct from the enzyme catalysing the activation of acetate.

Four assays for the activating enzyme, based on the characteristics of a CoA-dependent activation proceeding through an acyl-adenylate intermediate, were developed: the hydroxamate test for activated carboxylic groups in which hydroxylamine reacts with an activated intermediate to give the hydroxamic acid, detectable either spectrophotometrically by its ability to form a purple complex with ferric ions under acidic conditions, or chromatographically, using $[2\text{-}^{14}C]$ cinnamic acid as substrate; the measurement of the appearance of cinnamyl-CoA, which has a specific absorption maximum at 311nm.; the measurement of cinnamate-dependent pyrophosphate exchange, which may occur when $[^{32}P]$ pyrophosphate is added to the reaction system in the absence of CoA; and an estimation of the disappearance of the sulphydryl group of CoA, measured spectrophotometrically by the nitroprusside reaction.

Aqueous extracts of acetone powders, and preparations made by extraction of plant material in buffers containing ascorbate, when assayed by the spectrophotometric hydroxamate assay, showed very high activity towards acetate and other aliphatic acids, but little or no activity towards cinnamate. There was some indication that ammonium sulphate fractionation

gave partial seperation of acetate- and cinnamate-activating systems. The two activities also displayed a different pH-dependence and a different stability. They were widely distributed in plant tissues.

With enzyme preparations from leaves of spinach beet plants grown outside in a hot and dry summer, and extracted in the presence of polyvinylpyrrolidone, exceptionally high levels of cinnamate-activating enzyme were measured by the spectrophotometric hydroxamate assay. The activation was very sensitive to the presence of air, and to the presence and type of thiol reagents. Maximum activation occurred in the presence of mercaptoethanol and under an atmosphere of nitrogen. In contrast, the activation of acetate was unaffected by air and by thiol reagents.

After this successful series of experiments it was found impossible to repeat the extraction of high levels of cinnamate-activating enzyme from leaves of plants grown under a variety of conditions. The two sensitive radiochemical assays, and the assay for cinnamyl-CoA were therefore developed, and all plant material was grown under controlled conditions, in order to define its physiological state more exactly. Extracts low in mitochondrial content were prepared in the presence of sucrose to reduce the level of ATP-ase contamination.

The pyrophosphate exchange method measured cinnamate-dependent exchange catalysed by extracts of spinach beet leaves in a few cases. This exchange was completely inhibited by CoA, as in reports of other substrate-dependent exchanges catalysed by CoA-dependent enzymes.

The formation of [$^{14}$C] cinnamyl-hydroxamate was catalysed by extracts prepared from a variety of plant leaves (spinach beet, Beta vulgaris; spinach, Spinacia oleracea; Runner bean, Phaseolus vulgaris, and pea, Pisum sativum). The reaction appeared to be partially inhibited by p-coumaric acid, but not by other phenolic acids. There was a clear relationship between the age of a spinach beet seedling, and the activity extracted from its leaves. The development of the cinnamate-activating enzyme was inversely related to the level of betalain pigment in the leaf, and it was greater when the plant was grown under conditions of high illumination, containing a relatively high proportion of red light.

The cinnamyl-CoA assay showed activation with many of the extracts active with the [$^{14}$C] cinnamyl-hydroxamate method. It also demonstrated that the activating enzyme was not reactive towards the substituted cinnamic acids tested, that the reaction had a broad pH optimum between 7.5 and 8.0, and that the Km value for cinnamate was 4.25 x $10^{-3}$M. This assay measured the greatest activity in

enzyme preparations.

Comparison between the results obtained with the different assays is difficult because they measured the time-course and extent of activation differently. Nevertheless certain conclusions can be drawn. The preliminary studies showed clearly that the cinnamate-activating system is distinct from the acetate-activating system. The more sensitive assays showed that the cinnamate-activating system is dependent on CoA, and may be specific for cinnamic acid, although p-coumaric acid also may be activated. The enzyme probably enjoys a wide distribution in higher plants but its presence is dependent on the physiological state of the plant, and may be under light control.

Different assay methods measured maximum activation at different pH values, and measured different affinities of the enzyme for cinnamic acid. The pH optimum and the Km values measured by the cinnamyl-CoA assay are expected to give the true values of these parameters, since this assay measures the overall reaction in the absence of hydroxylamine, or excessive concentrations of pyrophosphate, and in the presence of CoA, which may be necessary for the full activity of the enzyme. It is also the only assay which measures an initial rate.

The hydroxylating enzyme.

The initial studies on cinnamic acid hydroxylase were leaf disc experiments, in which the formation of labelled p-coumaric acid from exogenous [2-$^{14}$C] cinnamic acid was demonstrated in material from spinach and spinach beet.

Preliminary studies on crude extracts of cinnamic hydroxylase from the leaves of plants in which the hydroxylation had already been established by leaf disc studies, showed that little activity was measured by two different spectrophotometric assays for phenols. A method using [2-$^{14}$C] cinnamic acid as substrate estimated [$^{14}$C] p-coumaric acid after chromatographic separation from the substrate. The formation of p-coumaric acid was dependent on NADPH or NADH and tetrahydrofolic acid. The preference for NADPH or NADH was dependent on the type of plant extract, but no absolute specificity was found.

Control values obtained with boiled extracts always gave greater radioactivity in p-coumaric acid than test values with untreated enzyme, even under optimum conditions of incubation. No completely adequate explanation for this can be offered, although some artifact in the experimental procedure is suspected, since a non-enzymic hydroxylation is unlikely. The activity which may be present, considering this limitation, is similar to the cinnamic hydroxylase activity described in several other tissues.

CONTENTS


| | | Page |
|---|---|---|
| Introduction | | 1 |
| Activation of cinnamic acids | | 2 |
| p-Hydroxylation of cinnamic acid | | 8 |
| Materials and Methods | | 12 |
| Materials : | 1. Chemicals | 12 |
| | 2. Plant materials | 14 |
| | 3. Chemical synthesis and properties of cinnamyl-hydroxamic acid | 16 |
| | 4. Preparation of insoluble polyvinylpyrrolidone for use in enzyme extraction | 17 |
| Methods | | 19 |
| Section 1. | The activation of cinnamic acid. Analytical methods and enzyme assay systems | 19 |
| Section 2. | The hydroxylation of cinnamic acid | 36 |
| | A. Leaf disc studies | 36 |
| | B. Analytical methods | 39 |
| | C. Enzyme assay systems | 42 |
| Section 3. | The determination of protein | 45 |
| Section 4. | The preparation of enzyme extracts | 46 |
| | A. The activating enzyme | 46 |
| | B. Cinnamic acid hydroxylase | 53 |
| Results | | 55 |
| The activating enzyme | | 55 |
| Section 1. | Acetate- and cinnamate-activation in crude enzyme preparations, assayed by the spectrophotometric hydroxamate method | 58 |

Section 2. The use of more sensitive methods for the assay of cinnamate-activating enzyme extracted from leaves of plants grown under controlled conditions ..... 74

The hydroxylating enzyme ..... 86

Section 1. Leaf disc studies ..... 86

Section 2. ~~p-coumaric~~ Cinnamic acid hydroxylation in crude extracts from spinach and spinach beet leaves ..... 88

Discussion ..... 94

The activating enzyme ..... 94

A. The evidence for a cinnamate-activating system distinct from the enzyme catalysing acetate-activation ..... 94

B. The properties of the cinnamate-activating system ..... 97

C. The variation of cinnamate-activating enzyme levels with physiological conditions 109

Cinnamic acid hydroxylase ..... 114

A. Leaf disc studies ..... 114

B. Studies with crude extracts ..... 116

Bibliography

# INTRODUCTION

The wide variety of phenolic compounds found in higher plants are in many cases known to be intimately concerned with the biochemistry, physiology and development of these plants. For example, lignin is a major structural component of higher plants, and the flavonoid pigments may determine the colour of flowers, fruits and leaves. Other phenolics of various types confer a characteristic taste on tubers, fruits and leaves, and have been implicated in the control of spore and seed germination (Van Sumere, 1960), and as components of systems acting in the varietal resistance of plants to infection (Cruickshank & Perrin, 1964).

Phenolic compounds have in common one or more ring systems derived from cinnamic acid (Fig.1). When plants are fed with particular cinnamic acids, they form derivatives with the same or closely similar ring patterns. Hess (1967) showed that the anthocyanins formed in Petunia petals were structurally related to the cinnamic acids which he supplied. Cinnamic acids may also be esterified, reduced, or undergo side-chain β-oxidation.

Thermodynamic considerations suggest that before these conversions can occur, the carboxylic group of the acid must be activated. Analogous reactions in plant and animal tissues have been shown to involve an activation step. The

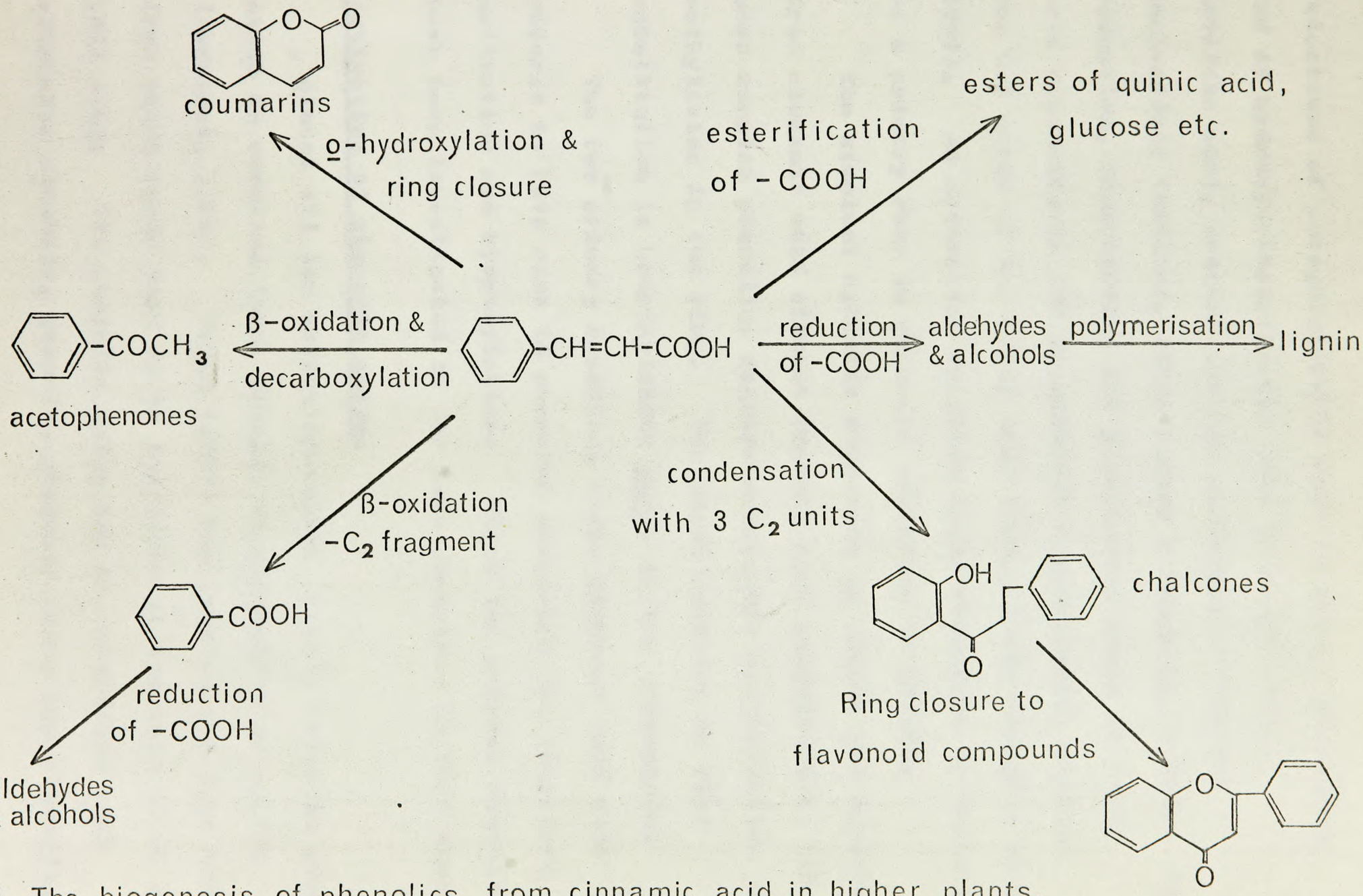



Fig.1. The biogenesis of phenolics from cinnamic acid in higher plants.

reductions of phosphoglyceric acid to triose phosphate, and of hydroxymethylglutaric acid to enzyme bound mevaldic acid, proceed through carboxylic-activated derivatives (Stiller, 1962; Porter & Anderson, 1967). The reduction, condensation and β-oxidation steps in fatty acid biosynthesis and degradation respectively, utilise the CoA ester of the fatty acid (Mudd, 1967; Stumpf & Bradbeer, 1959). An activation reaction would therefore by analogy, be a primary step in phenolic synthesis in plants.

The simplest aromatic compounds in plants are derived from cinnamic acid without further ring substitution, but more complex phenolics require successive hydroxylation and methylation in the ring. The first reaction in ring substitution is hydroxylation para- to the side-chain.

The two primary reactions which cinnamic acid must undergo to give rise to phenolic compounds are therefore activation and hydroxylation. These two enzymic reactions have been investigated in the work described in this thesis.

Activation of cinnamic acids.

Almost all low molecular-weight phenols occur in plant cells in combined form, usually as glycosides or esters (Harborne, 1964). Brown (1967) has pointed out that the free acids would tend to be insoluble at the acid pH of most cell saps. The phenolic acids may be esterified with aliphatic alcohols, phenols, phenolic acids and alcohols,

alkaloids, flavonoids, sugars and lignin. The glucose esters of phenolic acids are widely distributed and are formed when plants are fed with the appropriate uncombined acid (Towers, 1964). Free phenols are usually present only in storage organs or in dying or dead tissue.

It is likely that the intermediates of phenolic metabolism are more active compounds than these simple esters of cinnamic acids. The necessary activation of the carboxylic group has been suggested to require the formation of CoA esters (Grisebach, 1962; Neish, 1964; Towers, 1964).

Cinnamic acid may be the first, and normally the only acid to be activated before conversion. It is also the activation reaction which can be most easily investigated, since cinnamic acid contains no phenolic hydroxyl group, and is therefore not susceptible to either the cresolase or catechol oxidase activity of the phenolase system frequently found in crude extracts of plant material (Mason, 1957; Kertesz and Zito, 1962).

The occurrence of metabolically-active bound forms of cinnamic acid and its phenolic derivatives in acetone powders of wheat and barley plants has been reported by El-Basyouni & Neish (1966). They suggest the formation of cinnamyl-enzyme complexes, formed initially by the activation of cinnamic acid itself, which undergo hydroxylation and methylation before incorporation into esters and flavonoids, or reduction to alcohols. The increase in alkali-labile

esters of p-coumaric, ferulic, and sinapic acids which parallels the accumulation of lignin products in excised tissues of Sorghum vulgare (Stafford, 1967) supports this hypothesis. The co-distribution of phenylalanine and tyrosine ammonia lyases, cinnamic hydroxylase, and a weak polyphenolase activity in a particulate fraction from Sorghum (Stafford, 1969) is also in agreement with the proposed series of reactions. This type of complex bears some resemblance to the fatty acid synthetase or electron-transport particles studied in animal systems (Mudd, 1967; Green & Silman, 1967).

The role of CoA thiolesters of cinnamic acids in plant phenolic metabolism has been considered by Gross & Zenk (1966). They prepared several cinnamyl-CoA esters by the action of acyl- CoA synthetase prepared from bovine liver mitochondria and investigated their properties. They also described the preparation and properties of the hydroxamic acids of various cinnamic acids. Several assay methods for the enzymic activation of cinnamic acid, used in the work described in this thesis, have been developed from their observations.

There have been a few reports in the literature of the activation of cinnamic acid and its phenolic derivations. Zenk & Gross (1965), and Zenk (1966) described the preparation of a cell-free extract from Polystictus versicolor, L., which catalysed the reduction of [$^{14}$C] veratric acid (3, 4-dimethoxybenzoic acid) to the aldehyde and alcohol.

The reduction was ATP-dependent and required NADPH or NADH as reducing agent. CoA did not appear to take part in the reduction as its presence was not necessary for the reaction, and synthetic veratryl-CoA was not reduced. An activated intermediate could be trapped as the hydroxamate but its nature was not determined.

Gross, Bolkart & Zenk (1968) have also described the characterization of two distinct enzymes from Neurospora crassa which catalysed the reduction of a number of cinnamic acids to the corresponding cinnamaldehydes and cinnamyl alcohols. The first enzyme catalysed the reduction of aromatic acids to the corresponding aldehydes. It was purified 300-fold, and specifically required NADPH and ATP for activity. A reduced thiol was also necessary for maximum activity. The enzyme had a broad specificity towards aromatic acids of the $C_6$-$C_1$, $C_6$-$C_2$ and $C_6$-$C_3$ types. No evidence for activated intermediates was obtained. The second enzyme catalysed the reversible reduction of aromatic aldehydes to alcohols.

In these two reports, Gross and Zenk pointed out that there has been no report of corresponding enzymes in higher plants. They suggested that fungi might carry out cinnamic acid activation by a more primitive mechanism than higher plants, so that in the latter a method of activation involving CoA esters may operate.

There is abundant evidence in higher plants for CoA-dependent activation of aliphatic acids. Millerd and Bonner (1954) studied the activation of acetate, and the condensation of the acetyl-CoA formed to acetoacetate, using an acetone powder from spinach leaves. The enzyme which catalysed acetate-activation was found in all other plant tissues analysed, and had a wide specificity towards aliphatic acids. It was tentatively located in the mitochondria.

Most other studies on the activation of aliphatic acids have been concerned with fat metabolism, and in this respect, the activation of acetate (Hiatt, 1960, 1964; Rebeiz et al, 1965b), malonate (Hatch & Stumpf, 1962), propionate (Stumpf & Bradbeer, 1959) and several long-chain fatty acids (Rebeiz et al, 1964, 1965a) has been established. The presence of CoA was obligatory for activation in all cases. Rebeiz et al established that the activation and $\beta$-oxidation systems for long-chain fatty acids in peanut cotyledons was extra-mitochondrial. The activation system appeared to be particulate, whereas the $\beta$-oxidation system was soluble. The acetate-activating system was also soluble. The cellular location of short-chain fatty acid activating systems has not in general been examined, but several active preparations have been made from mitochondrial fractions.

The activation of cinnamic acids has been described only in a single report by Grisebach et al (1966), who used partially purified preparations from Cicer arietinum (chick pea) and Apium petroselinum (parsley). They measured the activation of a number of acids by the hydroxamate test, and found that the acids activated most readily were acetate and succinate. The reaction was more than 70 times as effective with these acids than with any cinnamic acids tested. Most other mono-, di-, and tri-carboxylic acids tested were also activated more effectively than the cinnamic acids. Considering the low specificity, insensitivity and unreliability of this assay method (Peterson, 1967), and the low activation figures reported for cinnamic acids, it seems unlikely that an enzyme specific for cinnamic acid activation was characterized in this work. The activating enzyme preparations did not promote a cinnamate-dependent pyrophosphate exchange, nor a transacylating activity from acetyl- or succinyl- CoA to cinnamic acids.

The work on the activation enzyme presented in this thesis will be considered in two sections:

1. Preliminary studies on cinnamate activation in crude extracts from spinach beet leaves and other plant material. This work led to a definition of the optimum conditions for extraction of the enzyme and for the assay of the enzyme by a spectrophotometric hydroxamate method. It also

furnished evidence for differences between the acetate - and cinnamate- activating systems.

2. The development of more precise assay methods for cinnamate-activation, including an estimation of the activation of [$^{14}$C] cinnamic acid by isolation of its radioactive hydroxamate, and the measurement of the appearance of cinnamyl- CoA. This work led to a definition of the conditions under which a cinnamate-activating enzyme can be extracted from plant tissue.

p-Hydroxylation of cinnamic acid.

During a long period of time it was not possible to obtain plant material containing the activation enzyme measured by the spectrophotometric hydroxamate method. Results obtained in this laboratory showed that feeding cinnamic acid to spinach and spinach beet leaf discs gave rise to p-coumaric acid. An alternative fate of cinnamic acid is therefore a para-hydroxylation. A possible explanation of the inability to find activating enzyme on various occasions may be that hydroxylation was at these times successfully competing with activation for the free acid available. Hydroxylation may normally take place after activation. This is probable since uncombined phenolic acids do not normally exist in the plant and the work of El-Basyoumi et al and of Stafford has shown the existence of active and bound forms of phenolic acids. Also, Levy

and Zucker (1960) have demonstrated that the second hydroxylation step from p-coumaric to caffeic acid can take place with these acids as esters of quinic acid, and Stafford (1969) has shown that chlorogenic acid was a more active substrate than unesterified phenols for polyphenolase action.

When this work was commenced, the only report of cinnamic acid hydroxylation was by Nair and Vining (1965a), who had prepared an aqueous extract of an acetone powder from spinach leaves capable of catalysing the conversion of cinnamic acid to p-coumaric acid. The enzyme was unstable, and could not be purified further or extracted consistently. The requirement for an external electron donor was absolute; NADH and NADPH were equally effective, and tetrahydrofolic acid, when added together with either of these electron donors, induced activity in excess of the sum of each acting separately. The pH optimum of the enzyme was 4.6, and it had no activity at pH 7.0.

Russell and Conn (1967) have since extracted a cinnamic acid hydroxylase from pea buds which had an absolute specificity for NADPH for which NADH would not substitute. Tetrahydrofolic acid again caused a greater stimulation in the presence of NADPH than when supplied alone. This enzyme appeared distinct from that described by Nair and Vining since the pH optimum was 7.5, and its

electron donor requirements were different.

Nair and Vining established that their crude enzyme preparation also possessed phenylalanine hydroxylating activity, and that the two activities were each inhibited by the other substrate. The phenylalanine hydroxylating activity, unlike the cinnamic acid hydroxylating activity, could be further purified, when it lost all its reactivity towards cinnamic acid (Nair & Vining, 1965b). The purified enzyme could still utilise both NADH and NADPH, and retained its low pH optimum and inactivity at and above pH 7.0. It seems probable that the reactivity towards cinnamic acid may be due to a broad specificity exhibited only by the crude enzyme preparation.

Amrhein and Zenk (1969) have found an enzyme similar to that of Russell and Conn in a preparation from buckwheat hypocotyls. Stafford (1969) has also found the enzyme in a preparation from Sorghum. In view of these reports it was interesting to establish whether spinach possesses a cinnamic acid hydroxylating enzyme with properties similar to the enzyme prepared by Russell and Conn, and whether its presence was in any way linked with that of the activation enzyme.

The work on the hydroxylation of cinnamic acid presented in this thesis is divided into two sections:

1. Studies with leaf discs from spinach and spinach beet to provide evidence for the conversion of cinnamic acid to p-coumaric acid in these tissues.
2. The preparation of crude extracts from these materials capable of catalysing the conversion of cinnamic acid to p-coumaric acid, assayed by spectrophotometric and radioactive methods.

# MATERIALS AND METHODS

## Materials

### 1. Chemicals

i) Substrates and antioxidants. Cinnamic acid (micro-analytical standard, British Drug Houses, Ltd.) was used without further purification. p-Coumaric, caffeic, ferulic and sinapic acids (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) were recrystallised from 50% ethanol until colourless. Ethyl cinnamate (laboratory reagent), hydroxylamine hydrochloride (analytical reagent), ascorbic acid (biochemical grade) and sodium diethyldithiocarbamate (analytical reagent) were obtained from British Drug Houses Ltd., and used without further purification.

ii) Thiol reagents. The following thiol reagents were used without further purification: glutathione, crystalline reduced form (Sigma London Chemical Co.,); dithiothreitol (Cleland's reagent), A grade (Calbiochem.Ltd., Los Angeles, U.S.A.); 2-mercaptoethanol (Koch-Light Laboratories). These reagents were all stored in a refrigerator, and all except 2-mercaptoethanol in a desiccator.

iii) Cofactors. The following cofactors were obtained from the Sigma London Chemical Co. in the forms and grades

indicated: ATP, disodium salt from equine muscle (Sigma grade, 99-100% pure); CoA, free acid from yeast (Grade III, approx. 98% pure); NADPH, tetrasodium salt, enzymatically reduced (Type II, 90-95% pure). These reagents were all stored in a desiccator at -10° to -15°. Tetrahydrofolic acid (Grade III, Sigma London Chemical Co.), and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (dimethyltetrahydropteridine), B grade (Calbiochem. Ltd.) were stored under an atmosphere of nitrogen at -10° to -15°.

iv) Preparative reagents. Polyvinylpyrrolidone was obtained in its insoluble form, Polyclar AT, from Fine Dyestuffs and Chemicals Ltd., Manchester, England, and washed by the procedure outlined on p. 17
Sephadex-G25 (coarse grade) was obtained from Pharmacia, Uppsala, Sweden.

v) Radioactive chemicals [2-$^{14}$C] cinnamic acid (Tracerlab Division Laboratory for Electronics Inc., Waltham, Mass., U.S.A.) was obtained as carrier-free solid, and dissolved either in 0.5% $NaHCO_3$ or 1% $KHCO_3$ to provide solutions containing 10 and 20 μc/ml. respectively. [$^{32}$P] tetrasodium pyrophosphate (The Radiochemical Centre, Amersham, England) was obtained as anhydrous solid and dissolved in water.

Where possible, all other reagents used were of analytical grade and solutions were made up in most cases in water which had been distilled twice, the second distillation over glass. Where this water was not used, deionized water was employed, which had been distilled before passing through an Elgastat (Elga Products Ltd., Lane End, Bucks).

2. Plant materials.

Spinach beet (Beta Vulgaris, L.) and Spinach (Spinacia oleracea, L.) were grown outside in beds of soil during the summer and early autumn months. Winter and spring growths were not used, as the plants were severely affected by frost. At other times, spinach beet was grown in the greenhouse in soil in boxes. Broad bean (Vicia faba, L., var. Seville Longpod), runner bean (Phaseolus vulgaris, L., var. Prizewinner), and pea (Pisum sativum, L., var. Alaska or Meteor) plants were also grown in the greenhouse.

Experiments using plants grown under controlled conditions were carried out with material grown either in a large growth room at 25°, with a 16hr.-day, or in a small growth cabinet, with the same temperature and day length. The character and intensity of the light was different in the two growth chambers. The large growth room was illuminated by "daylight" type fluorescent tubes, and the

average light intensity at soil height was 1083 foot-candles. The growth cabinet was lit by "natural" type fluorescent tubes, and the average light intensity at soil height was 1400 foot-candles. Both sets of measurements were taken with a Megatron light meter, which measures light intensity over the whole spectrum. The spectral characteristics of the two types of fluorescent tube are shown in Figs. 2 and 3. Fig.4 shows an average energy distribution curve for direct sunlight, for comparison. It can be seen that "natural" tubes emit a greater proportion of red light than "daylight" tubes, and that their spectral characteristics bear a closer resemblance to ordinary sunlight.

The conditions in the growth cabinet were much more defined than those in the growth room, since light was completely excluded during the dark period, and the plants grew in a continuous stream of air. In the grouth room, a low level of background daylight could penetrate during the dark period, and the air movement was low as it was achieved only by means of a small fan. The two different methods of air circulation tended to lead to excessive dehydration in the growth cabinet, and to excessive wetness in the growth room. Consequently, some form of damping-off disease sometimes killed the plants in the growth room, but this was largely eliminated by treating all seed boxes immediately after planting with Murphy's combined seed

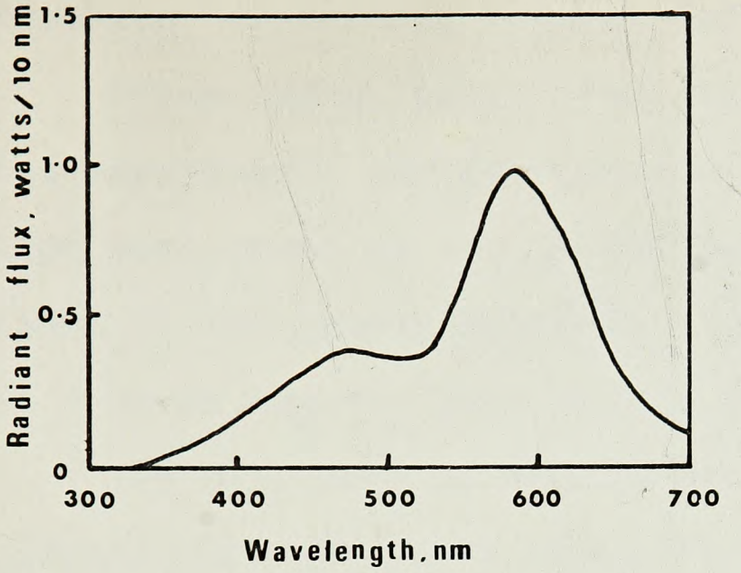



FIGURE 2

Spectral flux distribution of DAYLIGHT fluorescent tube

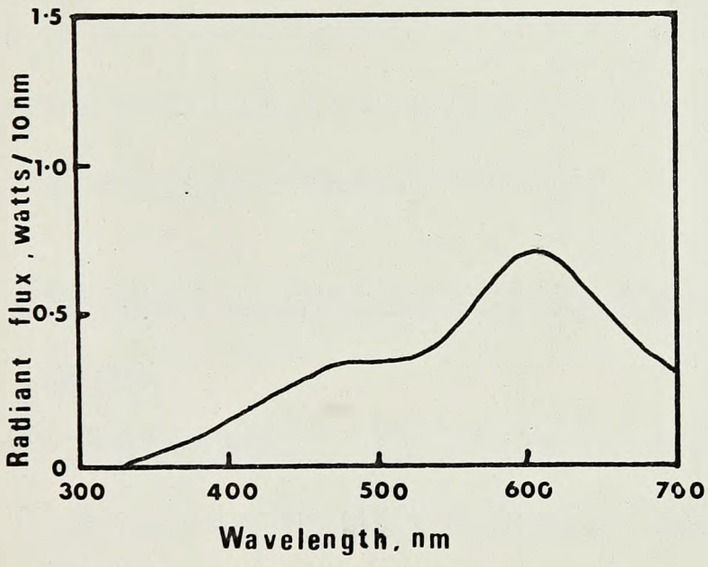



FIGURE 3

As Figure 2 for NATURAL fluorescent tube

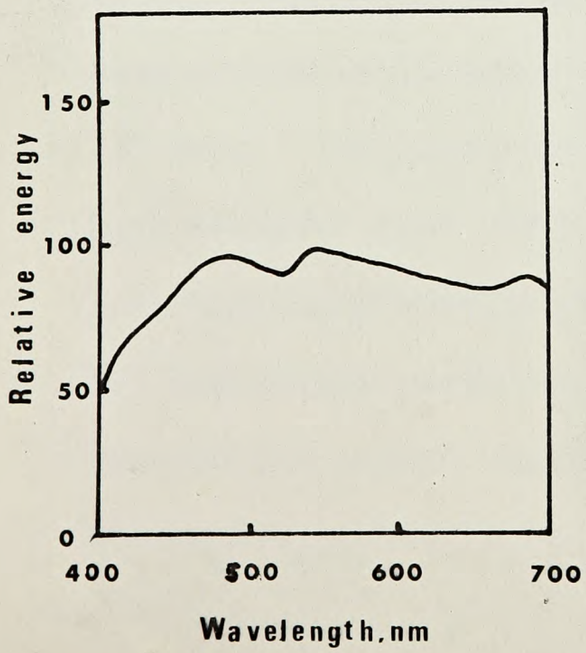



FIGURE 4

Average energy distribution curve for DIRECT SUNLIGHT

figs. 2 & 3 after Canham (1966)

fig. 4 after Rabinowitch (1951)

dressing (containing Lindane and Captan).

Runner bean, broad bean, cabbage (Brassica oleracea, L., var.Primo), pea (var.Alaska), spinach and spinach beet were grown in the growth room. Spinach beet only was grown in the growth cabinet. The plants were harvested at a known age and just before the end of the light period.

The only part of the plants used for experimental purposes was the leaf, except for epicotyls from pea seedlings (var.Meteor), grown in sand boxes for 4 to 6 days in a dark incubator at 25°; and roots from maize (Zea mays), grown between wet paper towels in trays for 4 to 5 days in a dark incubator at 30°.

3. Chemical synthesis and properties of cinnamyl-hydroxamic acid.

i) Synthesis. Cinnamyl-hydroxamic acid was prepared by treating ethyl cinnamate with hydroxylamine in alkaline solution, according to the method of Gross and Zenk (1966) who modified a corresponding synthesis for benzohydroxamic acid described by Hauser and Renfrow (1959). Attempts to recrystallise the crude cinnamyl-hydroxamate from 7.5% acetic acid as described by Gross and Zenk repeatedly yielded a red lower layer consisting of hydroxamic acid with impurities, and a white emulsified upper layer. The crude hydroxamate was therefore purified by recrystallisation from chloroform

three times, further impurities being removed each time by the addition of charcoal to the hot chloroform solution. Only about 20% of the original 55% yield was recovered from this operation. The purified material was stored in a desiccator at $-10^{\circ}$ to $-15^{\circ}$.

ii) Properties. The spectrum of the ferric complex of cinnamyl-hydroxamate was measured in a Beckmann-DB spectrophotometer, and is shown in Fig.5. It has a flat peak from 530nm to 560nm., the mid-point being at about 546nm. This wavelength was used to measure the concentration of cinnamyl-hydroxamate in the assay system described in a later section.

4. Preparation of insoluble polyvinylpyrrolidone (Polyclar AT) for use in enzyme extraction.

Purification of Polyclar AT was carried out according to the procedure outlined by Loomis and Battaile (1966). Samples of Polyclar AT were boiled in 10% HCl for 10 min. and then washed thoroughly with water until free of chloride ions detectable by the silver nitrate test.

The purified material was stored in a stoppered container at room temperature and used within three months. When it was stored for longer periods of time, fine particles separated out and interfered with the spectrophotometric estimation of hydroxamate. These

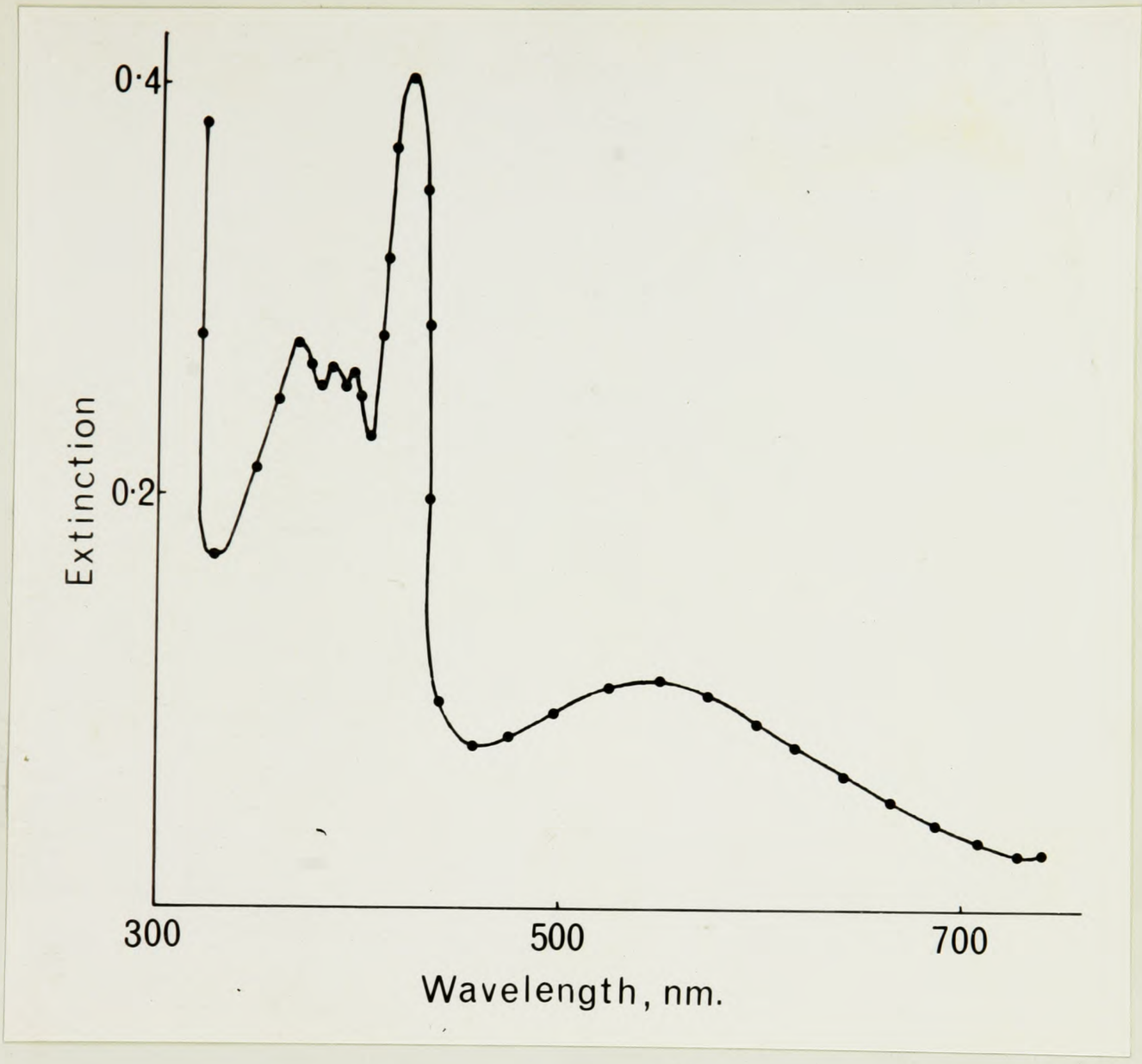



Fig. 5. Absorption spectrum of the ferric complex of cinnamylhydroxamate in acidic solution under the conditions of assay.

particles however did not seem to interfere with the effectiveness of the Polyclar in complexing with phenols in crude extracts of plant material.

## Methods

### Section 1. The activation of cinnamic acid. Analytical methods and enzyme assay systems.

A CoA-dependent enzymic activation of cinnamic acid would, by analogy with other activating systems, proceed through two stages involving an enzyme-bound acyl-adenylate intermediate, as shown in Fig.6.

An enzyme assay system must take into account the various requirements of activating enzymes that have already been established:

i) the pH optima of all systems so far studied lie above neutrality;

ii) a thiol reagent is necessary for the measurement of activation in many crude extracts (Millerd & Bonner, 1954; Peterson, 1967);

iii) $Mg^{++}$ ions are required for the first partial reaction (acetate-activating enzyme; Webster, 1965), or for both partial reactions (amino acid-activating enzymes; Peterson, 1967).

iv) $K^{+}$ ions stimulate both partial reactions of acetate - (Webster, 1966; Hiatt, 1960, 1964) and amino acid- (Peterson, 1967) activation.

Four assay procedures basdd on different characteristics of the enzyme reaction have been developed (Fig.6):

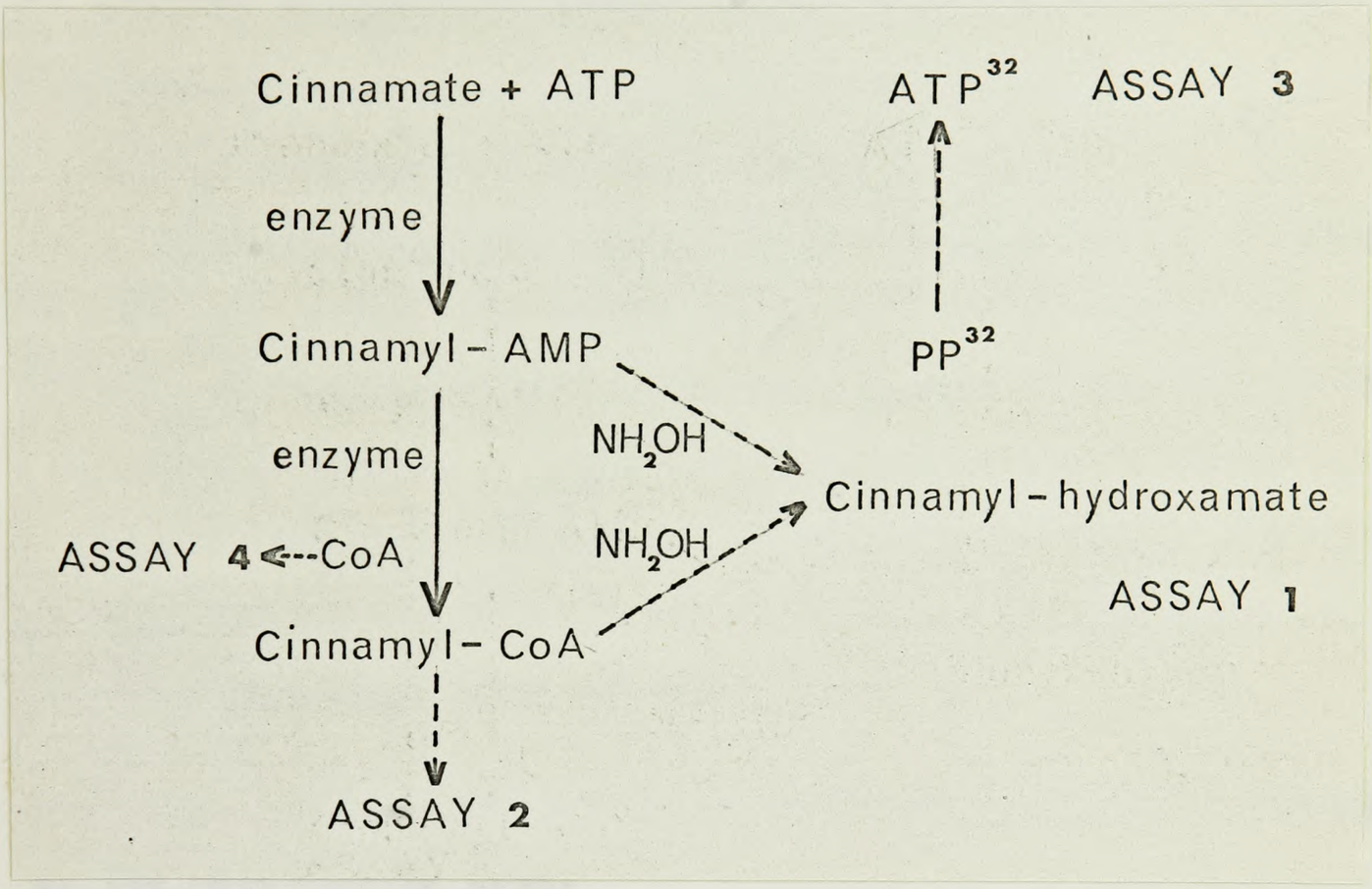



Fig.6. A mechanism for the enzymic activation of cinnamate, showing the various methods of assay used.

1. The hydroxamate assay, which is a non-specific test for activated carboxylic groups, in which hydroxylamine reacts with an activated group to give the hydroxamic acid. This is detectable either spectrophotometrically by its capacity to form a purple complex with ferric ions under acidic conditions, or chromatographically.
2. A spectrophotometric assay for the appearance of cinnamyl-CoA, which has a specific absorption maximum at 31inm.
3. An assay measuring the cinnamate-dependent pyrophosphate exchange occurring when $[^{32}P]$ pyrophosphate is added to the enzyme assay system in the absence of CoA.
4. A procedure for the estimation of the disappearance of the sulphydryl group of CoA.

Assay 1. The determination of activated carboxylic groups by the hydroxamate test.

a) the spectrophotometric method

This method was originally described by Lipmann and Tuttle (1945) for the determination of acyl phosphates. It was used to measure the activation of acetate and other aliphatic acids in plant extracts by Millerd and Bonner (1954).

Method of the assay

The assay was carried out in sealed 25ml. flasks containing an atmosphere of air or nitrogen, and shaken in a water bath

at 30°. The assay system contained: tris-HCl buffer (110 μmoles, pH 8.5); ascorbic acid (26 to 52 μmoles); cinnamic, acetic, or other acid (5 μmoles); $MgCl_2$ (5 μmoles); $NH_2OH$ (200 μmoles); thiol reagent (5 μmoles); ATP (5 μmoles); CoA (0.3 μmoles), and the enzyme sample in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0), in a total volume of 2.0ml. Suitable controls (the above system without either substrate or CoA) were included. The $NH_2OH$ solution was made up freshly by neutralising a solution of $NH_2OH.HCl$ (4M) with an equal volume of KOH (20%). The $NH_2OH.HCl$ solution was stored in a refrigerator and renewed frequently (at intervals of 14 days) because of the instability reported by Raacke and Bové (1960). The substrate was added in solution in tris-HCl buffer of the appropriate pH, or as the potassium salt.

After incubation for up to 6 hr., 1 ml. trichloracetic acid (12%) was added to each flask, and the insoluble material separated by centrifugation. The total volume of supernatant was assayed for hydroxamate by adding 1 ml. HCl (2.9M), followed by 1 ml. $FeCl_3$ (5% in 0.1M-HCl). The extinction was measured at 546nm. Standard curves were prepared for acetate activation by adding a fresh solution of acetyl phosphate to the mixture used for enzyme assay, and for cinnamate activation by adding cinnamyl-hydroxamate to the mixture used for enzyme assay. The colour was developed

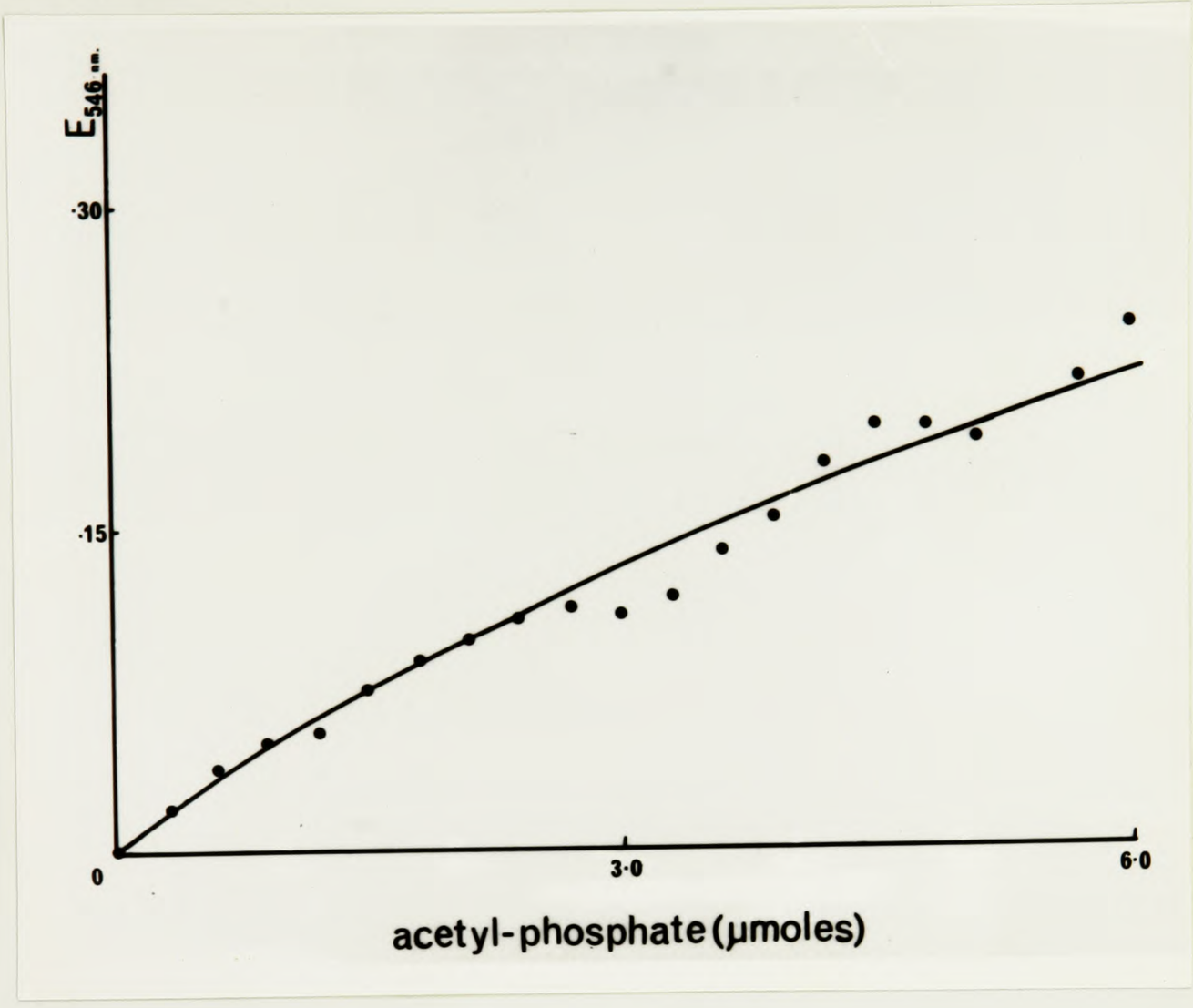



Fig.7. Calibration curve for acetyl-phosphate using the hydroxamate estimation under the conditions of enzyme assay (p. 21 ).

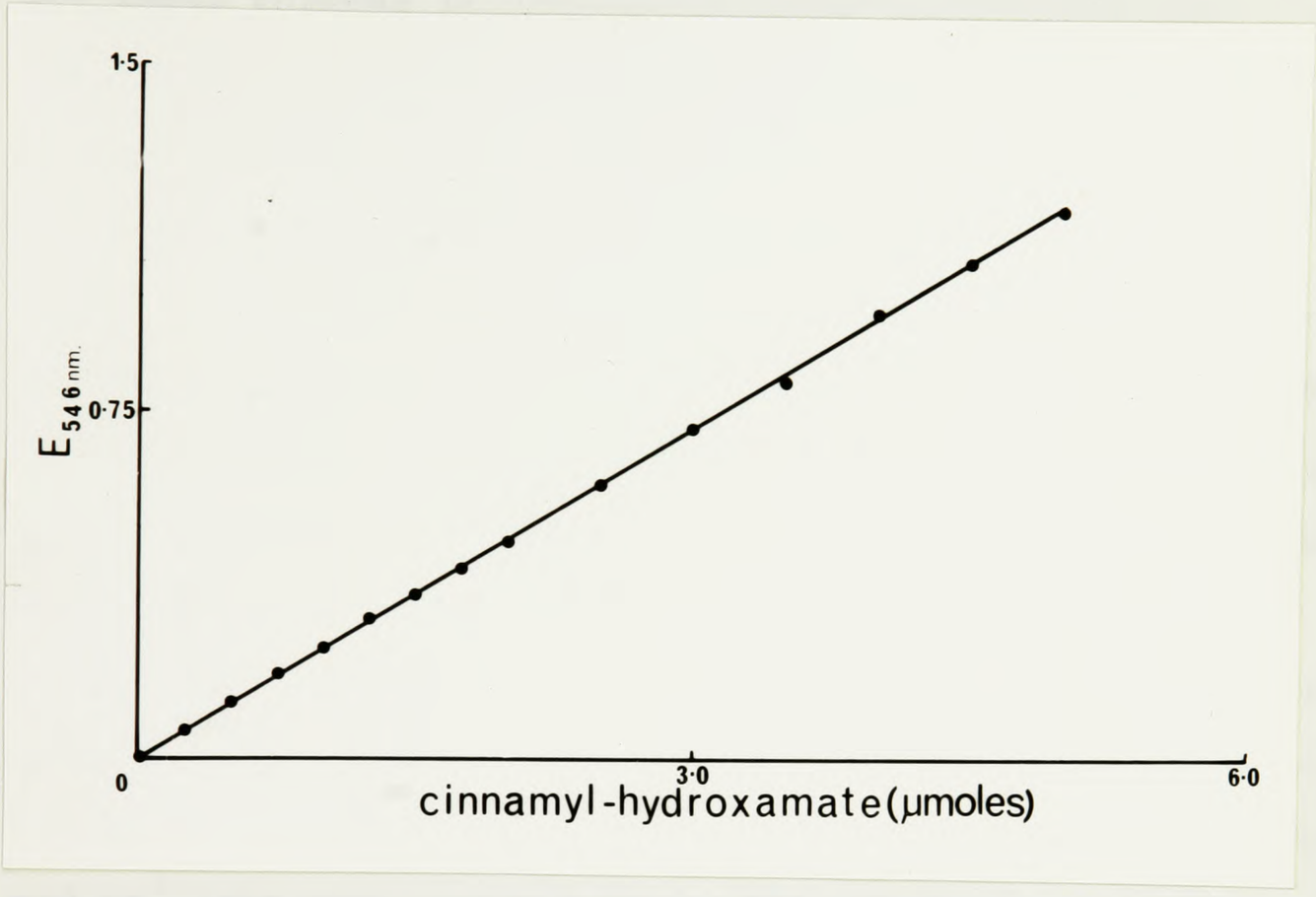



Fig. 8. Calibration curve for cinnamyl-hydroxamate under the conditions of enzyme assay (p. 21).

by adding successively trichloracetic acid, hydrochloric acid, and ferric chloride solutions. The calibration curves are shown in Figs.7 and 8 respectively. It can be seen that the small changes in extinction usually observed when the assays were carried out, represented much larger amounts of acetyl-hydroxamate than cinnamyl-hydroxamate. The standards were prepared under the conditions of enzyme assay because a number of anions liable to be present in the assay system (phosphate in particular), depress the colour development of the ferric complex (Lipmann & Tuttle, 1945).

In the early stages of the work, ascorbate was absent from the assay system, $Mg^{++}$ was added as the sulphate derivative, the thiol reagent used was glutathione, and the pH of the assay buffer varied from 7.4 to 8.5. The pH and buffer type used for enzyme extraction were also variable, and $Na^{+}$ ions were present since the substrate was often added as Na Salt, and the hydroxylamine hydrochloride was neutralised with NaOH instead of KOH. It was therefore important that fresh standard curves were prepared as the conditions were altered. In these early stages the control generally used was the complete system without CoA, since absence of CoA or substrate gave a similar value.

For some of the experiments carried out in an atmosphere of nitrogen, the time course of the reaction was studied by sampling in the apparatus shown in Fig.9. The total volume

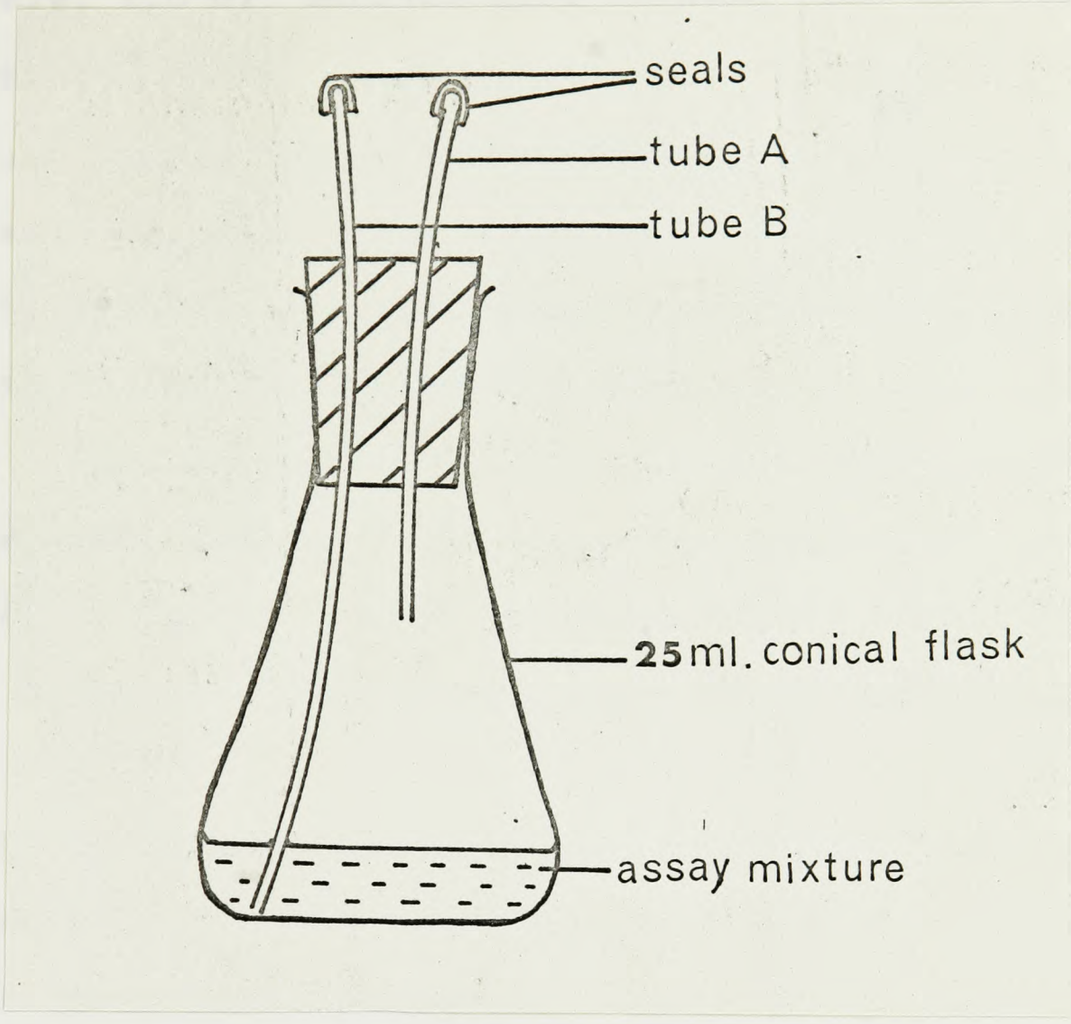



Fig. 9. Sampling apparatus for enzyme assay under nitrogen.

of assay mixture was then adjusted to a volume appropriate to the number of samples to be taken. After known intervals, 1.0 ml. samples were ejected from the flask through tube B, by blowing a stream of nitrogen through tube A. Any air drawn into tube B after the sample had been taken was removed by flushing the system with nitrogen through tube B. The sample was collected in a graduated centrifuge tube containing 0.5ml. trichloracetic acid (12%). The insoluble material was separated by centrifugation and the total supernatant assayed for hydroxamate by the addition of 0.5ml. HCl (2.9M), followed by 0.5ml. $FeCl_3$ (5% on 0.1M-HCl), and determination of the extinction at 546nm.

The spectrophotometric assay of activated groups by the hydroxamate test suffers from a number of disadvantages:

i) It gives high blanks, which as noted by Raacke and Bové (1960) are particularly marked with plant extracts.

ii) It is not specific, either for the acid activated, or for the type of activated group.

iii) It is ambiguous, in that hydroxylamine could react with the activated acid either as the adenylate or as the CoA ester, and so need not demonstrate CoA-dependence of the reaction. In practice the adenylate is tightly bound or inaccessible to external reagents (Kingdon et al, 1958), and CoA-dependence can be demonstrated.

iv) It is insensitive, requiring rather high concentrations of the hydroxamate for assay.

v) It is subject to the dependence of hydroxylamine reactivity and stability on pH, which is unlikely to be similar to the pH-dependence of the enzymic reaction (Stulberg & Novelli, 1962).

vi) The activating system may be inhibited by the high concentration of hydroxylamine necessary for the assay. Reports of such inhibition have been shown in several cases to be due to $Na^+$: inhibition occurs when $NH_2OH.HCl$ is neutralised with NaOH, but does not occur with salt-free hydroxylamine, KOH-neutralised hydroxylamine, or salt-free hydroxylamine in the presence of KCl. The latter reagent is most satisfactory since it overcomes the inhibitory effects of $Na^+$ normally present in extracts (Raacke & Bové, 1960).

vii) The assay cannot be used for the estimation of the activation of cinnamic acids with phenolic hydroxyl groups, since these acids themselves give a colour with $FeCl_3$ (Swain, 1953).

While it has these disadvantages the assay is very quick and easy to carry out, and can be used to measure the activation of a number of acids other than cinnamic acid. A comparison can therefore be made between the activation of cinnamic acid and other acids in the same system.

b) the chromatographic method

The hydroxamate test may also be used to measure cinnamic acid activation by the measurement of the conversion of [$^{14}$C] - labelled cinnamic acid to [$^{14}$C] cinnamyl-hydroxamate. A chromatographic method for the separation of [$^{14}$C] cinnamic acid from its [$^{14}$C] hydroxamate was devised. Gross and Zenk (1966) originally described the behaviour of cinnamyl-hydroxamate in three different solvent systems on Whatman No.1 chromatography paper, but only one of these, isopropanol: ammonia; water (8:1:1 v/v) gave any separation of the acid and its hydroxamate, and this was insufficiently complete for radioactive measurements in experiments with low enzymic activity. The separation of cinnamic acid and its hydroxamate on Whatman DEAE ion-exchange paper (DE 81) was achieved using 0.001M-NaCl as solvent. The paper was cut as shown in Fig.10 and 50 nmoles each of cinnamic acid and its hydroxamate spotted at the origin. The solvent dripped from the end of the paper after 2½hr., and after 21hr. cinnamic acid had moved only 1½-2cm., whereas cinnamyl-hydroxamate had moved 5-6cm. A complete separation by at least 3-4cm. was always achieved. The spots were detected by their u.v. absorbance, and distinguished by spraying with an acid ethanolic solution of ferric chloride*, when the hydroxamate appeared as a

---

*4% $FeCl_3$ in absolute ethanol acidified to 1M with conc. hydrochloric acid.

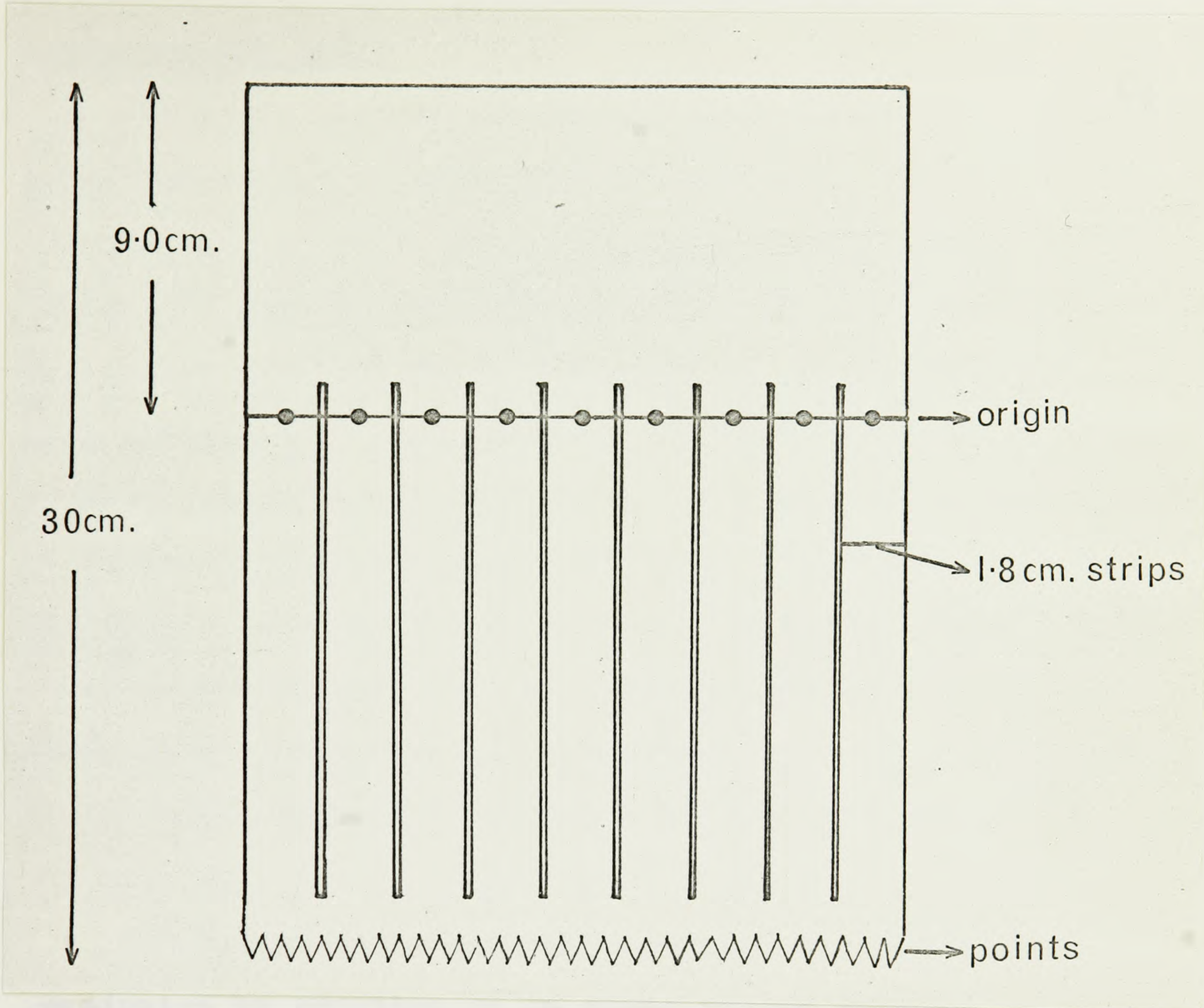



Fig.10. Pattern of the DEAE-paper chromatogram used for the separation of cinnamic acid from its hydroxamate.

purple spot.

Method of the assay

The assay was carried out in sealed plastic Eppendorf tubes containing an atmosphere of nitrogen, and shaken in a water bath at 30°. The assay system contained: tris-HCl buffer (13 μmoles, pH 8.5); ascorbic acid (2.7 μmoles); $MgCl_2$ (1.3 μmoles); $NH_2OH$ (20 μmoles); mercaptoethanol (0.5 μmoles); ATP (0.5 μmoles); CoA (0.03 μmoles); [2-$^{14}$C] cinnamic acid (0.1185 μmoles; 0.2 μc), and the enzyme sample in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0). The total volume was 0.2ml., and the final pH 7.8. Suitable controls (the above system without CoA, and without CoA and ATP), and a blank with boiled enzyme, were included. The hydroxylamine was prepared by neutralisation with KOH, and the [2-$^{14}$C] cinnamic acid was dissolved in 1% $KHCO_3$.

After incubation for up to 4hr., the reaction was terminated by addition of an equal volume (0.2ml.) of absolute ethanol. 50μl. of this solution, together with 50nmoles each of carrier cinnamic acid and cinnamyl-hydroxamate, were spotted onto DEAE-cellulose paper cut as shown in Fig.10, and the chromatogram was run for 21hr. At the end of this period, it was dried and the spots were detected under an ultra-violet lamp. The cinnamyl-hydroxamate spot was cut out and immersed in 4.0ml. CIBA-1 scintillation fluid (0.5% Butyl-PBD in Analar toluene) in a scintillation

vial. The radioactivity of each sample was counted for 10 min. in a Beckmann LS-200B scintillation counter. The radioactivity of the original sample was measured by spotting 50µl of the ethanolic reaction mixture directly onto a 1.8cm disc of DEAE-paper and counting as above.

By this method three of the major disadvantages of the spectrophotometric hydroxamate test were obviated: viz. non-specificity, high blank values, and insensitivity. Very small amounts (<1nmole) of substrate conversion were measurable, enabling enzyme preparations with a very much lower activity to be assayed. However the possibility of hydroxylamine inhibition of the enzyme still remained, and the method was relatively time-consuming. The method could be extended to measure the activation of acids other than cinnamic, if the appropriate chromatographic separating systems were devised, but as described, it is restricted to the measurement of cinnamic acid activation.

Assay 2. The spectrophotometric estimation of cinnamyl-CoA.

Gross and Zenk (1966) have described a sensitive optical test for the CoA esters of cinnamic acids based on the specific absorption maxima of these esters between 310 and 365 nm., in contrast to the absorption maxima of aliphatic CoA esters which lie between 230 and 240nm. Table 1 summarises the absorption maxima and extinction coefficients of the CoA esters of variously substituted

Table 1 . Absorption maxima and extinction coefficients of the CoA esters of cinnamic acids, (after Gross & Zenk, 1966).

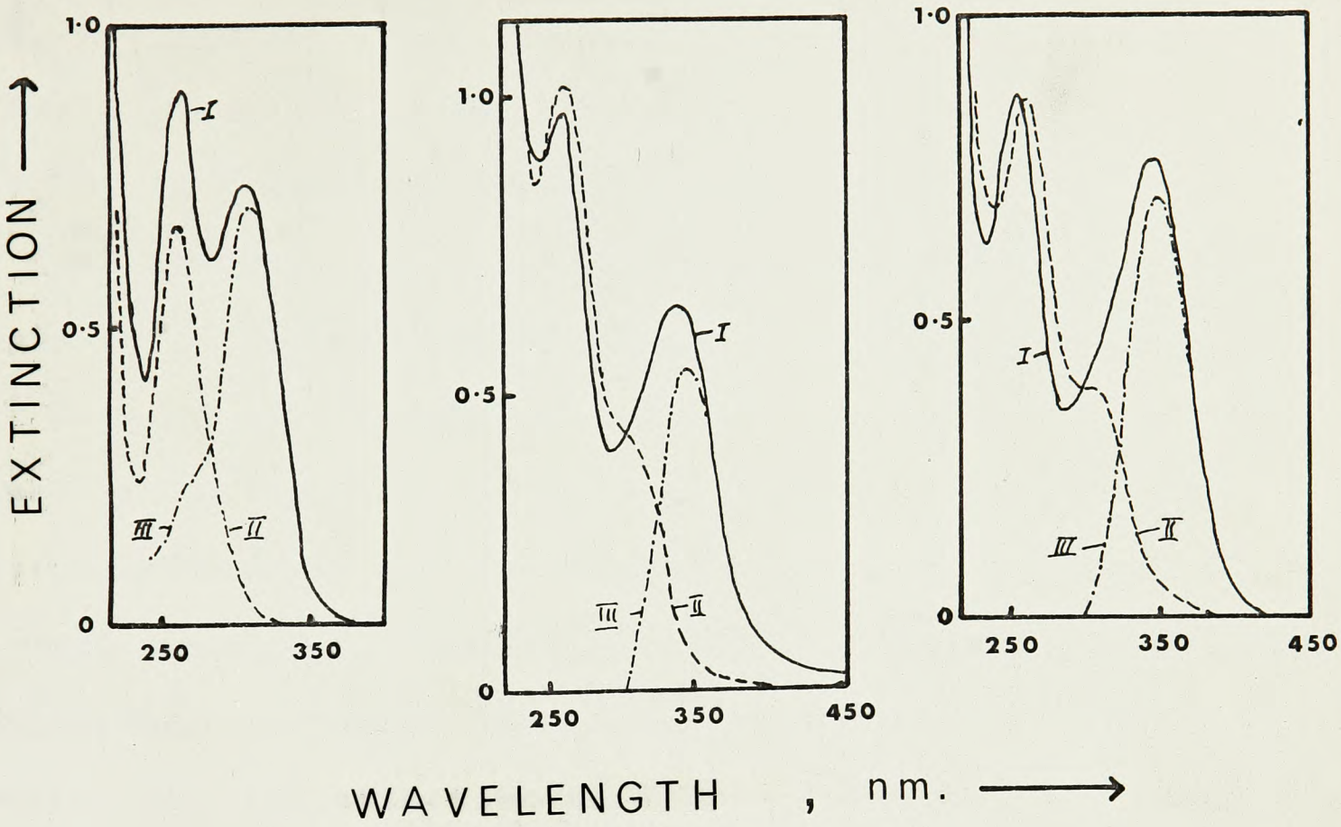
| CoA ester | Substitution pattern | $\lambda$ max (nm.) | $\epsilon \lambda$ max cm.$^2$/mol. $10^6$ |
|---|---|---|---|
| cinnamic acid | - | 311 | 22 |
| p-coumaric acid | 4-OH | 333 | 23 |
| caffeic acid | 3,4-OH | 363 | 13 |
| ferulic acid | 4-OH<br>3-$OCH_3$ | 345 | 19 |

(Sinapic acid was transformed so slowly by the activating enzyme preparation of Gross & Zenk that its extinction coefficient was not measurable).

---

cinnamic acids, and Fig.11 shows the ultra-violet spectra of cinnamyl-CoA and cinnamic acid, and the difference spectrum (after Gross & Zenk, 1966).

FIGURE 11 FIGURE 12 FIGURE 13




U.V.- SPECTRA OF CINNAMYL - CoA (FIG. 11)
p-COUMARYL - CoA (FIG. 12)
FERULYL - CoA (FIG. 13)

I CoA ester in phosphate buffer (0·1M, pH 8·0)

II after hydrolysis in NaOH (0·1M)

III difference spectrum

after Gross & Zenk (1966)

Method of the assay

For the measurement of the activation of cinnamic acid, two cuvettes were set up, each containing: tris-HCl buffer (130 to 140 μmoles, pH 8.5); ascorbic acid (20 μmoles); $MgCl_2$ (5 to 7 μmoles); mercaptoethanol (5 to 7 μmoles); ATP (5 to 7 μmoles); CoA (0.3 to 0.4 μmoles), and the enzyme sample in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0). The cuvettes were flushed with nitrogen, and then stoppered. They were placed in the cuvette holder of a Beckmann-DB recording spectrophotometer, and the reaction started by adding cinnamic acid (5 to 7 μmoles in 1% $KHCO_3$) to the test cuvette. The increase in extinction at 311nm. in the test cuvette, relative to the extinction at 311nm. in the control cuvette, was recorded directly, since the spectrophotometer subtracted the control values automatically. The final pH of the reaction mixture was 8.5, and the final volume was 2.0ml. CoA was present in the control cuvette so that corrections were made for any CoA-dependent changes at 311nm. due to bound cinnamic acids present in the crude enzyme extract.

This method could not be used for the measurement of the activation of substituted cinnamic acids, because the free acids have a much greater absorption at the λ max of their CoA esters (Figs.12 and 13). The acid was therefore included in the control cuvette, and in order to correct for extinction changes due to CoA and bound cinnamic acids

at the $\lambda$max of the relevant CoA ester, the estimation was carried out as follows: two cuvettes were set up, each containing tris-HCl buffer (130 to 140 µmoles, pH 8.5); ascorbic acid (20 µmoles); $MgCl_2$ (5 to 7 µmoles); mercaptoethanol (5 to 7 µmoles); ATP (5 to 7 µmoles), and the enzyme sample in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0). The cuvettes were flushed with nitrogen and then stoppered. They were placed in the cuvette holder of the spectrophotometer, and CoA (0.3 to 0.4 µmoles) was added to the test cuvette to start the reaction. Any change in extinction in the test cuvette at the $\lambda$ max of the cinnamyl-CoA ester to be estimated, was recorded directly, until it ceased. A cinnamic acid (5 to 7 µmoles in 1% $KHCO_3$) was then added to each cuvette and the extinction change in the test cuvette was followed at the appropriate $\lambda$ max. The final pH was 8.5 and the volume was 2.0 ml.

The rate of activation of unsubstituted cinnamic acid was the same when measured by either method but the first method was preferred as it was simpler and quicker to carry out. The amounts of cinnamyl-CoA formed were calculated from the recorded extinction changes and the extinction coefficients given in Table 1.

This assay has the advantages that it provides a sensitive, immediate, and continuous measurement of the complete reaction, and it is specific for both the cinnamic acid and for CoA. It also measures the activity of the

enzyme system in the absence of either an external reagent or an excess of any intermediate which could interfere with the enzyme.

Certain practical difficulties were however encountered in making the measurements, principally that the control cuvette must contain the same high concentrations of ultra-violet absorbing materials (mainly ascorbic and cinnamic acids), as the test cuvette in order to make measurement possible, but that the presence of these materials considerably decreases the sensitivity of the spectrophotometer.

Assay 3. The pyrophosphate exchange assay.

This assay relies on the reversible formation of acyl-adenylate with the release of pyrophosphate (Fig.6), and measures the incorporation of $[^{32}P]$ pyrophosphate into ATP.

Method of the assay, (modified methods of Anderson, 1968; Smith & Fowden, 1968).

The assay was carried out in sealed 25 ml. flasks, adapted for sampling under an atmosphere of nitrogen (Fig.9). The flasks were shaken in a water bath at 30° for up to 4 hr. and the assay system contained: tris-HCl buffer (110 μmoles, pH 8.5); ascorbic acid (20 μmoles); $MgCl_2$ (5 μmoles); cinnamic acid or other substrate (5 μmoles); mercaptoethanol (5 μmoles); ATP (5 μmoles); $[^{32}P]$ pyrophosphate (tetrasodium salt, 5 μmoles; 1.66 μc), and the enzyme sample in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0) in a total volume of

2.0ml. The volume of reaction mixture used was adjusted to allow for the removal of the required number of 1.0 ml. samples. A system without cinnamic acid was employed as control, and mixtures were also included which contained potassium fluoride (20 μmoles) as a phosphatase inhibitor, and CoA (0.3 μmoles) as an exchange inhibitor.

1.0 ml. samples were transferred at intervals to 0.5ml. trichloracetic acid (12%), and insoluble material was removed by centrifugation. 50 mg. Norit (activated charcoal) was added as 1.0ml. of a suspension in 0.1M-pyrophosphate (adjusted to pH 8.0 with phosphoric acid) to the supernatant. The mixture was shaken several times during 1 hr., and then left overnight at room temperature. It was assumed that all the ATP in solution had by then become absorbed to the charcoal, while the non-specific adsorption of $[^{32}P]$ pyrophosphate was minimised by adding the charcoal in pyrophosphate buffer. The charcoal was filtered onto 2.7 cm. Whatman No.1 paper discs in a Buchner funnel. Each disc was washed with 100ml. pyrophosphate buffer (0.1M, pH 8.0), and dried on a planchette under an infra-red lamp. The radioactivity of the charcoal, representing $[^{32}P]$ ATP, was measured using a thin end-window Geiger-Müller tube.

This assay has three advantages:

i) It involves exchange and hence does not measure the net formation of an activated product. It should therefore be sensitive.

ii) It avoids the use of hydroxylamine which is likely to be an enzyme inhibitor, and is an inefficient reagent for the measurement of activated groups.

iii) It can be used to measure the activation of any substrate.

The major disadvantages of the method are:

i) It measures only the first partial reaction of the activation sequence, and in the field of amino acid-activation, examples are documented (Peterson, 1967) where an amino acid promoting exchange, is excluded by the second partial reaction (the formation of amino acyl-tRNA). The competitive inhibition exerted by the terminal acceptor of the substrate (CoA or tRNA) in many systems is however some indication of whether or not the second partial reaction and the terminal acceptor are involved in the normal function of the enzyme.

ii) In the assay of crude extracts, there is a high background exchange rate due to endogenous substrates. These are only partially removed by dialysis or desalting. In such extracts there is also a high concentration of phosphatases, so that the concentration of reactants does not remain constant, and the measurement of activation is consequently not valid (Stulberg & Novelli, 1963). Fluoride has been used as a pyrophosphatase inhibitor but it can also inhibit the exchange reaction (Davis & Novelli, 1958; Peterson & Fowden, 1963).

Assay 4. The cinnamate-dependent disappearance of CoA, assayed by the nitroprusside method.

The substrate-dependent disappearance of the sulphydryl group of CoA has been used to measure the activation of a variety of fatty acids (Mahler et al, 1953), and to assay acetyl-CoA synthetase (Campagneri & Webster, 1963). The sulphydryl group was estimated by the nitroprusside reaction originally described by Grunert and Phillips (1951).

Method of theassay.

The assay was carried out in test tubes incubated at 30° for up to 2 hr. The assay system was modified from Campagnari and Webster (1963) and contained: tris-HCl buffer (35 μmoles, pH 8.0); ascorbic acid (2 μmoles); $MgCl_2$ (0.75 μmoles); ATP (0.6 μmoles); CoA (0.35 μmoles); cinnamic acid (0.75 μmoles); glutathione (0.75 μmoles), and the enzyme sample in a final volume of 0.325 ml. Systems without cinnamic acid were employed as controls, and mixtures were also set up to test the effect of the addition of KCl (37.5 μmoles) and the absence of glutathione.

The reaction was terminated by the addition of 0.06 ml. metaphospheric acid (30%), and insoluble material was removed by centrifugation. 0.03ml. samples were removed from the tubes containing glutathione, and 0.1ml. samples from those without glutathione. The samples were assayed by the nitroprusside reaction as described by Mahler et al (1953). Samples to which

water had been added to give a volume of 0.2ml., were added to tubes containing 0.4ml.* $Na_2CO_3$-NaCN solution, and 0.4ml. nitroprussside reagent (2.7g. $Na_2Fe(CN)_5.NO.2H_2O$ in 100 ml. solution stored in the dark). The extinction of the resulting solution was measured at 540nm. after 30 sec.

A standard curve was prepared using a range of glutathione concentrations in 0.2ml. solution (Fig.14). The relationship between extinction at 540nm. and glutathione concentration was linear up to 0.3 µmoles glutathione under these conditions.

This assay has the advantages of measuring the complete reaction, and of being dependent on both the added substrate and CoA. However, it measured a very small disappearance of CoA sulphydryl groups against a high sample content of sulphydryl groups and further, the non-specific disappearance of sulphydryl groups by oxidation in the presence of crude extracts at an alkaline pH obscured the measurement of CoA conversion to a thioL ester.

---

* $Na_2CO_3$-NaCN solution was made by dissolving 106g. anhydrous $Na_2CO_3$ in 450ml. solution, adding 2.2g. NaCN to this solution, and making the volume up to 500ml. The reagent was stored in the dark.

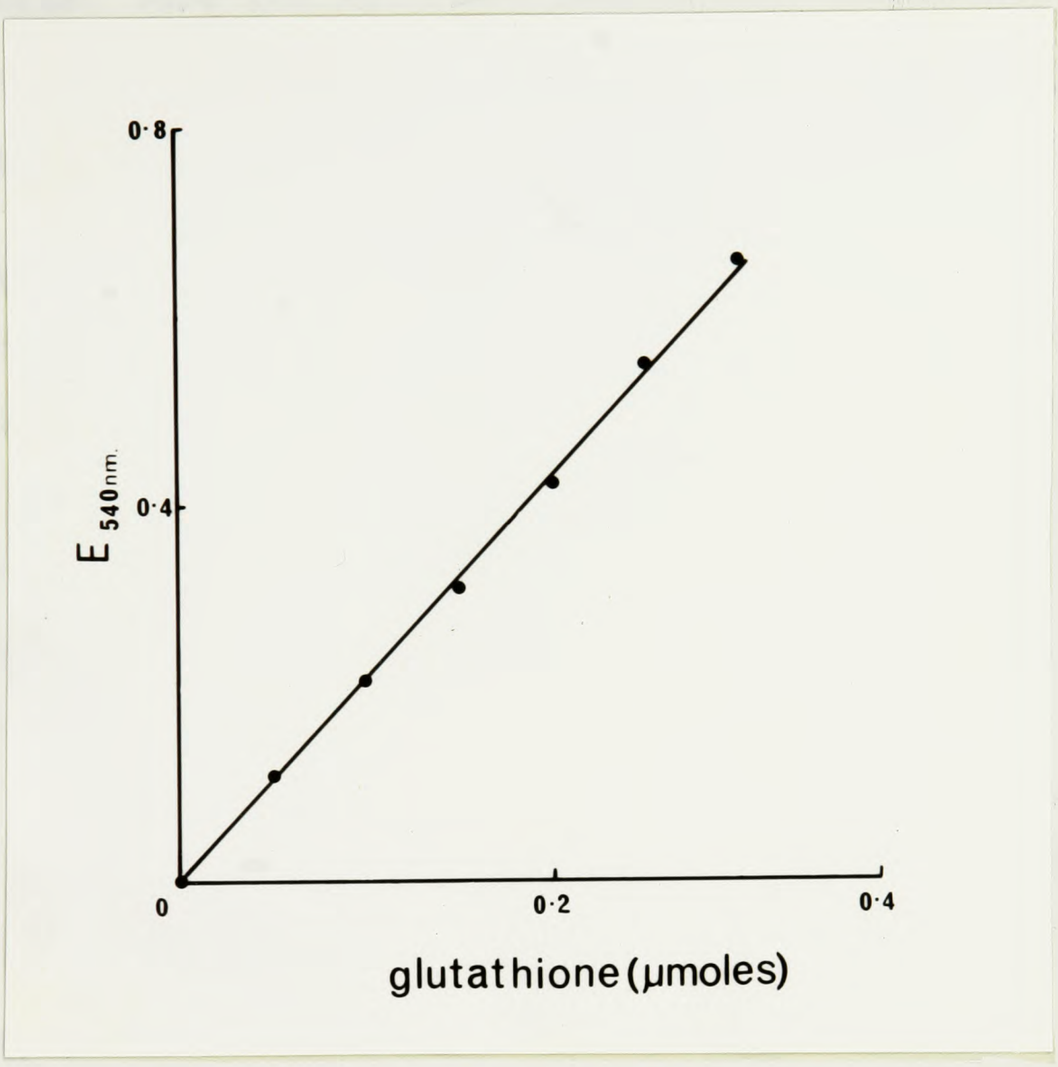



Fig. 14. Calibration curve for the estimation of sulphydryl groups by the nitroprusside assay. 0.2ml. standard glutathione solution was mixed with 4.0ml. $Na_2CO_3$-NaCN solution and 0.4ml. nitroprusside reagent, and the $E_{540}$nm. was measured after 30 sec.

## Section 2. The hydroxylation of cinnamic acid.

### A. Leaf disc studies

Discs were cut from the leaves of 4 to 8-week seedlings of spinach beet (Beta vulgaris, L.) and spinach (Spinacia oleracea, L.)

Incubation. The incubation procedure was based on the method of El-Basyouni et al (1964). 50 x 1cm. leaf discs of spinach beet or spinach were infiltrated with distilled water under vacuum, and incubated in petri dishes in 7.5ml. $NaH_2PO_4$-NaOH buffer (0.05M, pH 5.0) containing 0-15mM cinnamic acid for up to 24 hr. in darkness or under illumination. All dishes were placed 6 in. from a 150 watt light bulb, and those to be incubated in darkness were wrapped in silver paper. For experiments using [2-$^{14}$C] cinnamic acid, 2 µc of the acid (1.19 µmoles), was added to the incubation buffer, together with unlabelled cinnamic acid as required. Control incubations were carried out in the absence of cinnamic acid.

After incubation, the leaf discs were washed and extracted in 95% ethanol for 20 min. under reflux. A sample of freshly-cut leaf discs was also extracted as a further control. The ethanol was removed from the extracts in a rotary evaporator, and the residue resuspended in 10ml. 5% $NaHCO_3$. Alkaline treatment of the suspension was carried out in 2M-NaOH under nitrogen for 1 hr. The solution was then

acidified to pH 2.0 and extracted three times with 50ml. ether in a separating funnel. The ether extract was then extracted three times with 5 ml. 5% $NAHCO_3$ and the alkaline solution acidified to pH 2.0 and again extracted into ether.

The second ethereal extract was dried over anhydrous $Na_2SO_4$, and evaporated over a water bath. The residue was taken up in 75 µl. ethanol and applied to Whatman No.1 chromatography paper, with suitable standards. The chromatogram was developed in the upper phase of benzene: acetic acid: water (6:7:3, v/v) for 3 to 4 hr. It was then dried and examined under an ultra-violet lamp, before and after treatment with ammonia, and also by spraying with an ethanolic solution of diazotised sulphanilic acid. Spots were identified by their position ($R_F$), behaviour under the ultra-violet, and colour after treatment with diazotised sulphanilic acid. Table 2 shows these characteristics for a number of cinnamic acids. Ferulic acid could not be separated from sinapic acid in this solvent system.

When [2-$^{14}$C] cinnamic acid was used, the chromatograms were examined first under the ultra-violet, and then by cutting into 4 cm. strips, which were scanned using an I.D.L.1700 scaler and a double end-window automatic chromatogram scanner over $\frac{1}{2}$in. lengths, each counted for 100 sec.

Table 2. Identification of cinnamic acid and its derivatives

Cinnamic acid and its derivatives (0.1 umole or less) were applied to Whatman No.1 chromatography paper. The chromatograms were developed in the upper phase of benzene: acetic acid: water (6: 7: 3, v/v) for 3-4hr. The spots were located a) under an ultra-violet lamp, with and without exposure to $NH_3$ vapour, b) by spraying with an ethanolic solution of diazotised sulphanilic acid (a mixture of 12.5 ml. 0.3% sulphanilic acid in 8%HCl & 70% ethanol, and 0.75ml. 5% $NaNO_2$ in 70% ethanol), followed by an ethanolic solution of NaOH (10% NaOH in 70% ethanol).

| Acid | $R_F$ | Appearance under u.v. $-NH_3$ | $+NH_3$ | Colour with diazotised sulphanilic acid |
|---|---|---|---|---|
| caffeic | 0.10 | B. | G.B. fl. | brown |
| p-coumaric | 0.46 | b.fl. | B. fl. | dark red |
| sinapic | 0.70 | B.fl. | B. fl. | purple |
| ferulic | 0.73 | B.fl. | B. fl. | purple |
| cinnamic | 0.92 | a. | a. | no colour |

b. = faint blue; B. = blue; B. = bright blue; G. = green; a. = absorption; fl. = fluorescence.

## B. Analytical methods

The hydroxylation of cinnamic acid was followed by measuring the appearance of p-coumaric acid. This estimation was carried out in three different ways, only one of which proved useful.

### i) The diazotised sulphanilic acid assay for phenolic hydroxyl groups

This assay was used by Nair and Vining (1965a) for measurement of the activity of a cinnamic acid hydroxylase in an acetone powder prepared from spinach leaves. The procedure described by Nair and Vining was modified to give a more stable colour development, by decreasing the concentrations of alkali and diazotised sulphanilic acid reagents. In this way a satisfactory calibration curve for p-coumaric acid in solution in 1% $NaHCO_3$ (i.e. at a neutral pH) was obtained. A dilute diazotised sulphanilic acid solution was prepared by mixing 2ml. 0.2% sulphanilic acid in 0.1N-HCl with 0.32ml. 0.5% $NaNO_2$ at $0^{\circ}$, and diluting with 3 vol. (6.96 ml.) water. 1.0ml. of this reagent was added to 1.0ml. p-coumaric acid solution, followed 15 sec. later by 1.0ml. 5% $Na_2CO_3$. The extinction at 520nm. was measured 20 min. after the addition of alkali. The relationship between extinction and p-coumaric acid concentration under these conditions was linear over the range 0-0.2 μmoles (Fig.15).

However, solutions containing p-coumaric acid obtained

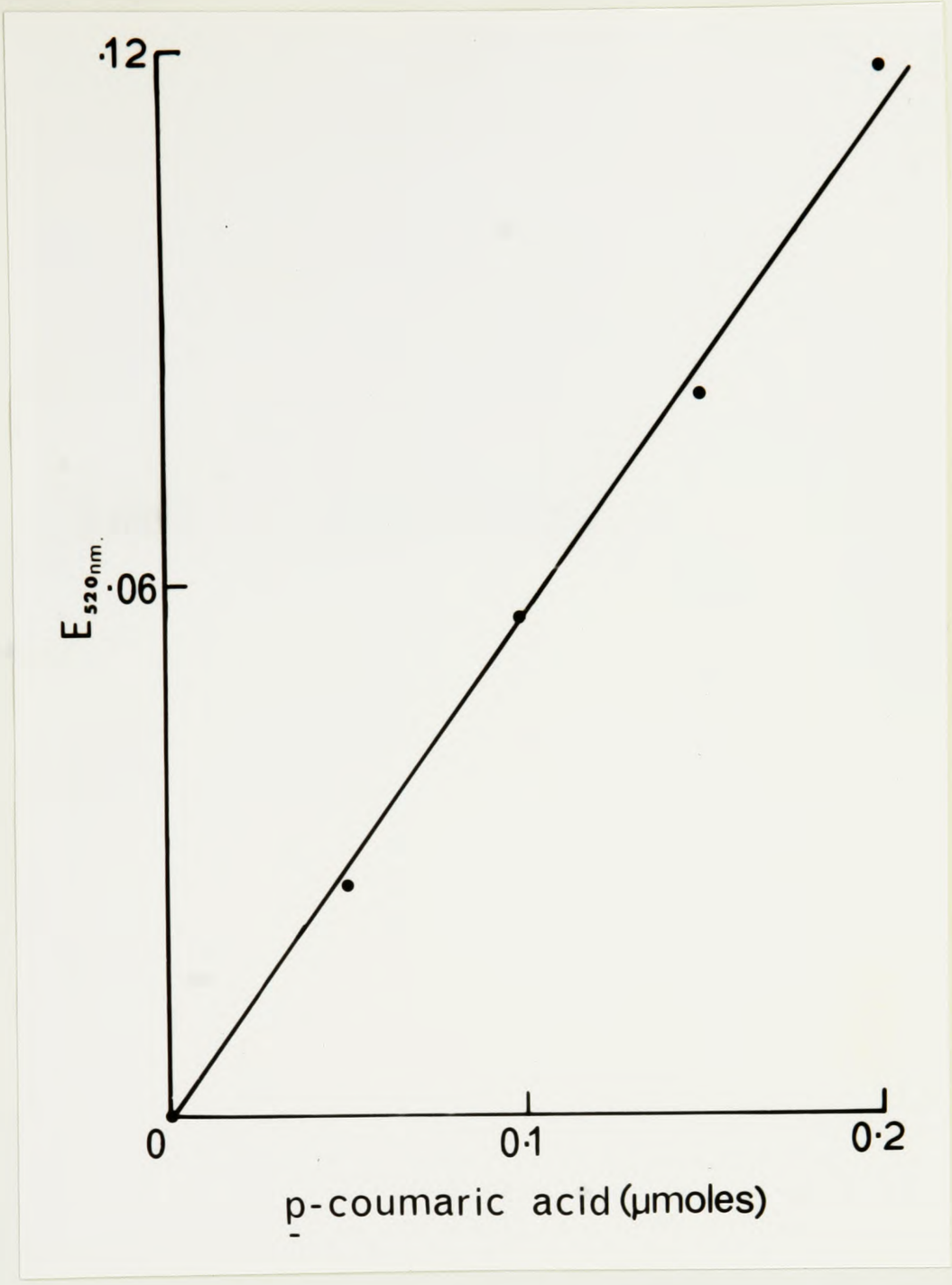



Fig. 15. Calibration curve for the estimation of p-coumaric acid by the diazotised sulphanilic acid method of Nair and Vining (1965a), using diluted reagents (p. 39 ). Solutions of p-coumaric acid in 0.5% $NaHCO_3$ were used as standards.

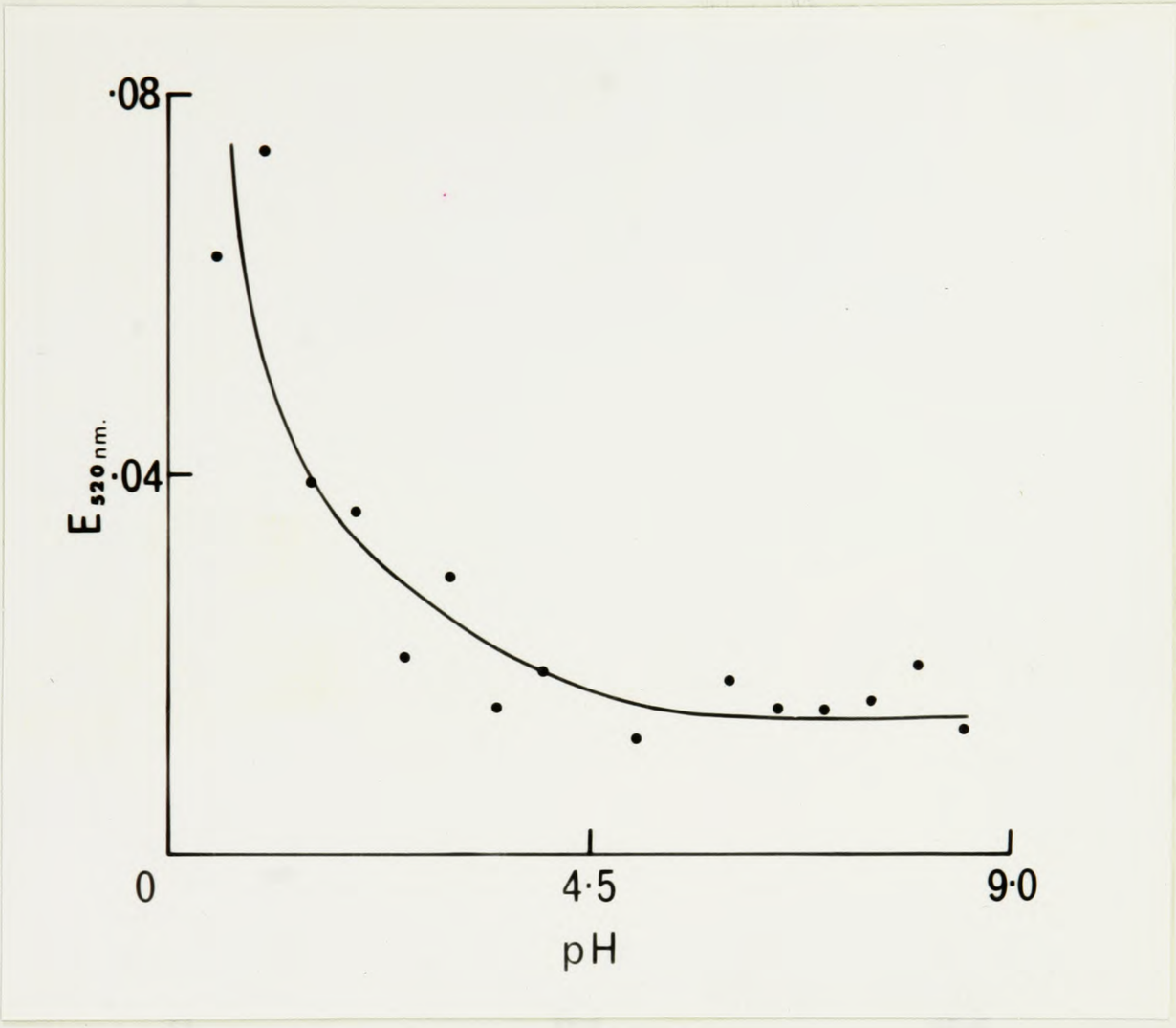



Fig.16. Effect of pH on the control value used in the estimation of p-coumaric acid by the diazotised sulphanilic acid method (cf.Fig.15). Samples prepared under the conditions of enzyme assay were treated at pH values from 0.5 to 8.5.

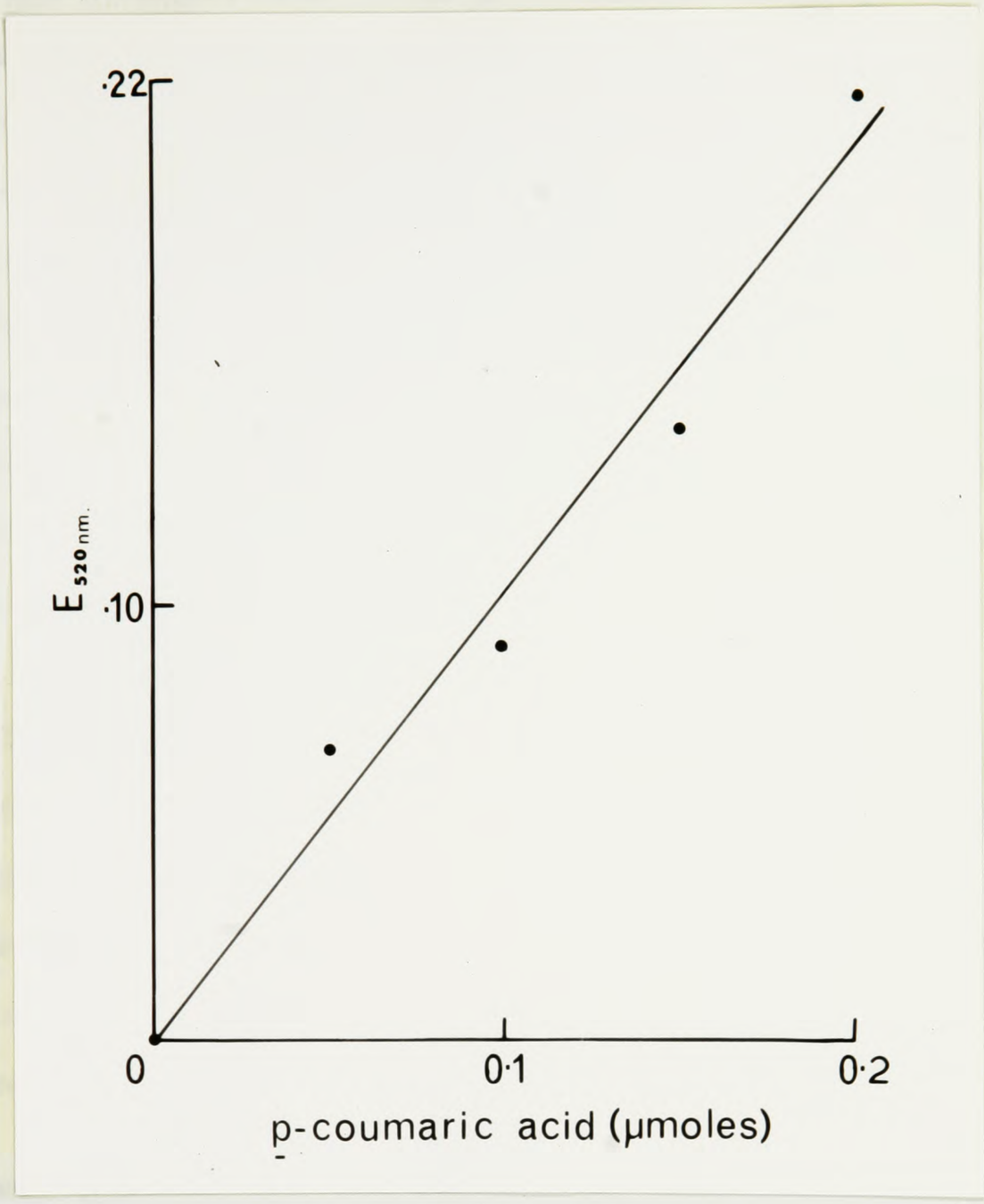



Fig.17. Calibration curve for the estimation of p-coumaric acid by a modified diazotised sulphanilic acid method. Samples of p-coumaric acid prepared under the conditions of enzyme assay were neutralised to pH 7.0 with NaOH, and estimated using diluted reagents as in Fig.15.

from the enzyme assay system, contained $NaH_2PO_4$-$Na_2HPO_4$ buffer and trichloracetic acid at a final pH of 1.7, and gave a high extinction value at 320nm. even in the absence of p-coumaric acid. The effect of pH on this control value (Fig.16) indicated that all solutions should be adjusted to pH 7.0 with NaOH before estimation of p-coumaric acid. A calibration curve for p-coumaric acid estimation derived in this way is shown in Fig.17. The relationship between extinction and p-coumaric acid concentration was again linear over a range of 0-0.2 µmoles, but was different from that shown in Fig.15.

This test estimates one or more phenolic hydroxyl groups, and is not specific for p-coumaric acid. It is therefore likely to give unreliable results since many enzyme preparations tested for cinnamic acid hydroxylating activity will also contain the phenolase complex, which could hydroxylate further any p-coumaric acid formed.

ii) Lugg's assay for the estimation of monophenols, (Lugg,1937).

2.0ml. solution containing 0-0.8 µmoles p-coumaric acid were added to 1.0 ml. 2.5M-$H_2SO_4$. Any insoluble material was removed by centrifugation, and the supernatant decanted and made up to 5.0 ml. with 2.5M-$H_2SO_4$, (Reagent A). 5.0ml. Reagent B (prepared by filtering a mixture of 75g. $HgSO_4$, 55g. $HgCl_2$ and 70g. anhydrous $Na_2SO_4$ in 1l. solution), was added and the mixture incubated for 30 min. at 60-65°.

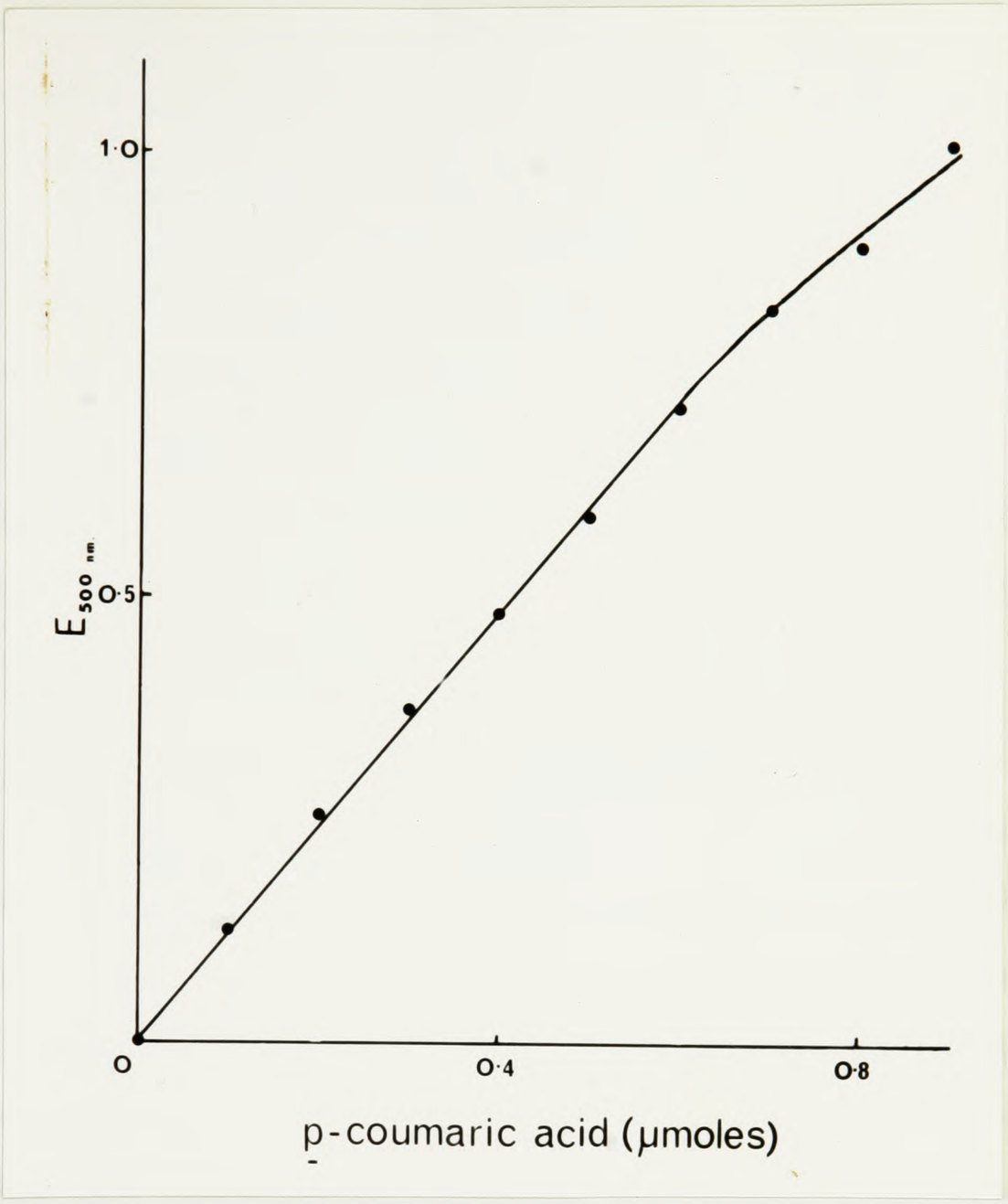



Fig.18. Calibration curve for the estimation of p-coumaric acid by the method of Lugg (1937), using solutions of p-coumaric acid in 0.5% $NaHCO_3$ as standards.

The samples were cooled to 1-2° below room temperature and any insoluble material removed by centrifugation. The supernatant was decanted and any precipitate washed with 10ml. Reagent C (1 vol. Reagent B diluted with 1 vol. 0.5 N-$H_2SO_4$). Washings and supernatant were combined and the volume made up to almost 25ml. with Reagent C. 0.5ml. N-$NaNO_2$ solution was added to a final volume of 25ml. and the solution mixed immediately. The extinction at 500nm. was measured 5 min. after mixing, and the relationship between extinction and p-coumaric acid concentration was linear over the range 0-0.8 μmoles (Fig.18).

iii) The estimation of $[2^{-14}C]$ p-coumaric acid.

6.0ml. samples at pH 2.0 containing $[2-^{14}C]$ p-coumaric acid were extracted three times with 18 ml. ether. The ethereal extracts from each sample were combined and dried over anhydrous $Na_2SO_4$ for 1hr. The dried ethereal solution was evaporated to dryness over a water bath. The residue was taken up in 50-70μl. 80% ethanol and applied to Whatman No.1 chromatography paper with 0.1 μmole each of unlabelled cinnamic and p-coumaric acids. The chromatogram was developed in the upper phase of benzene: acetic acid: water (6 : 7 : 3, v/v) for 3 to 4 hr. It was then dried and examined under an ultra-violet lamp for cinnamic and p-coumaric acids (Table 2). The radioactivity in p-coumaric acid was measured either after cutting the chromatogram into

4cm. strips and scanning with a double end-window automatic chromatogram scanner, or by cutting out the p-coumaric acid spots, immersing them in CIBA-I scintillation fluid, and measuring the radioactivity in an I.D.L. scintillation counter.

## C. Enzyme assay systems

The hydroxylating enzyme was assayed in one of the following systems:

### i) Method of Nair and Vining (1965a)

The reaction mixture contained: citric acid (100 μmoles) -$Na_2HPO_4$ (200 μmoles) buffer (pH 4.6); NADH (0.5 μmoles); tetrahydrofolic acid (0.2 μmoles); cinnamic acid (1.0 μmoles) and the enzyme sample in aqueous suspension, in a total volume of 3.0ml. in 25ml.flasks. The flasks were incubated in a shaking incubator at 30° for up to 1hr. and the reaction terminated by the addition of 0.5ml. 20% trichloracetic acid. The insoluble material was removed by centrifugation and 1.0ml. supernatant estimated for p-coumaric acid by the diazotised sulphanilic acid method.

### ii) Modified method of Russell and Conn (1967), using unlabelled cinnamic acid.

The reaction mixture contained: $Na_2HPO_4$-$NaH_2PO_4$ buffer (225 μmoles, pH 7.5); ascorbic acid (30 μmoles); cinnamic acid (5 μmoles); NADH or NADPH (2 μmoles); tetrahydrofolic

acid (0.6 μmoles), and enzyme sample in $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.0) in a total volume of 2.0ml. in 25ml. flasks. All cofactor solutions and volume adjustments were made with $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.5). Flasks without cinnamic acid or cofactors were used as controls. Quantities of reaction mixture were increased as necessary to provide the number of samples required for estimation of p-coumaric acid.

The flasks were incubated at 30° for up to 6hr. For p-coumaric acid estimation by the diazotised sulphanilic acid method, 1.0ml. samples were transferred to 0.25ml. 20% trichloroacetic acid, and for Lugg's method, 2.0ml. samples were transferred to 1.0ml. 2.5M-$H_2SO_4$.

iii) Modified method of Russell and Conn (1967), using [2-$^{14}$C] cinnamic acid.

The reaction mixture contained: $Na_2HPO_4$-$NaH_2PO_4$ buffer (75 μmoles, pH 7.5); ascorbic acid (22 to 33 μmoles); NADPH or NADH (4 μmoles); tetrahydrofolic acid (1.2 μmoles) or dimethyltetrahydropteridine (4 μmoles); [2-$^{14}$C] cinnamic acid (1.19 μmoles, 2μc), and the enzyme sample in $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.0) in a total volume of 4.0ml. in 25ml. flasks. All cofactor solutions and volume adjustments were made with $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.5). The flasks were incubated with shaking at 30° for 4hr., and the reaction was terminated by the addition of 0.2ml.

6M-HCl. Insoluble material was removed by centrifugation and washed twice with a small volume of water. The pH of the combined supernatant and washings (6.0ml.) was checked and adjusted to 2.0 if necessary. [2-$^{14}$C] p-coumaric acid was extracted and estimated.

Section 3. The determination of protein.

A. Protein was determined spectrophotometrically at 260 and 280nm. using the data of Warburg and Christian (1941), when the absence of ascorbate (which has a strong ultra-violet absorption) allowed.

B. Concentrated protein solutions were assayed by the biuret estimation described by Layne (1957), using an alkaline solution of $CuSO_4$ and sodium potassium tartrate as biuret reagent. A control was prepared with the same buffer as used for the protein sample, to correct for the presence of any interfering compounds such as tris or high concentrations of ammonium ions. The biuret colour was measured at 540nm. and gave an $E_{540}$ of 0.100 for 1mg. of protein, when calibrated with bovine serum albumin.

C. Dilute protein solutions were assayed by the Folin-Ciocalteau estimation as modified by Leggett-Bailey (1962). The method is essentially that of Lowry et al (1951), but sodium citrate replaces sodium tartrate to increase the stability of the reagent. A control was prepared with the same buffer as used for the protein sample, to correct for the presence of any interfering compounds such as ascorbate. A linear relationship was observed between extinction at 750nm. and concentration of bovine serum albumin over the range 0 to 40µg. (Fig.19).

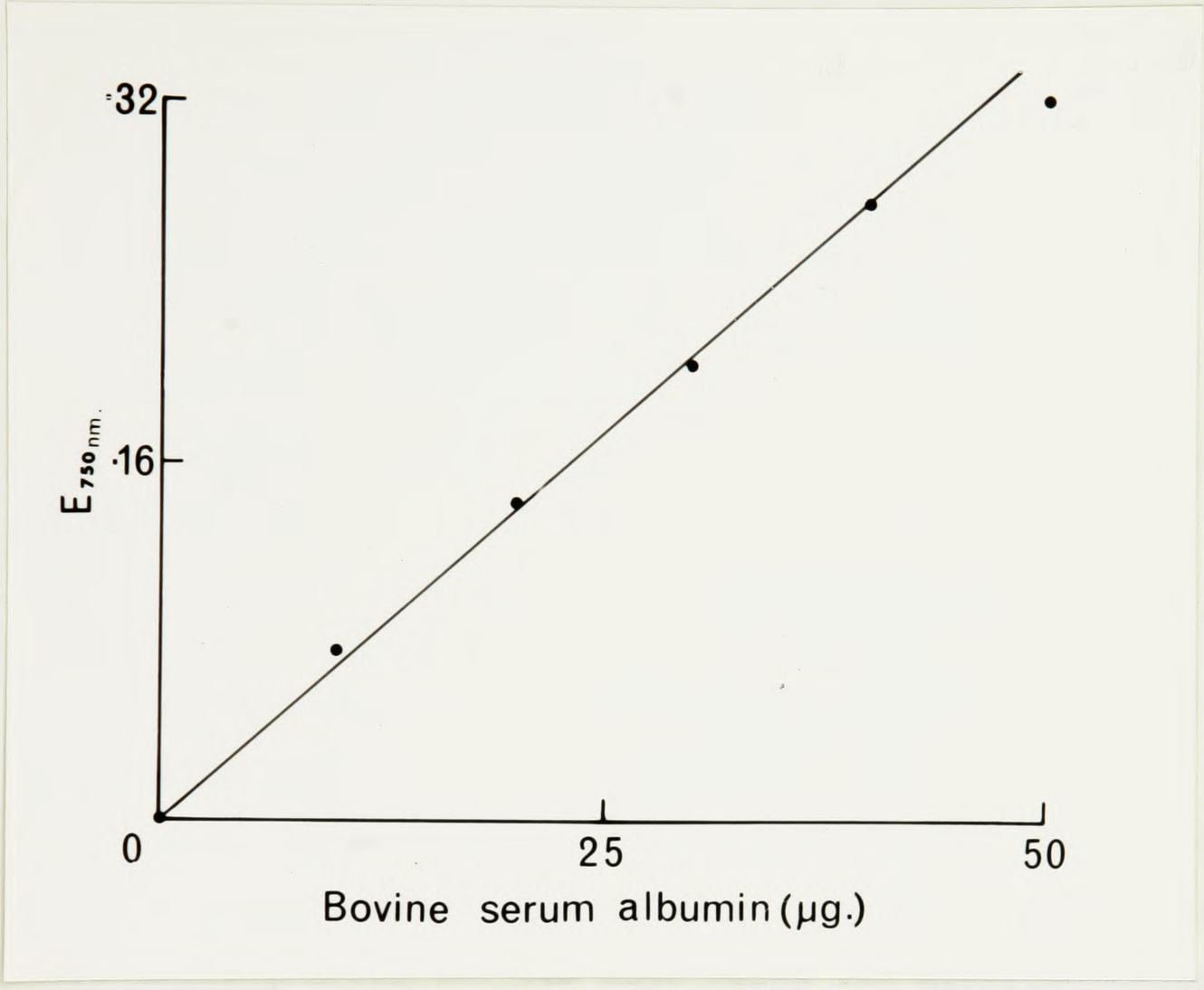



Fig.19. Calibration curve for estimation of protein by the Folin-Ciocalteau method, modified by Leggett-Bailey (1962). Solutions of bovine serum albumin were used as standards.

## Section 4. The preparation of the enzyme extracts.

Damage to the enzymes by any high concentrations of phenols and oxidising enzymes released during extraction was reduced by the following methods:

1. The use of acetone powders.
2. The inclusion of antioxidants such as ascorbate in the extracting media (Anderson, 1968).
3. The use of polyvinylpyrrolidone (PVP) as phenolic adsorbent (Loomis & Battaile, 1966). Any ~~remaining~~ remaining phenolics could be removed either by dialysis or by rapid desalting with Sephadex-G25.

### A. The activating enzyme

#### 1. Acetone powders

Acetone powders of spinach beet and runner bean leaves were prepared by the method of Nason (1955). The leaves were washed, dried and the main veins and mid-rib stripped out. The leaf remnants were macerated in 5 vol. acetone at 0° for 1 min. in a Waring blendor, and the suspension filtered through a Buchner funnel. The residue was macerated for 30sec. in another 5 vol. cold acetone, and the remaining acetone-insoluble material was washed twice with a total of 5 vol. cold acetone and dried in a vacuum desiccator at room temperature. It was stored at -10 to -15°.

A crude aqueous extract of the acetone powder was prepared by grinding 1g. in 20ml. tris-HCl buffer (0.1M, pH 7.7) at 0° in a glass homogeniser. In some experiments, the crude extract was filtered through cheesecloth and the filtrate centrifuged at 5000xg for 15min. in a Servall superspeed refrigerated centrifuge at 0°. The supernatant was decanted and various ammonium sulphate fractions (0-25%, 0-30%, 0-80%, 25-50%, 30-45%, 45-80%, 50-80%) were prepared by the addition of solid $(NH_4)_2SO_4$ (Analar recrystallised from 0.5mM-EDTA) to the appropriate saturation (Dixon & Webb, 1961), while the pH was maintained at 7.7 by the addition of ammonia solution. After each addition of $(NH_4)_2SO_4$ the solution was allowed to stand for 30min. with stirring, and the precipitated protein was removed by centrifugation at 10,000xg for 15 min. at 0°. It was resuspended in a minimum volume of tris-HCl buffer (0.1M, pH 7.7), and in some cases was either dialysed against 1.5L. of this buffer for 1.5hr. at 0°, or desalted by passing through a Sephadex-G25 column.

The Sephadex-G25 column was prepared by suspending 10g. coarse grade Sephadex-G25 in 500ml. water for at least 4hr., and then pouring the hydrated Sephadex suspension into a glass column (1cm. x 25cm.). The characteristics of the column were investigated by passing a 0.25% solution of blue dextran through it. The column was equilibrated at 0° by passing 100ml. tris-HCl buffer (0.1M, pH 7.7) through

it. 1.0-2.0ml. $(NH_4)_2SO_4$ fraction was applied to the column and eluted with this buffer.

All extracts and fractions were assayed by the spectrophotometric hydroxamate method.

2. Aqueous extracts prepared in the presence of ascorbate.

Aqueous extracts of a variety of plant organs (runner bean, broad bean, spinach beet and pea leaves; maize roots; pea epicotyls; and cauliflower florets) were prepared as follows:

The plant organs were washed, dried, and macerated in 2-3 vol. tris-HCl buffer (0.1M, pH 7.4; containing 0.02M-ascorbate) at 0° in a Waring blender, after removing the main veins and mid-ribs from the leaves. The macerate was filtered through cheesecloth, and the pH of the filtrate adjusted to 7.4 by the addition of ammonia solution. Cell-debris was removed by centrifugation at 5,000 x g for 15min. at 0°. The supernatant was decanted and various $(NH_4)_2SO_4$ fractions (usually 0-25%, 25-50% and 50-80%) were prepared from it by the addition of solid $(NH_4)_2SO_4$ to the appropriate saturation, the pH being maintained at 7.4 by the addition of ammonia solution. The $(NH_4)_2SO_4$ precipitated protein fractions were resuspended in a minimum volume of tris-HCl buffer (0.1M, pH 7.4; containing 0.02M-ascorbate).

This method was modified for pea epicotyls and cauliflower floret extracts. The washed and dried material

was macerated in 2-4 vol.tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA). The pea epicotyl macerate was further treated by grinding in a glass homogeniser. The crude extracts were filtered through cheesecloth, and the pH of the filtrates adjusted to 8.0 with ammonia solution. Cell debris was removed as before, and various $(NH_4)_2SO_4$ fractions prepared from the resulting supernatant, the pH of the solutions being maintained at 8.0 by the addition of ammonia solution.

The extracts and fractions were all assayed by the spectrophotometric hydroxamate method.

3. Aqueous extracts prepared in the presence of polyvinylpyrrolidone (PVP).

a) Soluble PVP

A small number of aqueous extracts of spinach beet leaves were prepared in the presence of 1% soluble PVP by a method in which the leaves were initially frozen, and then thawed into the extraction medium:

Spinach beet leaves were washed, dried, and stripped from the mid-rib and main veins. They were frozen in liquid nitrogen and then ground to a powder. The powder was stirred into 2 vol. tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate, 0.5mM-EDTA, and 1% soluble PVP) at 0° for 10-15 min., and then filtered through cheesecloth. The pH of the filtrate was checked and adjusted to 8.0 with ammonia solution.

if necessary. $(NH_4)_2SO_4$ fractionation was found impossible, since the soluble PVP was precipitated by $(NH_4)_2SO_4$ and interfered with the usual pattern of fractionation.

The extraction of spinach beet leaves by this method was also carried out in the absence of PVP, and into buffers of various composition and pH, in order to test the extractability and stability of activating activity under different conditions. The different buffers used were: tris-HCl (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA), and $KH_2PO_4$-KOH (0.1M, pH 7.0 or 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA).

The activity of the extracts was assayed by the spectrophotometric hydroxamate method.

b) Insoluble PVP (Polyclar AT).

Aqueous extracts of spinach beet leaves were initially prepared in the presence of insoluble PVP as outlined by Loomis and Battaile (1966):

10g. washed young leaves of spinach beet, from which the mid-ribs and main veins had been removed, were frozen in liquid nitrogen and ground to a powder. The powder was stirred into a suspension of washed Polyclar AT (10g.) in 60ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA) at 0°. On some occasions 0.02M-ascorbate was replaced by 0.1% mercaptoethanol in the buffer. The ascorbate was always added just before the leaf powder and

the pH was not adjusted to 8.0 until the PVP had been removed, since the complexing of phenolics by PVP takes place more readily near neutrality. The mixture was stirred for 10-15 min., and filtered through cheesecloth to give a filtrate at pH 7.1-7.2. The pH was adjusted to 8.0 with KOH, and the filtrate was centrifuged at 28,000xg for 15min. at 0°. The pale yellow supernatant was assayed by the spectrophotometric hydroxamate method.

The assay of extracts by the pyrophosphate exchange method requires the minimum contamination with ATP-ases. The extraction was therefore carried out by maceration of the plant material into a sucrose-containing buffer, in order to preserve mitochondrial integrity, prior to removal of these organelles by centrifugation:

15g. washed young leaves of spinach beet, spinach, runner bean, pea or cabbage from which the mid-ribs and main veins had been removed, were macerated in a suspension of Polyclar AT (10g.) in 60ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.25M-sucrose, 1.0mM-EDTA, and either 0.02M-ascorbate or 0.1% mercaptoethanol) at 0°. The macerate was stirred for a few minutes, and filtered through cheesecloth. The pH of the filtrate was adjusted to 8.0 with KOH, and then centrifuged at 28,000xg for 15 min. at 0°. The pale green supernatant, from which mitochondria had been removed, was low in ATP-ase contamination, and contained 3-7mg. of protein/ml. Low molecular-weight contaminants (such as phenolics) were

removed from a sample of this supernatant by passing it through a Sephadex-G25 column which was prepared and used as follows:

25g. coarse Sephadex-G25 was suspended in 1l. water for at least 4hr. The hydrated Sephadex suspension was poured into a glass column (1.5cm. x 50cm.). 250ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate or 0.1% mercaptoethanol) were passed through the column, and then 15ml. samples were introduced at the top of the column, and eluted with the equilibrating buffer. 3.0ml. samples were collected. Those which contained protein were clearly identifiable by their turbid appearence, but measurements of extinction at 260 and 280 nm. were used for confirmation. The concentration of protein in the combined eluate (measured by the Folin-Ciocalteau method) was 1-3.3mg./ml., which was approximately half the concentration in the undesalted sample. After use, the column was washed thoroughly with $KH_2PO_4$-KOH buffer (0.1M, pH 8.0), and between experiments the column was kept equilibrated with the washing buffer saturated with chloroform to prevent bacterial growth.

Some supernatants were subjected to an $(NH_4)_2SO_4$ fractionation before desalting, using BDH enzyme grade $(NH_4)_2SO_4$ (low in heavy metals). 0-25%, 25-50% and 50-80% $(NH_4)_2SO_4$ saturated fractions were prepared, resuspended in a minimum volume of $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing

0.02M-ascorbate or 0.1% mercaptoethanol), and desalted by passing through a Sephadex-G25 column, as above.

The supernatants were also investigated thoroughly by the [$^{14}$C] cinnamyl-hydroxamate assay, and the spectrophotometric assay for the appearance of cinnamyl-CoA.

B.Cinnamic acid hydroxylase

The first experiments with cinnamic acid hydroxylase employed an aqueous extract of an acetone powder of spinach beet leaves, prepared by the method of Nair and Vining (1965a).

250g. spinach beet leaves were frozen in liquid nitrogen and ground to a powder which was stirred into 250ml. water at 0°. The suspension was filtered through cheesecloth and 3 vol. cold acetone added slowly to the filtrate. The insoluble material was removed by centrifugation in an M.S.E. 4l. refrigerated centrifuge at 0°, and washed three times with cold acetone. The residue was dried in a vacuum desiccator and stored at -10 to -15°. An aqueous extract of the acetone powder was prepared by grinding 200mg. in 10ml. water in a glass homogeniser, immediately before enzyme assay.

An extract of spinach beet leaves was also prepared by a method similar to that used for the activating enzyme, using PVP and freezing the leaves before extraction, but the pH of the filtrate was adjusted to 4.6 instead of 8.0 before centrifugation.

Both extracts were assayed by the diazotised sulphanilic acid method.

Cinnamic acid hydroxylase was later extracted from leaves of spinach beet and spinach in the presence of insoluble PVP suspended in a buffer system similar to that described by Russell and Conn (1967):

10g. washed leaves of spinach beet or spinach, from which the mid-ribs and main veins had been removed, were frozen in liquid nitrogen and ground to a powder. The powder was stirred for 10-15min. into a suspension of Polyclar AT (10g.) in 80ml. $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.0; containing 0.02M-ascorbate and 0.5mM-EDTA) at 0°. The suspension was then filtered through cheesecloth and the pH of the filtrate adjusted to 7.0 with NaOH. Alternatively the leaves (10g.) were dispersed in a Waring blendor into the Polyclar suspension and stirred for 5min. before filtering and adjusting the pH. The filtrate was centrifuged at 500xg. and the supernatant assayed for cinnamic hydroxylase. In some cases the filtrate was centrifuged at 10,000xg and both the residue and the supernatant assayed for the enzyme.

# RESULTS

## The Activating Enzyme

The enzyme preparations are in general described in the order in which they were investigated. The first experiments tested the activity of acetone powders, used primarily because they have been freed during preparation from plant phenolics which might damage the enzyme. The drying procedure may also preserve enzymic activity and provides a simple method of storing an enzyme which may occur in plant material only at restricted times of the year.

When this method of preparation proved unsatisfactory, plant material was extracted by breaking it in buffers of various types, which contained an antioxidant to control phenolic oxidation. Extraction methods were further improved by carrying out the preparations in the presence of a phenolic adsorbent (polyvinylpyrrolidone, PVP) as well as the antioxidant.

The presence of activating systems in all these plant extracts was initially studied by the spectrophotometric hydroxamate method, which was used because it was simple, rapid, and could test the activation of a variety of acids. Acetate, as well as cinnamate, was used as substrate, since the activation of this acid by plant extracts is well known (Millerd & Bonner, 1954), and Grisebach et al (1966) have

prepared extracts which had a high activity in acetate activation but could also activate cinnamate. The enzyme catalysing the activation of acetate therefore seemed to be relatively easy to demonstrate, and as well as showing the characteristics of an activating system, might also indicate the presence of a cinnamate-activating enzyme. The spectrophotometric assay for cinnamyl-CoA and the nitroprusside assay for CoA sulphydryl groups were also tested at these early stages before the optimum conditions for enzyme assay and preparation had been established.

More precise assay methods were developed when it was shown that the spectrophotometric hydroxamate method was satisfactory only for preparations very active towards cinnamate. The first alternative assay used was the pyrophosphate exchange method, tested because it was sensitive and could demonstrate the activation of a variety of substrates.

When this assay failed to give positive results consistently, a specific radiochemical assay for cinnamate activation, by measurement of $[^{14}C]$ cinnamyl-hydroxamate formation, was employed. This method defined the physiological conditions under which a cinnamate-activating enzyme could be extracted from a variety of plant leaves. These enzyme preparations were also investigated by the spectrophotometric assay for cinnamyl-CoA, which was able

to establish properties of the enzyme that the [$^{14}$C] cinnamyl-hydroxamate method could not demonstrate.

## Section 1. Acetate - and cinnamate - activation in crude enzyme preparations, assayed by the spectrophotometric hydroxamate method.

### 1. Acetone powders

The levels of acetate activation found in various fractions of a crude aqueous extract of an acetone powder of spinach beet leaf which had been grown outside, are shown in Table 3. This table emphasises the very high values obtained with the control assay system (to which CoA had not been added), and against which the activation of acetate was measured. These control values increased progressively with the incubation period. However, in active $(NH_4)_2SO_4$ fractions, these values were considerably lower than those obtained with untreated acetone powder extract, and the percentage rise in extinction over the control value was higher. The 25-50% satd. $(NH_4)_2SO_4$ fraction was the most active, but the control value with this fraction was rather high. When divided further, the 30-45% satd. fraction contained the major proportion of the activity, and also showed a much lower control value than was obtained with the 25-50% satd. fraction. The time course of the reaction exhibited a variable lag period, which was not present at all with some preparations, (Figs.20,21).

The following properties of the enzyme were observed with the 30-45% satd. $(NH_4)_2SO_4$ fraction:

Table 3. The activation of acetate by preparations from spinach beet acetone powder.

Enzyme extracts were prepared from samples of 20g. of acetone powder which had been obtained from 200g. spinach beet leaves. The resuspended acetone powder was prepared by grinding acetone powder in 20 vol. tris-HCl buffer (0.1M, pH. 7.7). Debris was removed by centrifugation at 5,000xg. and $(NH_4)_2SO_4$ fractions were prepared from the supernatant (p. 47). 10ml. suspensions of the precipitates obtained between the stated saturations of $(NH_4)_2SO_4$ were prepared with the above buffer.

The activation catalysed by 0.5ml. resuspended acetone powder, and 0.3ml. $(NH_4)_2SO_4$ fraction, was measured in the following system: tris-HCl buffer (100 μmoles, pH 7.7); acetate (5 μmoles); $MgSO_4$ (5 μmoles); $NH_2OH$ (200 μmoles, neutralised with NaOH); GSH (5 μmoles); ATP (5 μmoles); CoA (0.3 μmoles), and enzyme in a total volume of 2.0ml. The system without acetate or CoA was used as control. The activation is expressed as μmoles hydroxamate formed in 4hr. at 30°.

| Enzyme preparation | Hydroxamate formed (μmoles) | Control $E_{546}$ value | Extinction increase in test solution (%) |
|---|---|---|---|
| Resuspended acetone powder | 0.52 | .216 | 21 |
| 0-80% satd.$(NH_4)_2SO_4$ppt. | 0.51 | .174 | 26 |
| 0-25% satd.$(NH_4)_2SO_4$ppt. | 0.05 | .056 | 7 |
| 25-50% satd.$(NH_4)_2SO_4$ ppt. | 0.65 | .152 | 38 |
| 50-80% satd. $(NH_4)_2SO_4$ ppt. | 0.07 | .184 | 3 |
| 0-30% satd.$(NH_4)_2SO_4$ppt. | 0 | .043 | 0 |
| 30-45% satd.$(NH_4)_2SO_4$ ppt. | 0.51 | .062 | 73 |
| 45-80% satd. $(NH_4)_2SO_4$ ppt. | 0.28 | .250 | 11 |

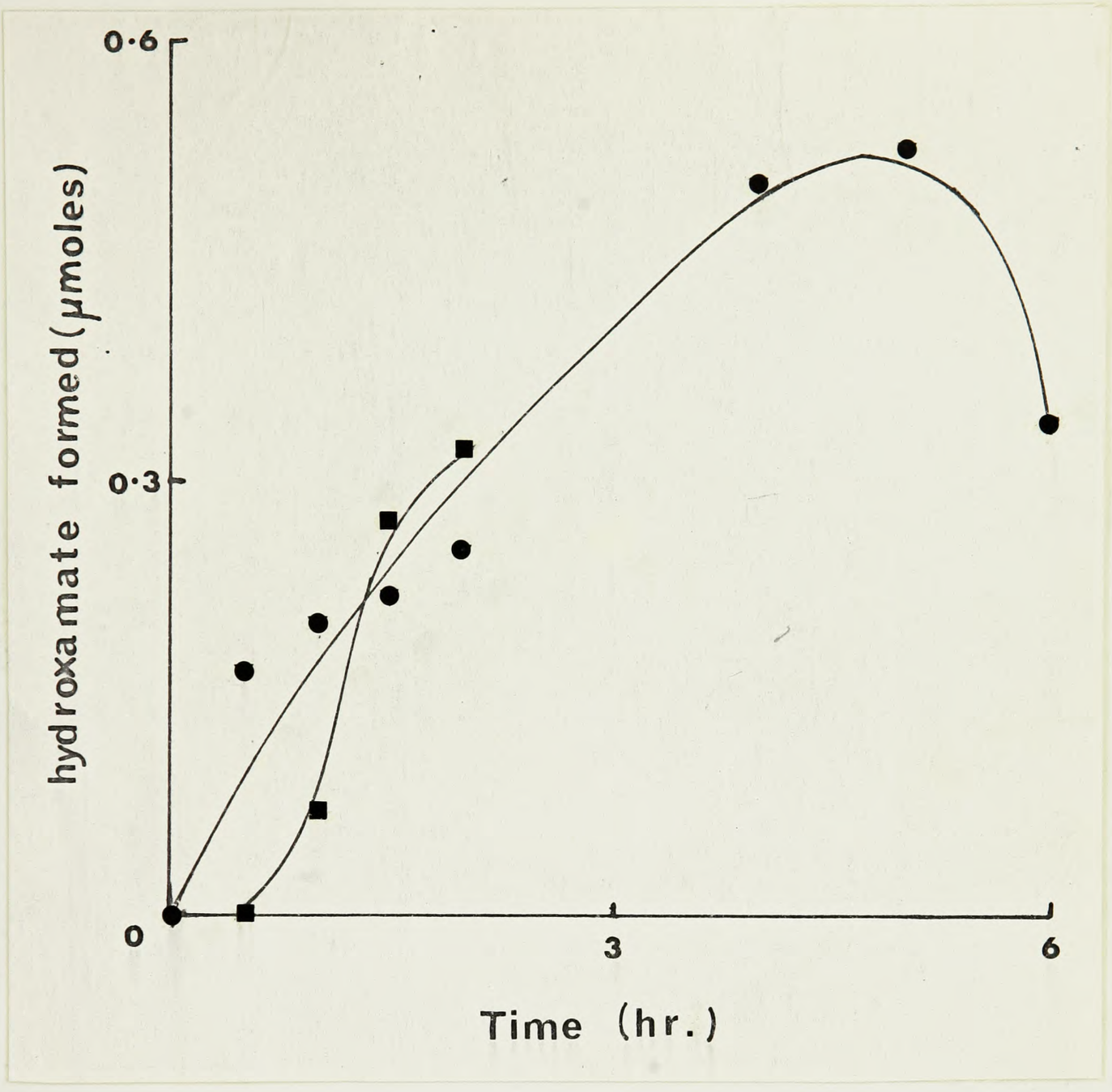



Fig.20. Activation of acetate with resuspended acetone powders of spinach beet leaves. The enzyme extract was prepared by grinding 1g. acetone powder in 20ml. tris-HCl buffer (0.1M, pH 7.7) in a glass homogeniser. The conditions of assay are described in Table 3. The progress of the reaction is expressed as µmoles hydroxamate formed at 30° by 0.5 ml. resuspended acetone powder (■, acetone powder 1, where 12g. acetone powder were obtained from 200g. leaves; ●, acetone powder 2, where 20g. acetone powder were obtained from 200g. leaves).

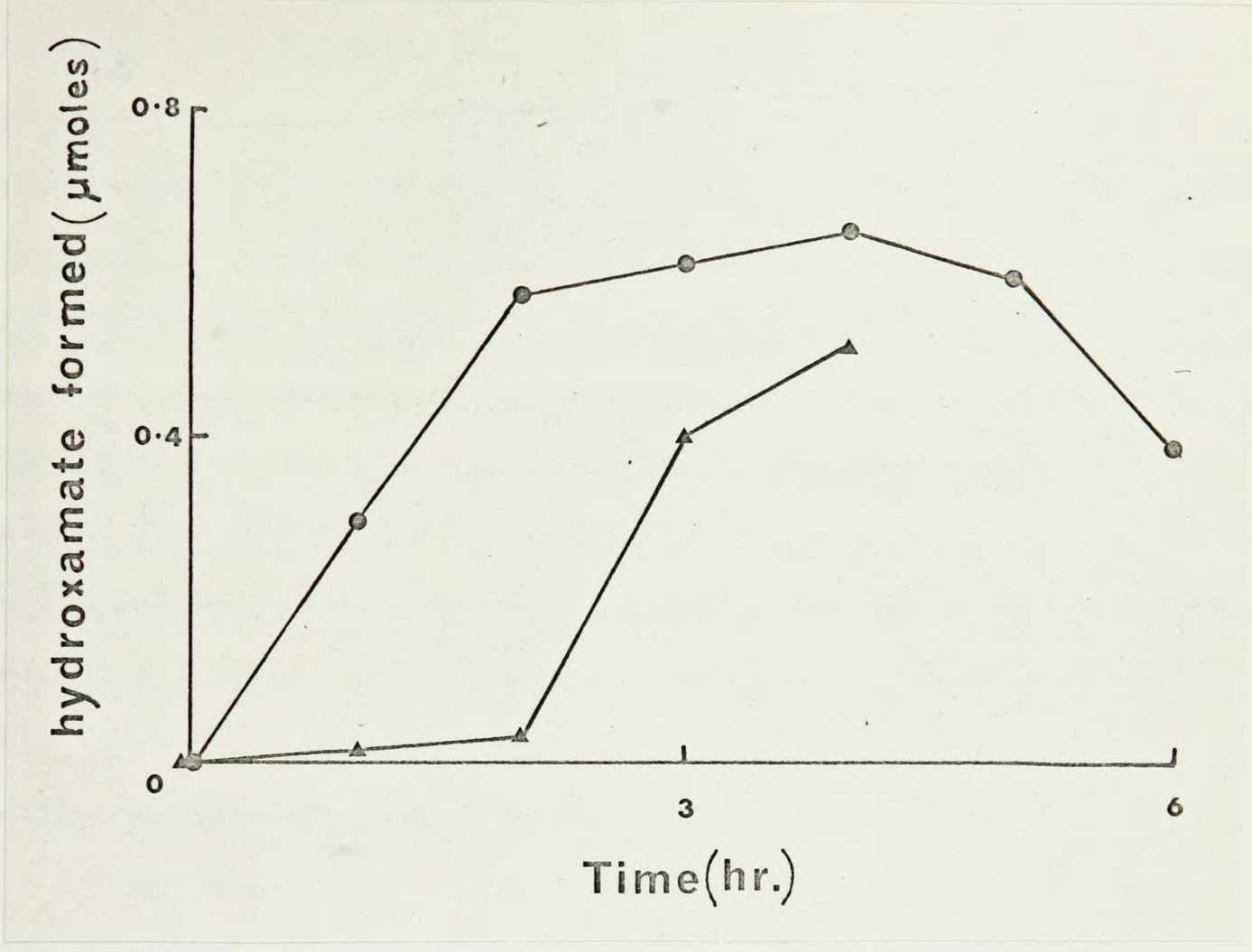



Fig. 21. Activation of acetate with $(NH_4)_2SO_4$ fractions from an acetone powder of spinach beet leaves. Preparation of $(NH_4)_2SO_4$ fractions and conditions of assay are described in Table 3. The progress of the reaction is expressed as μmoles hydroxamate formed at 30° by 0.3ml. each of 25-50% satd. $(NH_4)_2SO_4$ fraction (●), and 30-45% satd. $(NH_4)_2SO_4$ fraction (▲).

The extent of activation increased with enzyme concentration but above a certain value decreased (Fig.22). This suggested that there was an inhibitor in the preparation which reduced enzyme activity as its concentration was increased. On storage in tris-HCl buffer (0.1M, pH 7.7) the enzyme lost 50% of its activity in 40hr. (Fig.23). The hydroxylamine concentration for optimum measurement of activity was only 0.1M, which was normally used (Fig.24). The shape of the graph, rising steeply only at low concentrations of hydroxylamine, showed that hydroxylamine was inhibitory to the enzyme system, but since the experiments were carried out using hydroxylamine neutralised with NaOH, the effect could have been due to the increase in the concentration of sodium ions.

The activation of acetate was entirely dependent on the presence of ATP, CoA and $Mg^{++}$. This is demonstrated in Table 4 for the 0-80% satd. $(NH_4)_2SO_4$ fraction; other fractions gave similar results. The enzyme activity was completely lost on dialysis, or on desalting by passing through a Sephadex-G25 column. The pH-dependence of acetate activation, using a range of buffers from pH 4.0 to 9.2 is shown in Fig.25. The presence of hydroxylamine, and possibly of other components of the assay system, reduced the pH range over which activation could be measured to 5.8-8.0. The optimum pH was near 7.0, which corresponded to tris-HCl and imidazole-HCl buffers of pH 7.4. From a

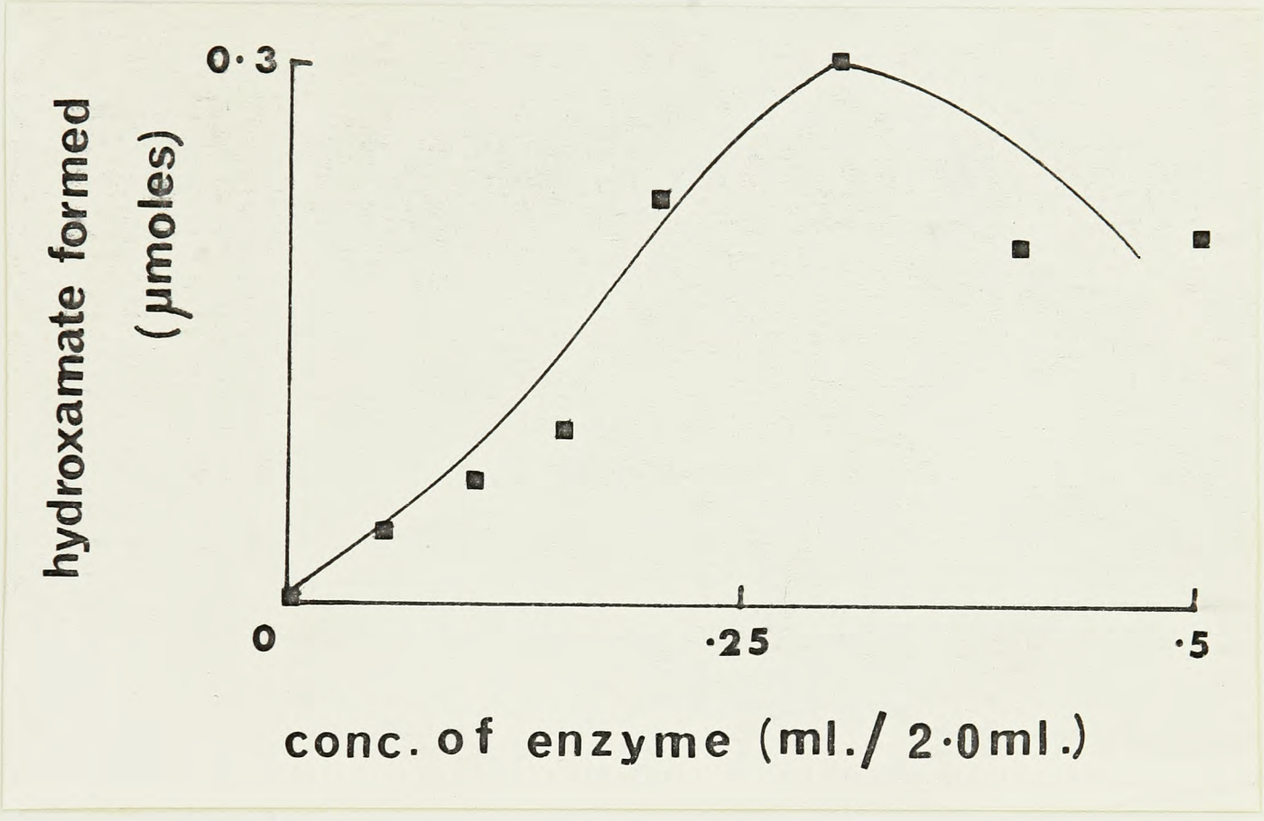



Fig.22. Relationship between acetate activation and enzyme concentration in a preparation from an acetone powder from spinach beet leaves. The activation catalysed by 0.05 to 0.3ml. 30-45% satd. $(NH_4)_2SO_4$ fraction, prepared as described in Table 3, was measured as described in Table 3, and is expressed as µmoles hydroxamate formed in 1hr. at 30°.

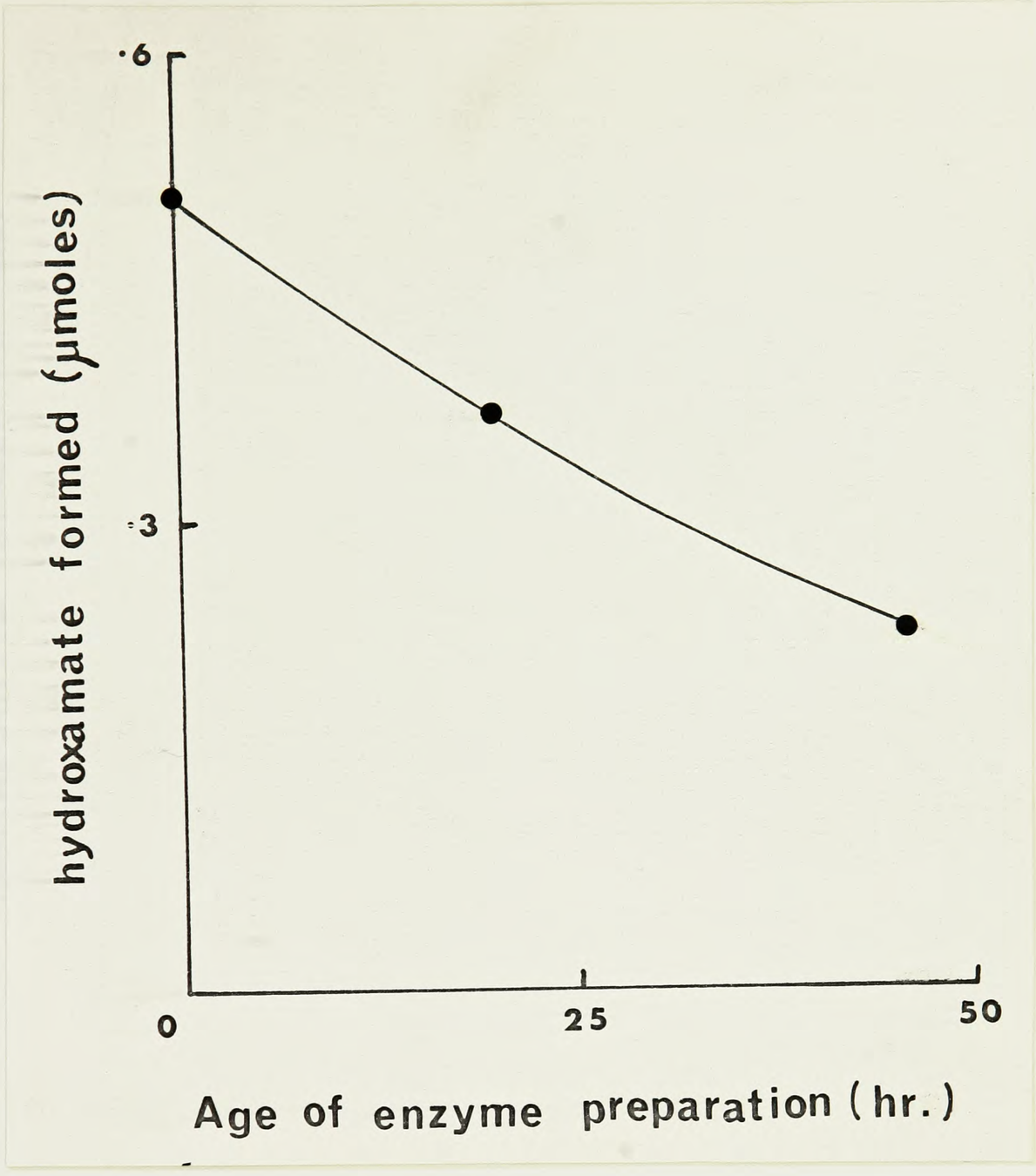



Fig. 23. Stability of an acetate-activating preparation from an acetone powder from spinach beet leaves. The activation catalysed by 0.2ml. 30-45% satd.$(NH_4)_2SO_4$ fraction, prepared as described in Table 3, was measured as described in Table 3, and is expressed as µmoles hydroxamate formed in 4hr. at 30°.

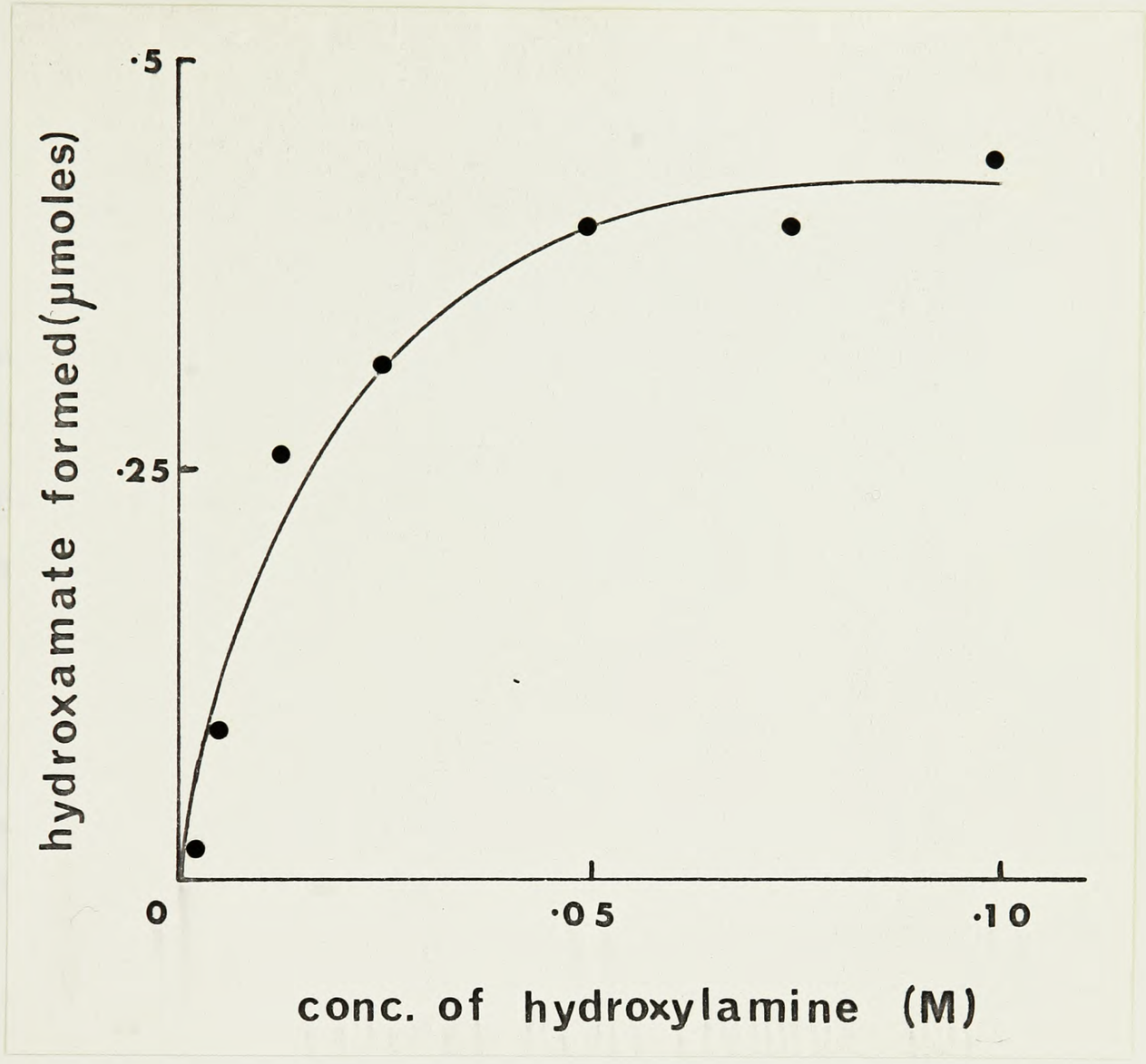



Fig. 24. Effect of hydroxylamine concentration on the activation of acetate. The activation catalysed by 0.2ml. 30-45% satd. $(NH_4)_2SO_4$ fraction, prepared from an acetone powder from spinach beet leaves as described in Table 3, was measured at hydroxylamine concentrations up to 0.1M in the system described in Table 3, and is expressed as µmoles hydroxamate formed in 4hr. at 30°.

Table.4. The requirements of the acetate-activating system from spinach beet acetone powder.

The activation catalysed by 0.5ml. 0-80% satd. $(NH_4)_2SO_4$ fraction, prepared as described in Table 3, was measured in the system described in Table 3. The system without acetate was used as control. The activation is expressed as μmoles hydroxamate formed in 4 hr. at 30°.

| System | Hydroxamate formed (μmoles) |
|---|---|
| Complete | 0.62 |
| No CoA | 0.10 |
| No ATP | 0.06 |
| No CoA or ATP | 0 |
| No $Mg^{++}$ | 0.05 |

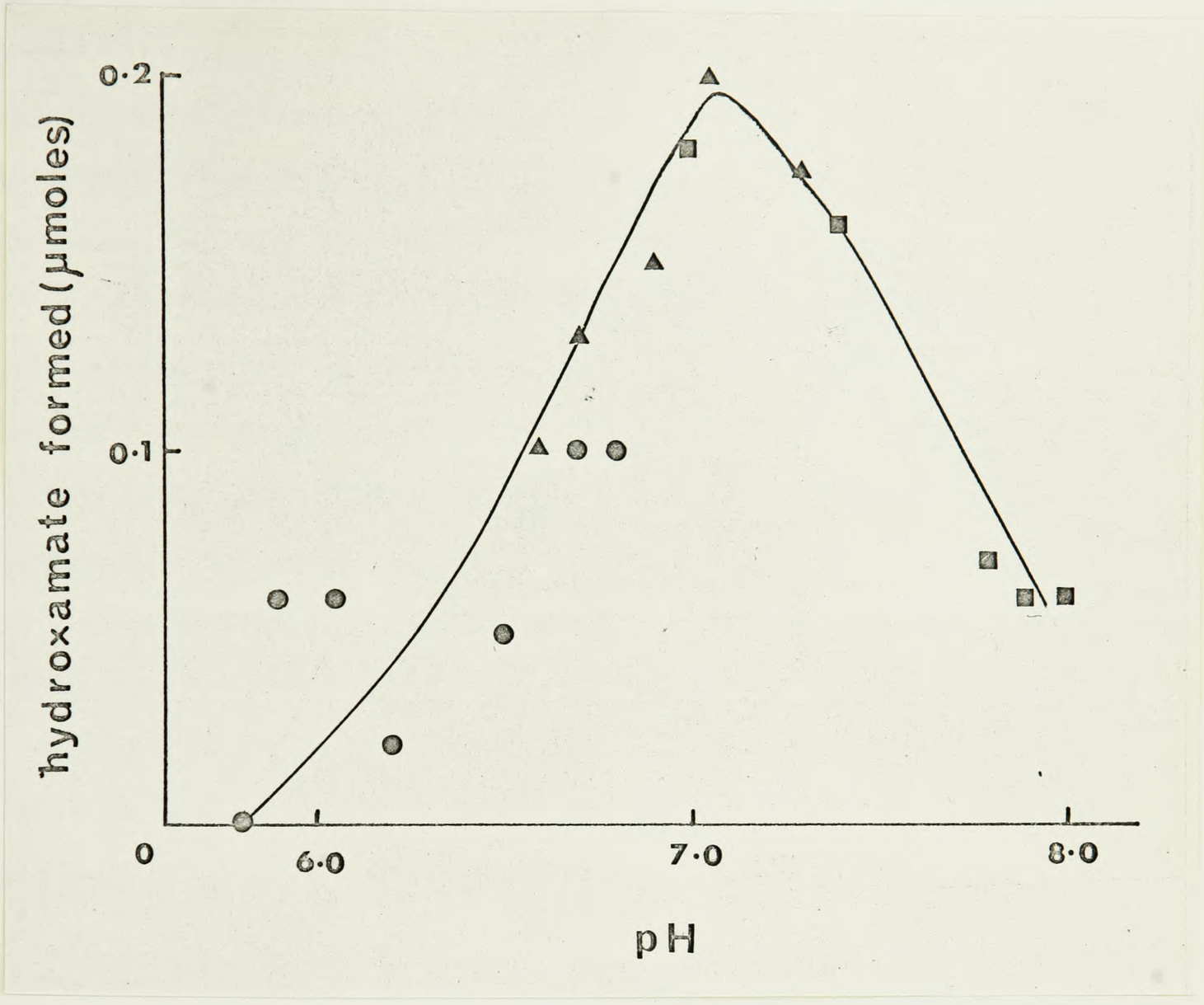



Fig. 35. pH-activity relationship of acetate activation catalysed by a preparation from an acetone powder from spinach beet leaves. The activation catalysed by 0.2ml. 30-45% satd. $(NH_4)_2SO_4$ fraction, prepared as described in Table 3 was measured as described in Table 3, but using 120 µmoles each of phthalate-NaOH buffer, pH 4.0 to 6.2 (●); imidazole-HCl buffer, pH 6.2 to 7.8 (▲); or tris-HCl buffer, pH 7.4 to 9.1 (■), instead of 100 µmoles tris-HCl buffer, pH 7.7. The pH of each system was measured after preparation. The activation is expressed as µmoles hydroxamate formed in 4hr. at 30°.

comparison of the effectiveness of these two buffers in extraction and assay of the enzyme, tris-HCl buffer (0.1M, pH 7.4) was subsequently preferred, since it offered a wider useful pH range even though no marked difference between the two buffers was observed.

The activity of the enzyme system towards several different acids is shown in Table 5. Although the system, in both an acetone powder suspension, and in the 30-45% satd. $(NH_4)_2SO_4$ fraction, was active towards some aliphatic acids, it showed negligible activity towards cinnamate.

The activity of suspensions of acetone powders prepared from spinach beet leaves as the winter advanced fell. Aqueous extracts of acetone powders of runner bean leaves from plants grown in the greenhouse, showed very little activity towards acetate.

2. Aqueous extracts prepared in the presence of ascorbate.

Aqueous extracts of a variety of plant organs were prepared by macerating the material in tris-HCl buffer (0.1M, pH 7.4) containing 0.02M-ascorbate, and removing cell-debris by centrifugation at 5,000xg. The pH of 7.4 was used not only because of earlier results with acetone powder extracts, but also because ascorbate is more stable in neutral or acid solutions.

Table 5. The activation of different acids by preparations from spinach beet acetone powder.

The activation catalysed by 0.5ml. resuspended acetone powder, and 0.3ml. 30-45% satd. $(NH_4)_2SO_4$ fraction, prepared as described in Table 3, was measured in the system described in Table 3, using as substrates 5 μmoles each of various acids. To avoid the difficulty of different extinction coefficients for each acyl-hydroxamate, the activation is expressed as the extinction difference at 546nm. between the complete system, and the system without CoA, developed in the stated time, at 30°.

| Enzyme preparation | Substrate | Activation ($\Delta E_{546}$ nm.) |
|---|---|---|
| Resuspended acetone powder (2hr.incubation) | acetate | .114 |
| | propionate | .115 |
| | succinate | .130 |
| | oxalate | .100 |
| | malonate | .085 |
| | maleate | .078 |
| | citrate | .062 |
| | cinnamate | 0 |
| 30-45% satd.$(NH_4)_2SO_4$ ppt. (3hr. incubation) | acetate | .019 |
| | propionate | .002 |
| | succinate | .031 |
| | cinnamate | .005 |

i) Spinach beet.

A crude extract, and various $(NH_4)_2SO_4$ fractions, prepared from spinach beet leaves obtained from plants subjected to frost in November, and then transferred to pots in the greenhouse, showed no activity in acetate-activation. In contrast, leaves from young spinach beet plants raised entirely in the greenhouse in wintertime, gave extracts with very high activity towards acetate (Table 6). All fractions, whether crude extracts, or $(NH_4)_2SO_4$ fractions, gave low control values, comparable with those from the active 30-45% satd. $(NH_4)_2SO_4$ fraction prepared from an acetone powder extract. As with the acetone powder, the most active fraction here was obtained by saturating to 25-50% with $(NH_4)_2SO_4$, but some activity passed into the 50-80% satd. fraction. The time course of the reaction with active fractions, is shown in Fig.26, and the effect of varying the enzyme concentration in Fig.27. No lag in the development of activation was observed but again, there was evidence of an inhibition of the enzyme at high concentrations of the preparation.

The activity of the two most active fractions towards several different acids was investigated (Table 7). The activation of cinnamate was very low, and present in both the 25-50% and the 50-80% satd.fractions.

Table 6. The activation of acetate by aqueous extracts from leaves of spinach beet, made in the presence of ascorbate.

A macerate of spinach beet leaves was prepared from 100g. of leaf material and 200ml. tris-HCl buffer (0.1M, pH 7.4; containing 0.02M-ascorbate). It was filtered through cheesecloth and the filtrate centrifuged at 5,000xg, 40ml. suspension was prepared from the residue with the above buffer, and $(NH_4)_2SO_4$ fractions were prepared from the supernatant (p. 48). 10ml. suspensions of the precipitates obtained between the stated saturations of $(NH_4)_2SO_4$ were prepared in the above buffer.

The activation catalysed by the stated amount of each preparation was measured in the following system: tris-HCl buffer (100 μmoles, pH 7.4); ascorbate (24-28 μmoles); acetate (5 μmoles); $MgSO_4$ (5 μmoles); $NH_2OH$ (200 μmoles, neutralised with NaOH); GSH (5 μmoles); ATP (5 μmoles); CoA (0.3 μmoles) and enzyme in a total volume of 2.0ml. The system without CoA was used as control. The activation is expressed as μmoles hydroxamate formed in 3 hr. at 30°.

| Enzyme preparation | Vol. used (ml.) | Hydroxamate formed (μmoles) | Control $E_{546}$ value | Extinction increase in test solution (%) |
|---|---|---|---|---|
| Filtrate of macerate | 0.4 | 0.33 | .080 | 31 |
| Resuspended residue | 0.4 | 0.13 | .070 | 14 |
| 0-25% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | 0.18 | .076 | 18 |
| 25-50% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | 0.10 | .080 | 200 |
| 50-80% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | 0.97 | .070 | 100 |
| 80% satd. $(NH_4)_2SO_4$ supernatant | 0.4 | 0 | .092 | 0 |

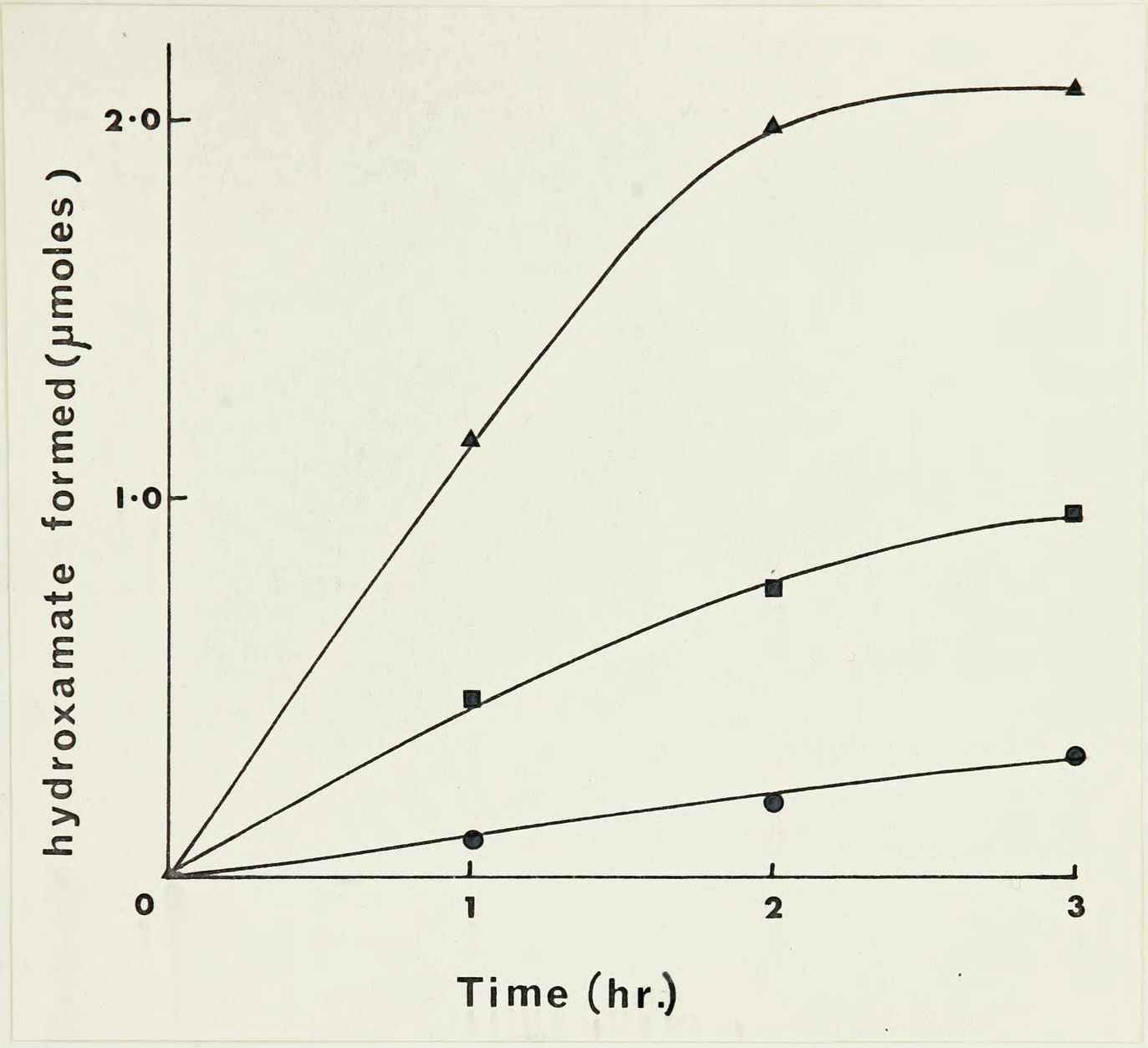



Fig. 26. Activation of acetate with extracts from spinach beet leaves prepared in the presence of ascorbate. The activation catalysed by fractions prepared as described in Table 6, was measured in the system described in Table 6. The progress of the reaction is expressed as μmoles hydroxamate formed at 30° by 0.4ml. filtrate of macerate (●), and by 0.2ml. each of 25-50% satd. $(NH_4)_2SO_4$ fraction (▲), and 50-80% satd. $(NH_4)_2SO_4$ fraction ( ■ ).

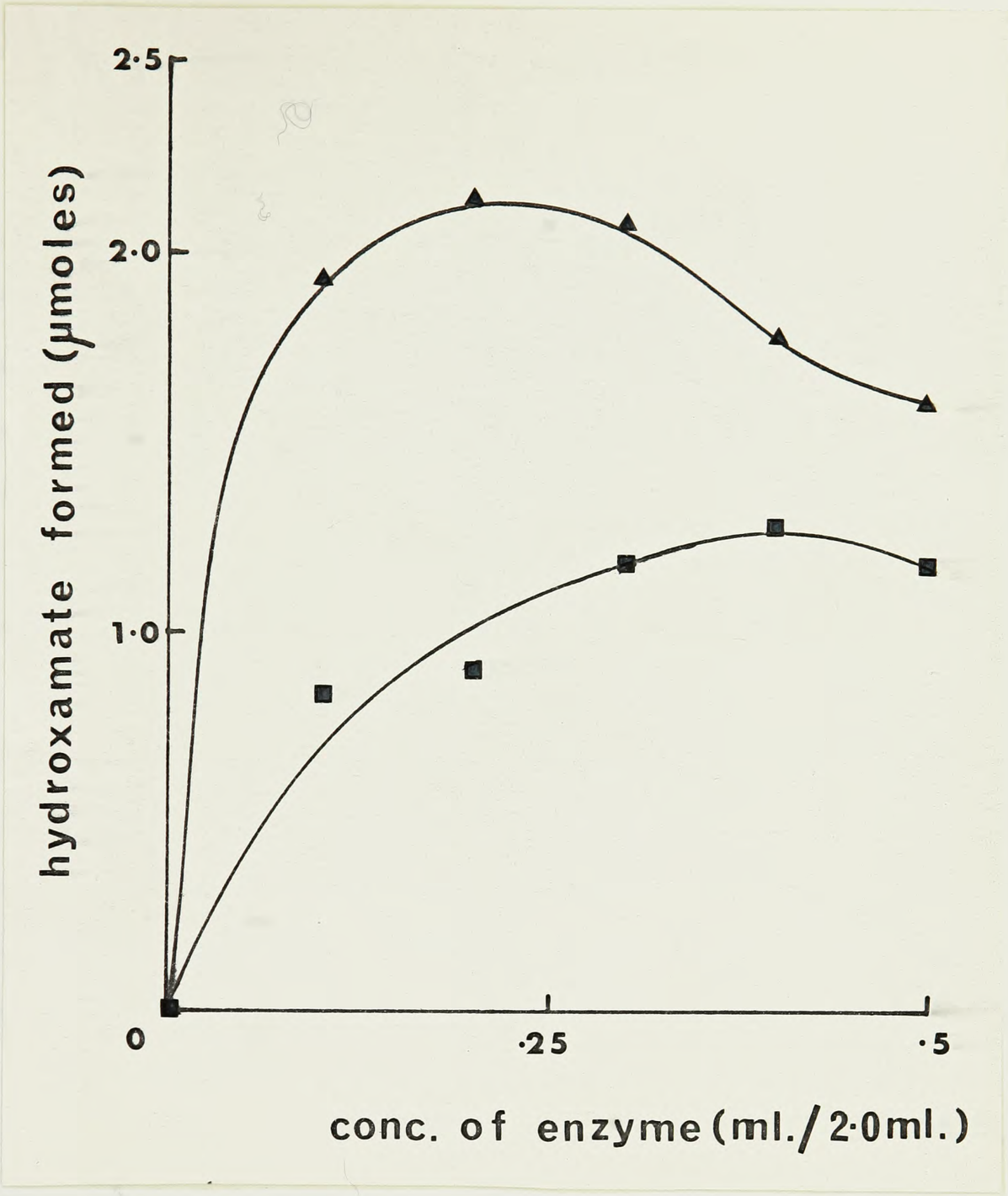



Fig. 27. Relationship between acetate activation and enzyme concentration. The activation catalysed by $(NH_4)_2SO_4$ fractions prepared from spinach beet leaves extracted in the presence of ascorbate as described in Table 6, was measured in the system described in Table 6 using 0.1 to 0.5ml. each of 25-50% satd. $(NH_4)_2SO_4$ fraction (▲), and 50-80% satd. $(NH_4)_2SO_4$ fraction (■). The activation is expressed as µmoles hydroxamate formed in 2hr. at 30°.

ii) Other plants.

Aqueous extracts of the organs from several other plants (leaves of broad bean and pea from plants grown in the greenhouse in winter; and roots of maize grown in a dark incubator at 30°) prepared in tris-HCl buffer (0.1M, pH 7.4) containing 0.02M-ascorbate, catalysed acetate-activation. The activity of $(NH_4)_2SO_4$ fractions prepared from these extracts towards several different acids is shown in Table 7. In all cases $(NH_4)_2SO_4$ fractionation achieved some separation of activity towards these acids.

In extracts of spinach beet, broad bean, and pea leaves, the acetate-activation catalysed by the 25-50% satd.$(NH_4)_2SO_4$ fraction was always greater than that catalysed by the 50-80% satd.fraction. Conversely, the activation of succinate was almost completely isolated in the 50-80% satd. $(NH_4)_2SO_4$ fraction. The activation of propionate was low, and variably distributed between the two fractions. In extracts of broad bean and pea leaves, the cinnamate-activation was low, but confined to the 50-80% satd.$(NH_4)_2SO_4$ fraction; for spinach beet leaf it was low and distributed equally between the 25-50% and 50-80% satd.fractions.

In maize root extracts, however, the distribution pattern of activation was different. The 50-80% satd. $(NH_4)_2SO_4$ fraction was inactive in catalysing acetate-activation; the activity towards this acid was mainly in

Table 7. The activation of different acids by $(NH_4)_2SO_4$ fractions from aqueous macerates of plant materials made in the presence of ascorbate.

Spinach beet leaf extracts were prepared as described in Table 6. Other extracts were prepared similarly using the following amounts of material: 150g. broad bean leaves were macerated in 260ml. buffer, and 10ml. suspensions were prepared from the $(NH_4)_2SO_4$ ppts., excepting the 25-50% satd. ppt. from which 16 ml. suspension was prepared; 52g. pea leaves were macerated in 100 ml. buffer, and 10 ml. suspensions were prepared from the $(NH_4)_2SO_4$ ppts.; 10g. maize roots were ground in 20ml. buffer in a glass homogeniser, and 5 ml. suspensions were prepared from the $(NH_4)_2SO_4$ ppts.

The reactivity of the enzyme preparations towards acetate, succinate, propionate, and cinnamate was measured in the system described in Table 6, using 5 μmoles of each substrate. The system without CoA was used as control. The activation is expressed as the extinction difference at 546nm. (cf. Table 5), developed in the stated time at 30°.

| | | | Activation ($\Delta E_{546nm.}$) | | | |
|---|---|---|---|---|---|---|
| Preparation | Vol. used (ml.) | Time of incubation (hr.) | Acetate | Succinate | Propionate | Cinnamate |
| Young spinach beet leaf | | | | | | |
| 25-50 | 0.1 | 2 | .126 | 0 | .050 | .002 |
| 50-80 | 0.1 | 2 | .035 | .015 | .018 | .002 |
| Broad bean leaf | | | | | | |
| 25-50 | 0.3 | 1 | .030 | .014 | .018 | 0 |
| 50-80 | 0.3 | 1 | .015 | .022 | 0 | .025 |
| Pea leaf | | | | | | |
| 25-50 | 0.3 | 1 | .040 | 0 | .010 | 0 |
| 50-80 | 0.3 | 1 | .035 | .015 | .005 | .009 |
| Maize root | | | | | | |
| 0-25 | 0.2 | 2 | .009 | .004 | .007 | .010 |
| 25-50 | 0.2 | 2 | .012 | .007 | .012 | 0 |
| 50-80 | 0.2 | 2 | 0 | 0 | 0 | 0 |

0-25, 25-50, and 50-80 denote ppts. formed between particular $(NH_4)_2SO_4$ saturations. The 0-25 fractions from spinach beet, broad bean and pea were inactive.

the 25-50% satd. fraction, but there was also a little in the 0-25% satd.fraction. Activity towards succinate and propionate was also highest in the 25-50% satd. $(NH_4)_2SO_4$ fraction, but detectable in the 0-25% satd. fraction. The levels of activation of these acids in maize root extracts were comparable to those of acetate, whereas in other tissue extracts they were lower. Cinnamate-activation was low, but limited to the 0-25% satd.fraction. Maize root extracts showed a generally low activating activity.

The activity towards cinnamate was not very stable overnight, and often disappeared altogether, whereas the activity towards acetate always survived overnight, but often lost 50% of its original activity. Succinate-activation showed a similar stability to that of acetate.

iii) Variation of extraction conditions.

These results demonstrated that an enzyme which activated cinnamate could be extracted in aqueous macerates of various plant organs in the presence of ascorbate. The levels of activation were very low, but higher than any obtained from the extracts of acetone powders, and it was possible in three cases to confine the activity towards cinnamate to a specific $(NH_4)_2SO_4$ fraction. Preparations were further examined by different extraction methods, and by the use of altered pH or buffers during the extraction procedure. The possible inhibitory effects of $Na^+$ ions on

the assay of enzymic activity (Hiatt, 1964; Webster, 1966; Peterson, 1967) were taken into account by neutralising hydroxylamine with KOH (instead of NaOH), and by adding substrates either as potassium salts, or in solution in tris-HCl buffer (instead of as Na salts). The extracts were also checked for activity by the spectrophotometric assay for cinnamyl-CoA formation, and by the nitroprusside assay for CoA sulphydryl group disappearance.

Extracts of pea epicotyls and cauliflower florets were made by maceration in tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA). The supernatant from the removal of cell debris by centrifugation at 5,000 xg was fractionated with $(NH_4)_2SO_4$. Acetate-activation was easily measurable, and spread over two or three $(NH_4)_2SO_4$ fractions. Cinnamate-activation was higher than in previous extracts, but spread over two or three $(NH_4)_2SO_4$ fractions. (Table 8). It was not stable overnight. The pea epicotyl fractions were not active in cinnamate-activation when assayed for cinnamyl-CoA formation or by using the nitroprusside estimation.

In order to check that higher levels of cinnamate-activating activity were extracted at pH 8.0, than at a lower pH, extracts of spinach beet leaves from plants grown in the greenhouse in winter were prepared simultaneously in tris-HCl and $KH_2PO_4$-KOH buffers of different pH, containing 0.02M-ascorbate and 0.5mM-EDTA. The extracts were prepared

Table 8. The extraction of acetate - and cinnamate -activating enzymes from pea epicotyls and cauliflower florets.

Enzyme extracts were prepared by the method described in Table 6, but using tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA). The following amounts of material were used: 99g. 4½-day pea epicotyls were macerated in 396ml. buffer, and 10ml. suspensions were prepared from the residue from centrifugation at 5,000xg., and from the $(NH_4)_2SO_4$ ppts.; 200g. cauliflower florets were macerated in 400ml. buffer, 30ml. suspension was prepared from the residue from centrifugation at 5,000xg, and 10ml. suspensions from the $(NH_4)_2SO_4$ ppts. excepting the 25-50% satd.ppt. from which 30ml. suspension was prepared.

The activation catalysed by the stated amount of preparation was measured in the following system: tris-HCl buffer (100 μmoles, pH 8.0); ascorbate (24-26 μmoles); acetate or cinnamate (5 μmoles); $MgCl_2$ (5 μmoles); $NH_4OH$ (200 μmoles, neutralised with KOH); GSH (5 μmoles); ATP (5μmoles); CoA (0.3 μmoles), and enzyme in a total volume of 2.0ml. The system without CoA was used as control. The activation is expressed as the extinction difference at 546nm. developed in 2hr. at 30°, (cf. Table 5).

| Plant Organ | Enzyme preparation | Vol. used (ml.) | Activation ($\Delta E_{546nm.}$) Acetate | Cinnamate |
|---|---|---|---|---|
| Pea epicotyls | 1. Filtrate of macerate | 0.4 | .022 | .005 |
| | 2. Resuspended residue | 0.4 | .014 | 0 |
| | 3. 0-25% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | .006 | .010 |
| | 4. 25-50% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | .023 | .013 |
| | 5. 50-80% satd. $(NH_4)_2SO_4$ ppt. | 0.2 | .009 | .027 |
| | 6. 80% satd. $(NH_4)_2SO_4$ supernatant | 0.4 | 0 | .005 |
| Cauliflower florets | 1. | 0.4 | .004 | 0 |
| | 2. | 0.4 | .010 | .005 |
| | 3. as above | 0.2 | 0 | 0 |
| | 4. | 0.2 | .014 | .014 |
| | 5. | 0.2 | .047 | .016 |
| | 6. | 0.4 | .006 | 0 |

by freezing the leaves in liquid nitrogen, and after grinding, stirring the powdered leaves into the buffer. The filtrate of this extract was assayed immediately, and after 2½hr. storage at 0°, for activation of both cinnamate and acetate. (Table 9). A greater amount of cinnamate-activating activity was extracted at pH 8.0 into either $KH_2PO_4$-KOH buffer (0.1M), or tris-HCl buffer (0.1M), than at pH 7.0 into $KH_2PO_4$-KOH buffer (0.1M). The amount of acetate-activating activity extracted was similar at pH 7.0 or pH 8.0 in either buffer. The cinnamate-activating activity was most stable at pH 8.0 in $KH_2PO_4$-KOH buffer; the acetate-activiting activity appeared to lose no activity during storage under these conditions for 2½hr. All extractions were subsequently carried out in $KH_2PO_4$-KOH buffer (0.1N, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA).

A few of the characteristics of the cinnamate-activating system extracted under these conditions were investigated. Fig.28 shows the relationship between the pH of the assay mixture and the amount of cinnamate-activation. The optimum pH was about 7.8, which corresponded to the use of tris-HCl assay buffer, pH 8.5. This is higher than the pH optimum for acetate-activation catalysed by an $(NH_4)_2SO_4$ fraction from an acetone powder extract (Fig.25). Fig.29 shows the relationship between enzyme concentration and cinnamate-activation. The optimum volume of extract was 0.4-0.5ml./

Table 9. The extraction and stability of activating enzymes from frozen spinach beet leaves under different conditions.

Enzyme extracts were prepared by stirring 25g. frozen and ground spinach beet leaves in 100ml. buffer for 15 min. The suspension was filtered through cheesecloth and the pH of the filtrate was adjusted to the approximate value with KOH (for $KH_2PO_4$-KOH buffers), and ammonia solutions (for tris-HCl buffers).

The activation catalysed by 0.5ml. enzyme preparation was measured in the system described in Table 8. The system without CoA was used as control. The activation is expressed as the extinction difference at 546nm. developed in 2 hr. at 30°. (cf. Table 5).

| Buffer and pH used for extraction and storage | Activation ($\Delta E_{546nm.}$) | | | |
|---|---|---|---|---|
| | Fresh Preparation | | Preparation after 2½hr. storage | |
| | Acetate | Cinnamate | Acetate | Cinnamate |
| 0.1M $KH_2PO_4$-KOH buffer, pH 7.0, containing 0.02M-ascorbate & 0.5mM-EDTA | .090 | .014 | .070 | .011 |
| 0.1M $KH_2PO_4$-KOH buffer, pH 8.0, containing 0.02M-ascorbate & 0.5mM-EDTA | .070 | .026 | .086 | .020 |
| 0.1M tris-HCl buffer, pH 8.0, containing 0.02M-ascorbate & 0.5mM-EDTA | .085 | .030 | .069 | .015 |

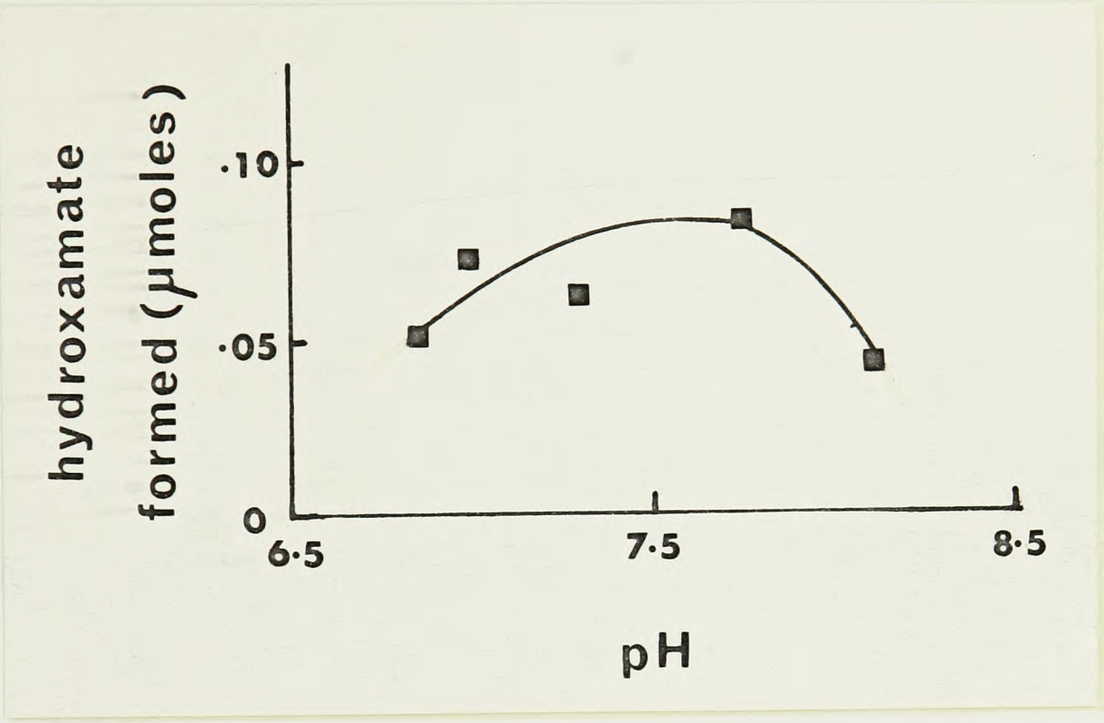



Fig.28. pH-activity relationship of cinnamate activation catalysed by an extract from spinach beet leaves prepared in the presence of ascorbate. The enzyme extract was prepared by stirring 50g. frozen and ground spinach beet leaves in 200ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA) for 15 min. at 0°. The filtrate of the extract, at pH 8.0, was assayed in the following system: tris-HCl buffer (100 µmoles, pH 7.0 to 9.0); ascorbate (30 µmoles); cinnamate (5 µmoles); $MgCl_2$ (5 µmoles); $NH_2OH$ (200 µmoles, neutralised with KOH); GSH (5 µmoles), ATP (5 µmoles); CoA (0.3 µmoles), and 0.5 ml. enzyme sample in a total volume of 2.0ml. The system without CoA was used as control, and the pH of each system measured after preparation. The activation is expressed as µmoles hydroxamate formed in 2 hr. at 30°.

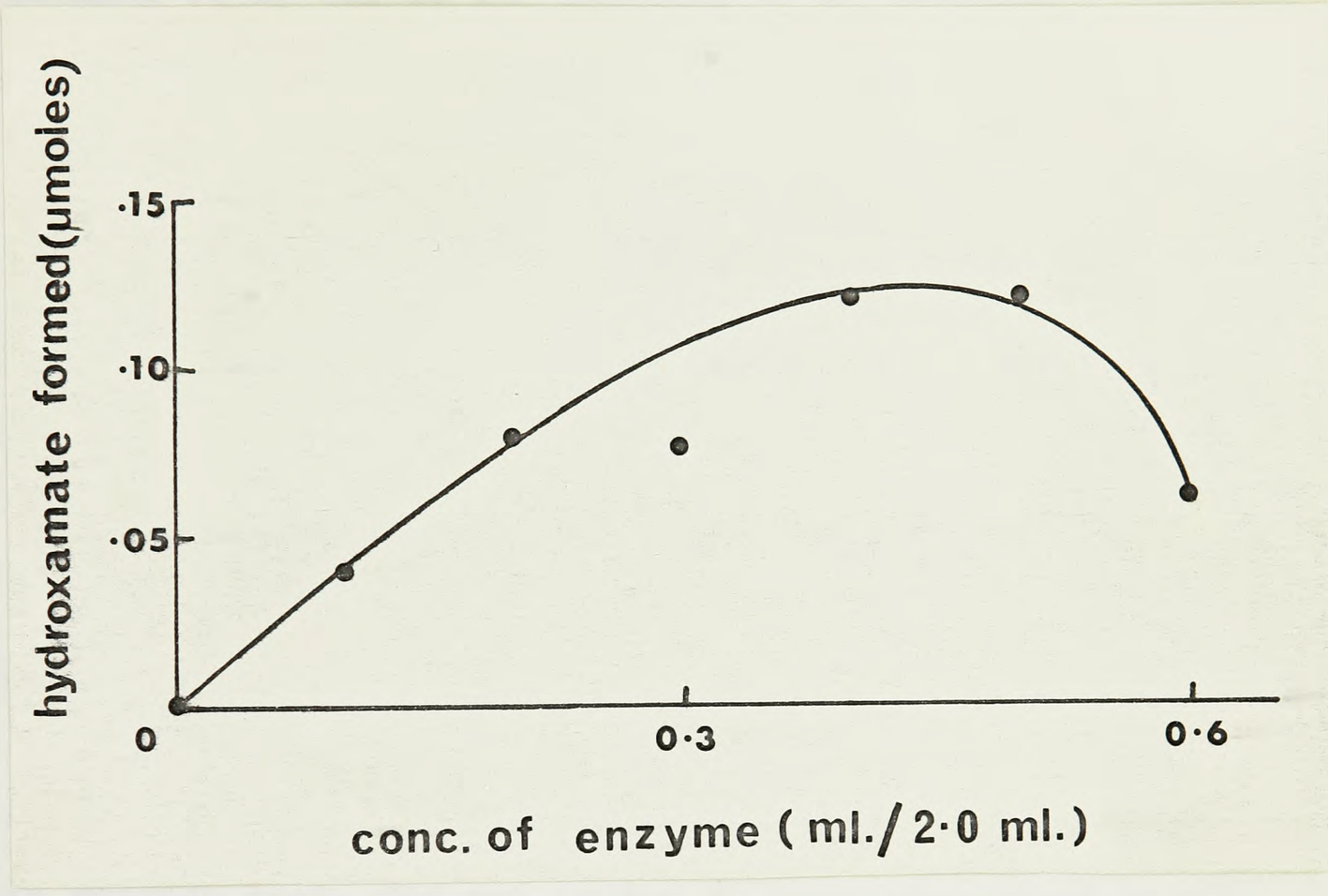



Fig.29. Relationship between cinnamate activation and enzyme concentration. The activation catalysed by an enzyme extract prepared as described in Fig.28, was measured in the system described in Fig.28, but using tris-HCl buffer at pH 8.5, and enzyme concentrations up to 0.6ml./2.0ml. The activation is expressed as µmoles hydroxamate formed in 2 hr. at 30°.

2.0ml. assay mixture. The activation fell above this concentration, and as in other crude extracts, this fall is assumed to be due to the presence of an inhibitor, which becomes apparent at high concentrations of extract. The cinnamate-activating activity was not stable overnight.

3. Aqueous extracts prepared in the presence of soluble PVP and ascorbate.

In the extractions in which the effect of soluble PVP was examined, the most satisfactory extraction buffer ($KH_2PO_4$-KOH, pH 8.0) was not used. Tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA) was employed.

The presence of 1% soluble PVP in this extraction buffer, which was used to make an extract of frozen leaves of spinach beet, did not appear to have any effect on the amounts of acetate- and cinnamate-activating activities extractable (Fig.30). It had no effect either on the stability of the two activities over a period of 6hr. (Fig.31). The presence of soluble PVP failed to reduce the control values obtained in the assay of the enzymes, and interfered with $(NH_4)_2SO_4$ fractionation. Its use was therefore not continued.

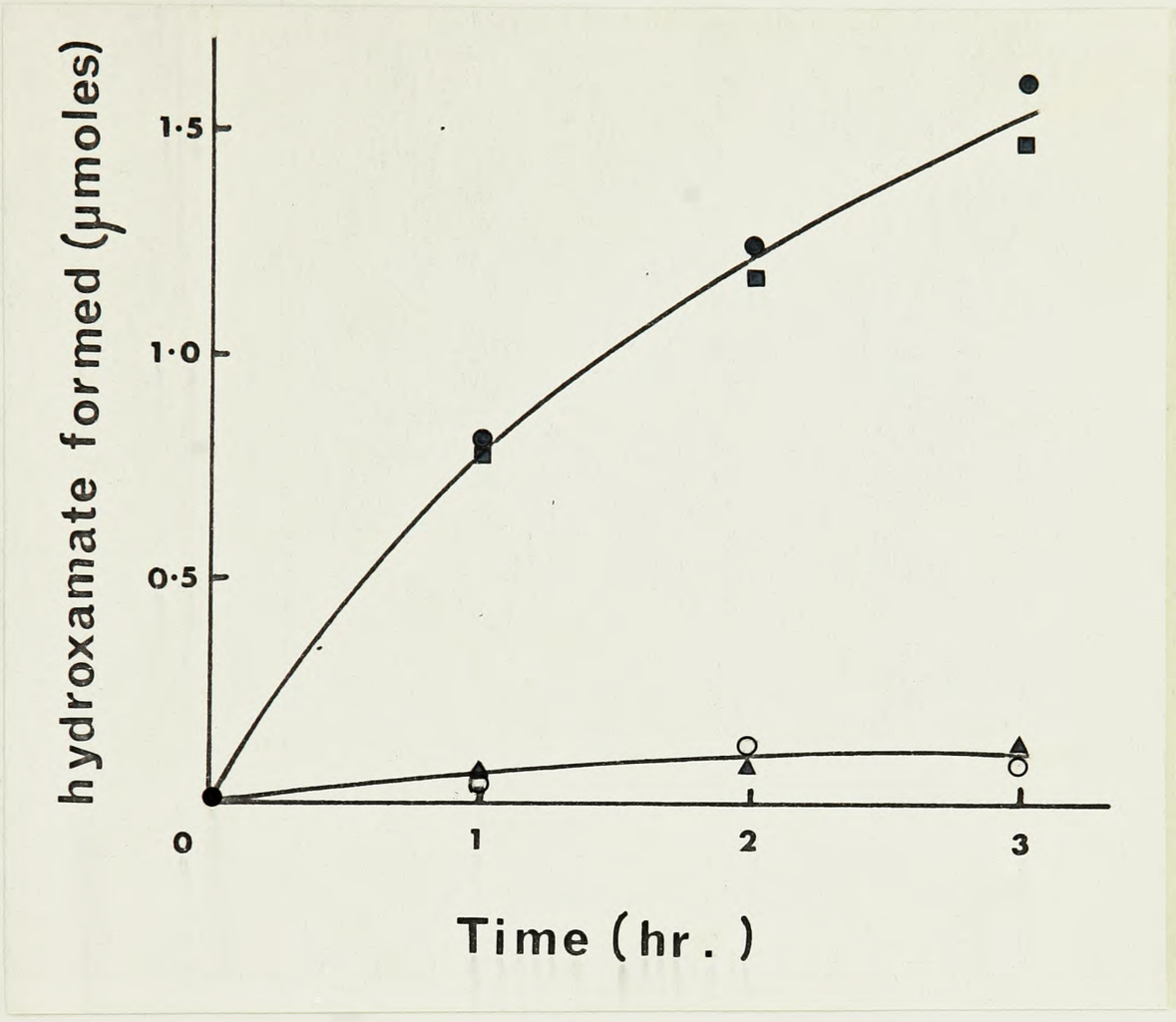



Fig. 30. Effect of soluble polyvinylpyrrolidone on the extraction of activating enzymes from spinach beet leaves in the presence of ascorbate. The enzyme extract was prepared from frozen leaves (cf. Fig. 28) but using tris-HCl buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA), ( ●,○ ); or the same buffer also containing 1% soluble polyvinylpyrrolidone ( ■,▲ ). The activation catalysed by 0.5ml. enzyme sample was measured as described in Fig. 28, but using tris-HCl buffer at pH 8.5, and 5 μmoles of acetate or cinnamate as substrate. The progress of the reaction is expressed as μmoles hydroxamate formed at 30° in the presence of acetate ( ●,■ ), or cinnamate ( ▲,○ ).

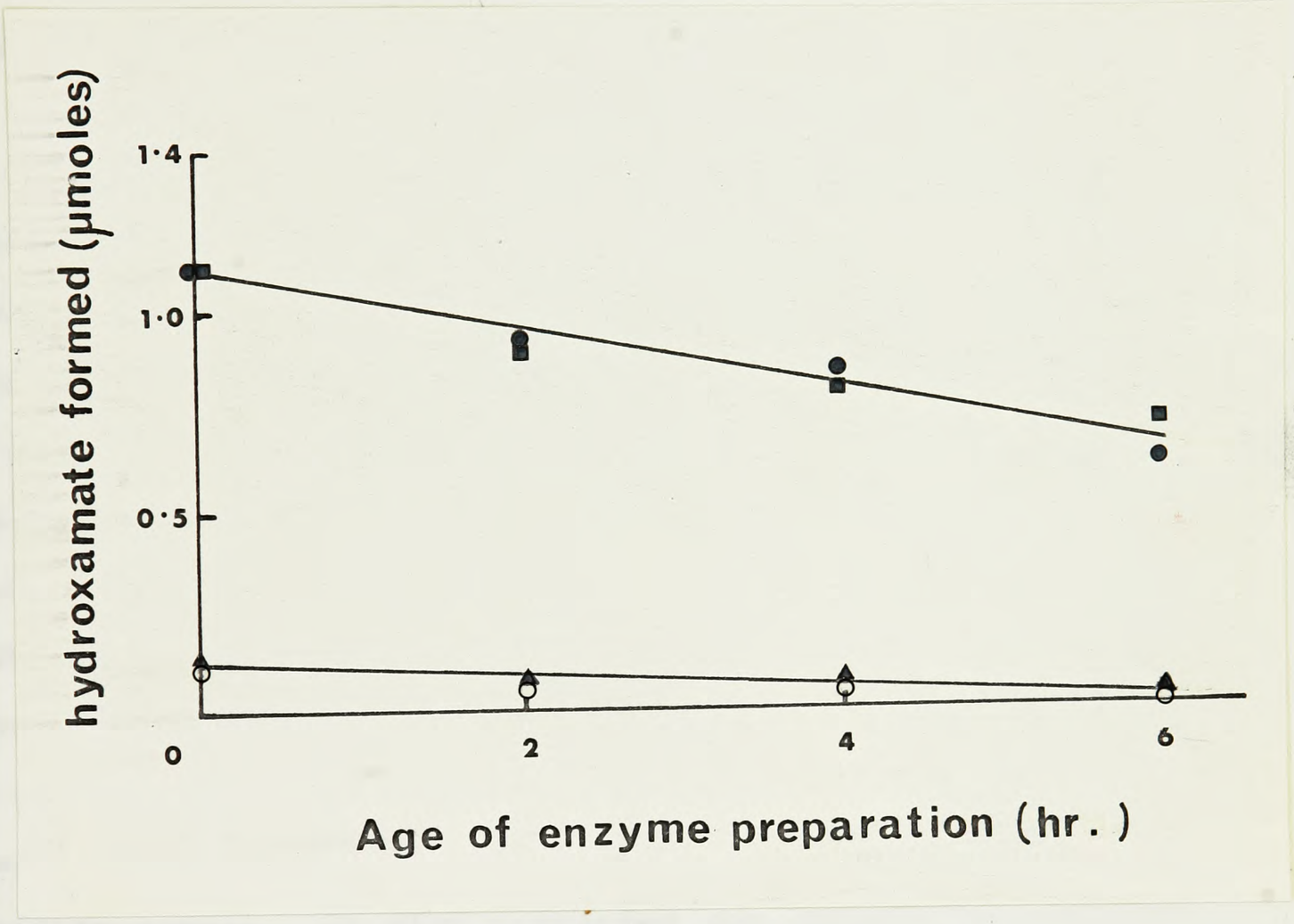



Fig. 31. Effect of 1% soluble polyvinylpyrrolidone on the stability of activating enzymes prepared from spinach beet leaves in the presence of ascorbate. The activation catalysed by 0.5ml. enzyme sample, stored for up to 6hr. after preparation as described in Fig.30, was measured in the system described in Fig.30. The activation is expressed as µmoles hydroxamate formed in 2 hr. at 30° by the preparation containing PVP (■, acetate; ▲, cinnamate), and by the preparation without PVP (●, acetate; ○, cinnamate).

## 4. Aqueous extracts prepared in the presence of insoluble PVP, and ascorbate or other antioxidants.

### i) The effect of insoluble PVP on the extractability and stability of the cinnamate-activating enzyme.

Extraction of frozen and ground leaves from spinach beet plants which had been grown in the greenhouse in spring was carried out in a suspension of insoluble PVP in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA). The pellet sedimented by centrifugation of the filtrate from this extract at 28,000xg, contained no cinnamate-activating activity, but the supernatant was active in this respect. The activity was not affected by the inclusion of PVP in the extraction medium, nor was the stability of the enzyme assayed after 4 hr.storage. However, insoluble PVP had a marked action in reducing the control values obtained in the enzyme assay. It also prevented the extract from browning on storage in the refrigerator for 24 hr. A decay of the control values with time was apparent in extracts prepared in the presence of insoluble PVP (Table 10).

Insoluble PVP was therefore used in all subsequent extractions, and the activity of the 28,000xg supernatant was investigated without further purification or fractionation.

Table 10. **The effect of insoluble PVP on the extraction and stability of cinnamate - activating enzyme from frozen spinach beet leaves.**

The enzyme extract was prepared by stirring 10g. frozen spinach beet leaf powder in a suspension of 10g. Polyclar AT in 60ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate and 0.5mM-EDTA) for 15 min. at 0°. The filtrate of this extract was adjusted to pH 8.0 with KOH, and centrifuged at 28,000 x g for 15 min. at 0°. A similar preparation was made in the absence of Polyclar AT.

The activation catalysed by 0.4ml. each of the supernatant, and the residue (as 10 ml. of a suspension prepared in the above buffer) was measured in the system described in Fig.30. The system without CoA was used as control. The activation is expressed as μmoles hydroxamate produced in 2hr. at 30°.

| Enzyme preparation | Hydroxamate formed (μmoles) | Control $E_{546}$ value | Extinction increase in test solution (%) |
|---|---|---|---|
| 1. Supernatant from centrifugation at 28,000 x g | 0.08 | 0.110 | 11 |
| 2. As (1) but prepared in the absence of PVP | 0.09 | 0.118 | 14 |
| 3. Residue from (1) | 0 | 0.132 | 0 |
| 4. Residue from (2) | 0 | 0.132 | 0 |
| 5. (1) stored for 4hr. at 0° | 0.07 | 0.096 | 10 |
| 6. (2) stored for 4hr. at 0° | 0.06 | 0.118 | 5 |

(ii) The effect of growth conditions on the extractability of cinnamate-activating enzyme from spinach beet.

The preparations from spinach beet leaves so far described have contained very low levels of cinnamate-activating enzyme. They were all made from leaves of plants raised outside, and grown through the winter in pots in the greenhouse, or from plants raised entirely in the greenhouse during winter and spring. When fresh growths of spinach beet become available outside in summer (1967) the leaves from these plants were shown to contain very much higher levels of cinnamate-activating enzyme.

The use of the assay system without CoA as control was recognized to be invalid since, when these highly active extracts were assayed by the spectrophotometric hydroxamate method, the extinction at 540nm given by this control was lower than the extinction at 540nm. given by the assay system without substrate (Table 11). This difference indicated that not all CoA-dependent hydroxamate formation occurring in the presence of a substrate, was due to activation of that substrate. It also showed that substrate-dependent activation was likely to be CoA-dependent.

Compared with the high cinnamate-dependent activity obtained when the extraction of frozen spinach beet leaves was carried out in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0), no cinnamate-dependent activity was extracted in tris-HCl buffers

Table 11. The cinnamate-activating activity in extracts from spinach beet leaves from a fresh growth of young plants raised outside.

An enzyme extract was prepared in the presence of insoluble PVP as described in Table 10, and assayed as described in Fig.30. Systems without CoA or without cinnamate were used as controls. Hydroxamate formed in the presence of cinnamate (i.e. using the latter control) by 0.6 ml. enzyme at 30° was determined.

a, complete system; b, without CoA; c, without cinnamate.

| Time of incubation (hr.) | System | $E_{546nm}$ | $\Delta E_{546}$ without CoA | $\Delta E_{546}$ without cinnamate | Hydroxamate formed (μmoles) |
|---|---|---|---|---|---|
| 1 | a | .091 | | | |
| | b | .075 | .016 | .011 | .05 |
| | c | .080 | | | |
| 2 | a | .130 | | | |
| | b | .105 | .025 | .010 | .05 |
| | c | .120 | | | |
| 3 | a | .188 | | | |
| | b | .149 | .039 | .023 | .12 |
| | c | .165 | | | |
| 4 | a | .233 | | | |
| | b | .198 | .035 | .021 | .11 |
| | c | .212 | | | |
| 5 | a | .292 | | | |
| | b | .248 | .044 | .027 | .13 |
| | c | .265 | | | |
| 6 | a | .345 | | | |
| | b | .315 | .030 | .027 | .13 |
| | c | .318 | | | |

(0.1M) over a range of pH from 8.0 to 9.0, even though CoA-dependent formation of hydroxamate was obtained in the presence of cinnamate.

There was no visible lag in the formation of cinnamate-dependent hydroxamate. The optimum concentration of the enzyme extract was 0.6ml./2.0ml. assay mixture; at higher concentrations the amount of activation fell.

iii) The effect of air, and of different thiol reagents, on activation.

When assayed under an atmosphere of nitrogen, rather than under air, a considerable increase in cinnamate-activation catalysed by the active extracts described in part (ii), was observed. A lag in the development of this activation over the first 2 hr. of incubation was observed, but activation then continued for 6½ hr. after the start of the incubation. In an atmosphere of air, the comparatively small amount of cinnamate-dependent hydroxamate formation had ceased to increase by ½ hr. (Fig.32).

When, as well as using an atmosphere of nitrogen, mercaptoethanol was substituted for glutathione as thiol reagent in the assay system, the activation of cinnamate was further increased. Dithiothreitol was less effective in protecting cinnamate-activation than either mercaptoethanol or glutathione. When no thiol reagent was included in the assay system, but an atmosphere of nitrogen retained, the

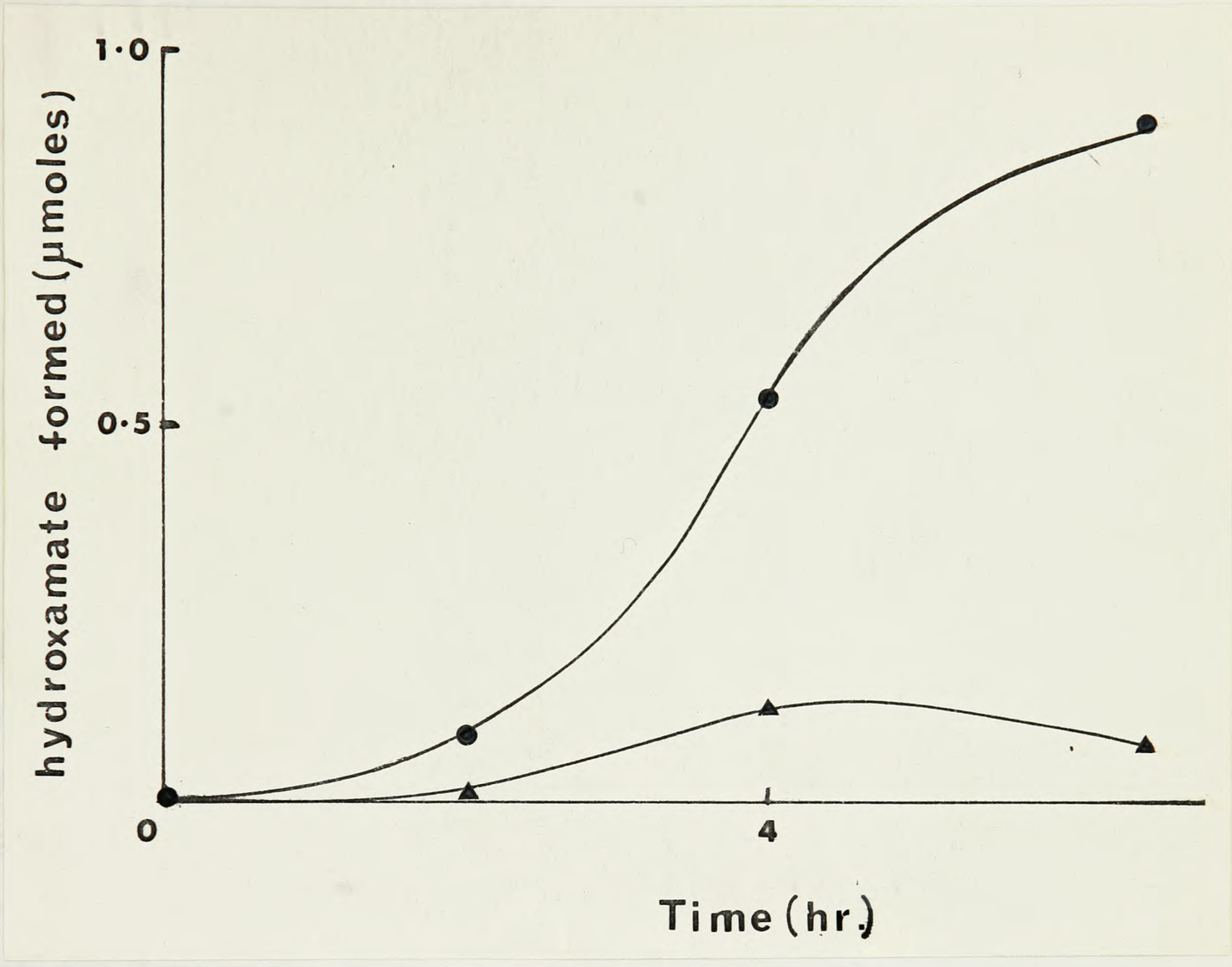



Fig. 30. Effect of air on the activation of cinnamate. The activation catalysed by an extract prepared in the presence of insoluble PVP as described in Table 10, was measured as described in Fig.30, except that the incubation was carried out under air or nitrogen and 5 μmoles mercaptoethanol was used instead of GSH as thiol reagent. The system without cinnamate was used as control, and the progress of the reaction is expressed as μmoles hydroxamate formed at 30° by 0.6ml. enzyme sample under air (▲) or nitrogen (●).

level of cinnamate-activation decreased to that measured in an assay system containing glutathione or mercaptoethanol, but incubated in air. An atmosphere of nitrogen, and thiol reagents therefore appeared to exert similar effects in their stimulation of cinnamate-activation, but maximum activity required both (Table 12). They also had a similar effect in reducing the value of the control (Table 13).

The system catalysing the activation of acetate responded quite differently to both an atmosphere of nitrogen, and to variation in thiol reagents. When an anaerobic atmosphere was substituted for air, there was no change in the level of acetate-activation (Table 12). This level also seemed to be insensitive not only to the type of thiol reagent, but even to the presence of the thiol reagent. 2-Mercaptoethanol may have stimulated acetate-activation, but the figures for this reaction were too variable to demonstrate this unequivocally.

In all the assays described here, the formation of hydroxamate in the control system without CoA was always lower than the formation of hydroxamate in the control system without substrate, for both cinnamate and acetate. This again indicates that both activation reactions were CoA-dependent.

Table 12. The effect of different thiol reagents on the activation of acetate and cinnamate.

An enzyme extract was prepared from a fresh growth of young spinach beet leaves in the presence of insoluble PVP as described in Table 10. It was assayed as described in Fig.30, except that the incubation was carried out under air or nitrogen, and 5 μmoles of GSH, mercaptoethanol, or dithiothreitol were used as thiol reagents. The system without substrate was used as control and the activation is expressed as μmoles hydroxamate formed in 1 hr. at 30° by 0.6ml. enzyme preparation.

| Thiol reagent | Cinnamate activated (μmoles) | | Acetate activated (μmoles) | |
|---|---|---|---|---|
| | air | $N_2$ | air | $N_2$ |
| GSH | 0.13 | 0.36 | 1.47 | 1.85 |
| mercaptoethanol | 0.13 | 0.63 | - | 2.55 |
| dithiothreitol | - | 0.23 | - | 1.42 |
| NONE | - | 0.13 | - | 1.80 |

Table.13. The effect of thiol reagents and of nitrogen in decreasing the extinction given by the control system.

The enzyme preparation and the control systems were as described in Table 12. The incubations were carried out under air or nitrogen at 30°, and the results are expressed as extinction at 546nm. in the presence of various thiol reagents.

| Thiol reagent | Control $E_{546}$ value | |
|---|---|---|
| | air | $N_2$ |
| GSH | .265 | .162 |
| mercaptoethanol | .285 | .132 |
| dithiothreitol | - | .142 |
| NONE | - | .174 |

iv) The activity of spinach beet leaves from ageing plants towards cinnamate.

The active extracts described in parts (ii) and (iii) above were all prepared from a fresh growth of young spinach beet leaves raised outside in a very warm and dry summer. The leaves were large enough to harvest by mid-June, and the experiments were carried out from that time until mid-July, when the leaves were becoming fairly large. Attempts to prepare active extracts from leaves of the same plants after that time, yielded extracts which showed either very low, or no activity towards cinnamate. By the end of July the spinach beet plants had bolted. A fresh growth of spinach beet leaves was harvested about six weeks later from young plants. These yielded extracts with some activity, but this was invariably less than the activity of the extracts described above. Several experiments performed to test the effect of different concentrations of mercaptoethanol, both in the extract and in the assay system, gave contradictory results. There was however, some indication that the addition of mercaptoethanol to the extract decreased the loss of activity normally occurring during storage of the enzyme, and that 2.5 to 5mM-mercapto-ethanol represented the optimum concentration in the assay system.

By the end of September only a very low level of cinnamate-activating enzyme could be extracted from the spinach

beet leaves grown outside. The levels of activity extracted from the leaves were comparable to those measured in air with very active extracts. Throughout the following winter and spring it was impossible to detect the activating enzyme at higher levels than those initially studied, in extracts from either spinach beet or spinach leaves, whether raised in the greenhouse, or transferred to the greenhouse from outside.

v) Extraction of the cinnamate-activating enzyme from fresh summer growths of spinach beet, and from spinach beet grown under controlled conditions.

During the following summer (1968) high levels of cinnamate-activating enzyme were extracted only on two or three occasions. There was no demonstrable pattern in extracted activity; it could not be correlated either with the age or the size of the leaves as in the previous summer. In most cases the activity was either low or absent, and in a long sequence of experiments it proved impossible to predict the activity of any preparation. The colder, wetter conditions during this summer implied that the growth conditions of the spinach beet leaves were critical. The spinach beet plants were therefore grown under controlled conditions, in order to define more closely the physiological condition of the leaves from which measurable amounts of the activating enzyme could be extracted.

The spinach beet was at first raised in a large growth room run for a 16hr.-day at 20°, with lighting strength equivalent to a bright spring day ("daylight" fluorescent tubing, 1083 foot-candles illumination at soil height). The growth rate of the plants under these conditions was very slow, so that after 1½ months, the temperature was raised from 20° to 25°, but the daylength and lighting conditions were left unaltered. The spinach beet plants grew rapidly under these conditions, the leaves reaching a maximum length of 10cm. after 42 days. Thereafter the growth of the largest leaves ceased.

Even occasionally high levels of cinnamate-activating enzyme could not be extracted from these leaves although 0.01M-mercaptoethanol as well as 0.02M-ascorbate was often included in the extraction medium. All extracts were either inactive or feebly active; no predictable pattern was again observed. In the absence of any other growth facilities, more sensitive assays of cinnamate-activation were developed, so that low levels of activity could be consistently and accurately measured.

Section 2. The use of more sensitive methods for the assay of cinnamate-activating enzyme extracted from leaves of plants grown under controlled conditions.

1. The pyrophosphate exchange assay.

In order to reduce the level of contamination with ATP-ases to the minimum, the pyrophosphate exchange assays were carried out using enzyme preparations which had been centrifuged at 28,000xg. for 15 min. at 0° to remove mitochondria. The extracts were prepared from a macerate of spinach beet leaves in a suspension of insoluble PVP in $KH_2PO_4$-KOH buffer (0.1M, pH 8.0), containing 0.35M-sucrose, 0.02M ascorbate (or 0.1% mercaptoethanol) and 1mM-EDTA. In some cases the 28,000xg. supernatant was fractionated with $(NH_4)_2SO_4$. The fractions were each desalted before assay by passing them through a Sephadex-G25 column, in order to lower the concentration of endogenous exchange-promoting substances found in crude extracts.

Several extracts prepared using ascorbate as antioxidant from spinach beet leaves raised in the growth room at 25° with a 16hr.-day, were active by the pyrophosphate exchange assay, which was carried out under nitrogen (Table 14). The presence of CoA in the assay system completely inhibited the cinnamate-dependent pyrophosphate exchange. In the presence of 0.01M-KF, the total exchange rate was increased seven-fold

**Table 14.** **Characteristics of the pyrophosphate exchange catalysed by an extract of spinach beet leaves.**

The enzyme extract was prepared by macerating 15g. leaf material in a suspension of 15g. Polyclar AT in 90ml. $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.25M-sucrose, 0.02M-ascorbate and 1mM-EDTA). The extract was stirred for a few minutes and then filtered through cheesecloth. The filtrate, at pH8.0, was centrifuged at 28,000xg for 15 min. at 0°. The supernatant was assayed either directly, or after desalting.

Pyrophosphate exchange was measured in the following systems, incubated under an atmosphere of nitrogen: tris-HCl buffer (55 μmoles, pH 8.5); ascorbate (17 μmoles); mercaptoethanol (2.5 μmoles); $MgCl_2$ (2.5 μmoles); ATP (2.5 μmoles); [$^{32}$P] pyrophosphate (2.5 μmoles, 0.83 μc), and 0.35ml. desalted enzyme preparation, in a total volume of 1.0ml. This system measured the background exchange value (B). The cinnamate-promoted exchange (C) was measured by incubating the system in the presence of cinnamate (2.5 μmoles), and calculating the extra [$^{32}$P] ATP formed under these conditions. Incubations were also carried out in the presence of CoA (0.15 μmoles), and KF (10μmoles).

The exchange is expressed as μmoles [$^{32}$P] ATP formed in the absence of cinnamate (B), and the presence of cinnamate (C) at 30°, under nitrogen.

| Conditions | Exchange | Time of incubation (hr.) | [$^{32}$P] ATP formed (μmoles) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | ½ | 1 | 1½ | 2 | 3 | 4 |
| System | B | | .04 | .63 | .58 | .45 | .38 | .38 | .40 |
| | C | | .01 | .06 | .17 | .12 | .20 | .14 | .10 |
| + CoA | B | | .03 | .49 | .40 | .35 | .33 | .30 | .28 |
| | C | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| +KF | B | | .13 | .63 | .80 | 1.00 | 1.20 | 1.70 | 2.00 |
| | C | | 0 | 0 | .10 | 0 | .18 | .27 | 0 |
| { +KF | B | | .04 | .45 | .73 | .85 | 1.10 | 1.60 | 1.70 |
| { +CoA | C | | .03 | 0 | 0 | 0 | 0 | 0 | 0 |

and again there was no cinnamate-dependent exchange in the presence of CoA. However, in the presence of KF, the cinnamate-dependent exchange measured in the absence of CoA was erratic; over the course of a 4hr. assay, it seemed to rise and fall at least twice. CoA inhibited the total exchange rate as well as the cinnamate-dependent exchange, both in the presence and absence of KF. The time course of the cinnamate-dependent exchange under different conditions is shown in Fig. 33. Maximum exchange was reached in 2 hr. in the absence of KF and CoA, and thereafter declined. An appreciable lag in the development of exchange was observed only in the presence of KF.

When $(NH_4)_2SO_4$ fractions were examined, the cinnamate-dependent pyrophosphate exchange, measured in the absence of KF and CoA, was found in the 25-50% satd. fraction, but no activity was found in the 0-25% and 50-80% satd. fractions (Table 15). However the bulk of the exchange activity not requiring cinnamate also fell in the active fraction. The cinnamate-dependent exchange activity, but not the other exchange activity, was lost overnight.

Cinnamate-dependent exchange was also measured in desalted supernatants prepared from spinach beet leaves in the presence of 0.1% mercaptoethanol instead of 0.02M-ascorbate. The activity extracted in the presence of mercaptoethanol was as high as that extracted in the presence of ascorbate, but

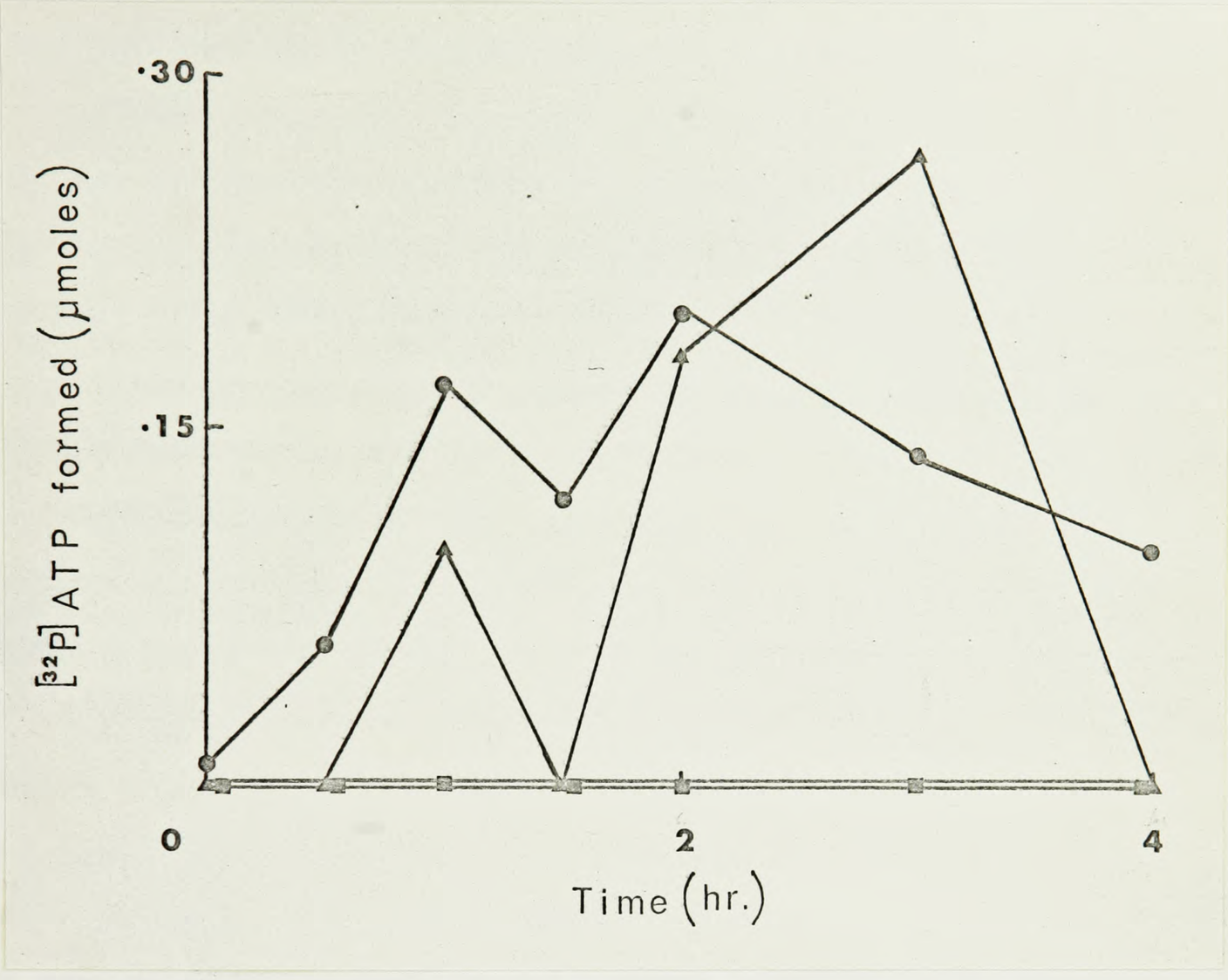



Fig. 33. The measurement of cinnamate activation by the pyrophosphate exchange method. The exchange activity was measured as described in Table 14, using a similar enzyme preparation. The course of the reaction is described by umoles $[^{32}P]$ ATP formed in the presence of cinnamate under nitrogen at 30° by the system as described in Table 14 (●), + CoA and +CoA, +KF (■), and +KF(▲).

it was lost on $(NH_4)_2SO_4$ fractionation (Table 15). No cinnamate-dependent exchange was measured if the extraction was carried out in the absence of any antioxidant.

These preparations which were active towards cinnamate by the pyrophosphate exchange assay, showed barely detectable activity by the spectrophotometric hydroxamate assay, after the usual lag period of two hours.

Despite the success obtained in several experiments, in demonstrating cinnamate activation by the exchange assay, a corresponding number of experiments gave completely negative results. The failures could not be correlated with conditions such as the age of the plant. Extracts from leaves gathered from plants of the same age, and of similar size, were only occasionally active in this assay. Some variable factor seemed to be operating in either the extract or the assay. Extracts which were inactive in catalysing a cinnamate-dependent pyrophosphate exchange when prepared in the presence of 0.02M-ascorbate or 0.1% mercaptoethanol, were also inactive when prepared in the presence of 10 or 25mM-thioglycollate, or 2 or 5mM-diethyldithiocarbamate as antioxidants. 10mM-thioglycollate was completely ineffective in controlling browning of the extract, and the extract containing 2mM-diethydithiocarbamate browned visibly over a few hours.

Table 15. $(NH_4)_2SO_4$ fractionation of the pyrophosphate exchange activity catalysed by extracts from spinach beet leaves.

An enzyme extract was prepared as described in Table 14, using 20g. leaf material instead of 15g. $(NH_4)_2SO_4$ fractions were prepared as described on p.52, and the following volumes of suspensions were prepared from precipitates obtained between the stated saturations of $(NH_4)_2SO_4$, using $KH_2PO_4$-KOH buffer (0.1M, pH 8.0; containing 0.02M-ascorbate): 10ml. (0-25% ppt.), 12 ml. (25-50% ppt.) and 5ml. (50-80% ppt.). After desalting, the supernatant and resuspended $(NH_4)_2SO_4$ fractions were approximately twice their original volume, and contained half the concentration of protein.

A similar preparation was carried out in the presence of 0.1% mercaptoethanol instead of 0.02M-ascorbate. The volumes of the resuspended $(NH_4)_2SO_4$ ppts. were (in the same order as above): 7ml., 6ml., 4ml.

The exchange was measured in the system described in Table 14, using 0.33ml. desalted supernatant, or 0.085 ml. desalted $(NH_4)_2SO_4$ fraction, and is expressed as μmoles $[^{32}P]$ ATP formed in the absence of cinnamate (B), and the presence of cinnamate (C), at 30°, under nitrogen.

| Enzyme preparation | Exchange | Time of incubation (hr.) | $[^{32}P]$ATP formed (μmoles) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 |
| Supernatant prepared in 0.02M-ascorbate | B | | .07 | .38 | .38 | .35 | .35 |
| | C | | .04 | .06 | .07 | .06 | .14 |
| 0-25% satd. $(NH_4)_2SO_4$ ppt. | B | | .03 | .05 | .08 | .09 | .11 |
| | C | | 0 | 0 | 0 | 0 | 0 |
| 25-50% satd. $(NH_4)_2SO_4$ ppt. | B | | .01 | .43 | .35 | .33 | .33 |
| | C | | 0 | .03 | .10 | .07 | .08 |
| 50-80% satd. $(NH_4)_2SO_4$ ppt. | B | | .02 | .07 | .09 | .08 | .08 |
| | C | | 0 | 0 | 0 | 0 | 0 |
| Supernatant prepared in 0.1% mercapto-ethanol | B | | .03 | .45 | .48 | .43 | .43 |
| | C | | 0 | .08 | .06 | .09 | .08 |
| 0-25% satd. $(NH_4)_2SO_4$ ppt. | B | | .03 | .05 | .06 | .08 | .09 |
| | C | | 0 | 0 | 0 | 0 | 0 |
| 25-50% satd. $(NH_4)_2SO_4$ ppt. | B | | .03 | .53 | .51 | .51 | .49 |
| | C | | 0 | 0 | 0 | 0 | 0 |
| 50-80% satd. $(NH_4)_2SO_4$ ppt. | B | | .03 | - | .60 | .60 | - |
| | C | | 0 | - | 0 | 0 | - |

In the extracts which did not show cinnamate-dependent exchange, exchange was suppressed by cinnamate by up to one-third of the amount measured in its absence. Generally, these latter rates were lower than those measured in extracts active in cinnamate-dependent exchange. Reduction of the pH of the tris-HCl buffer used in the assay system to pH 8.0, 7.5 or 7.0 did not reveal any cinnamate-dependent exchange if it was absent when tris-HCl buffer at the normal pH of 8.5 was used. No exchange was stimulated under these conditions by p-coumaric or ferulic acids. All three acids partially suppressed the exchange activity measured in their absence, and ferulic acid in the presence of tris-HCl buffer at pH 7.0, completely inhibited the exchange.

2. The chromatographic method of measuring activation of [2-$^{14}$C] cinnamic acid by formation of its [2-$^{14}$C] hydroxamate.

Since desalted preparations low in ATP-ase activity from spinach beet leaves grown under controlled conditions, had sometimes shown activity by the pyrophosphate exchange assay, the activity of similar extracts was tested under an atmosphere of nitrogen in the presence of hydroxylamine, for the formation of [2-$^{14}$C] cinnamyl-hydroxamate from added [2-$^{14}$C] cinnamate.

The activity of preparations from leaves of spinach beet plants of different ages, grown in a growth room, and a growth cabinet, was investigated. Ascorbate (0.03M) was

used as antioxident during the extraction in all cases.

i) The relationship between age and enzyme activity.

Leaves grown under either of these conditions showed a relationship between the age of the plant and the extracted activity measured by the formation of [$^{14}$C]cinnamyl-hydroxamate during a period of 4hr. under nitrogen (figs. 34 a and b). These figures also show the amount of redness visible on the leaves at various ages, this coloration being due to the development of the betalain pigments.

The coloration is formed first on the underside of the young leaf only. In samples of leaves grown in both chambers, the colour reached a maximum at 8 days after planting of the seeds. The intensity of the colour found in leaves from the growth cabinet was however at least twice that in leaves from the growth room. The colour had disappeared from both types of leaf within 24 days from planting the seeds. However, in the leaves from the growth cabinet, it began to develop again immediately as a coloration on both surfaces of the leaf, at first around the edges, and gradually spreading to cover the whole of both leaf surfaces. This secondary development of colour may have been related to the higher illumination level in the growth cabinet, and also to the tendency to dehydration under these conditions, which was particularly evident around the edges of the older leaves.

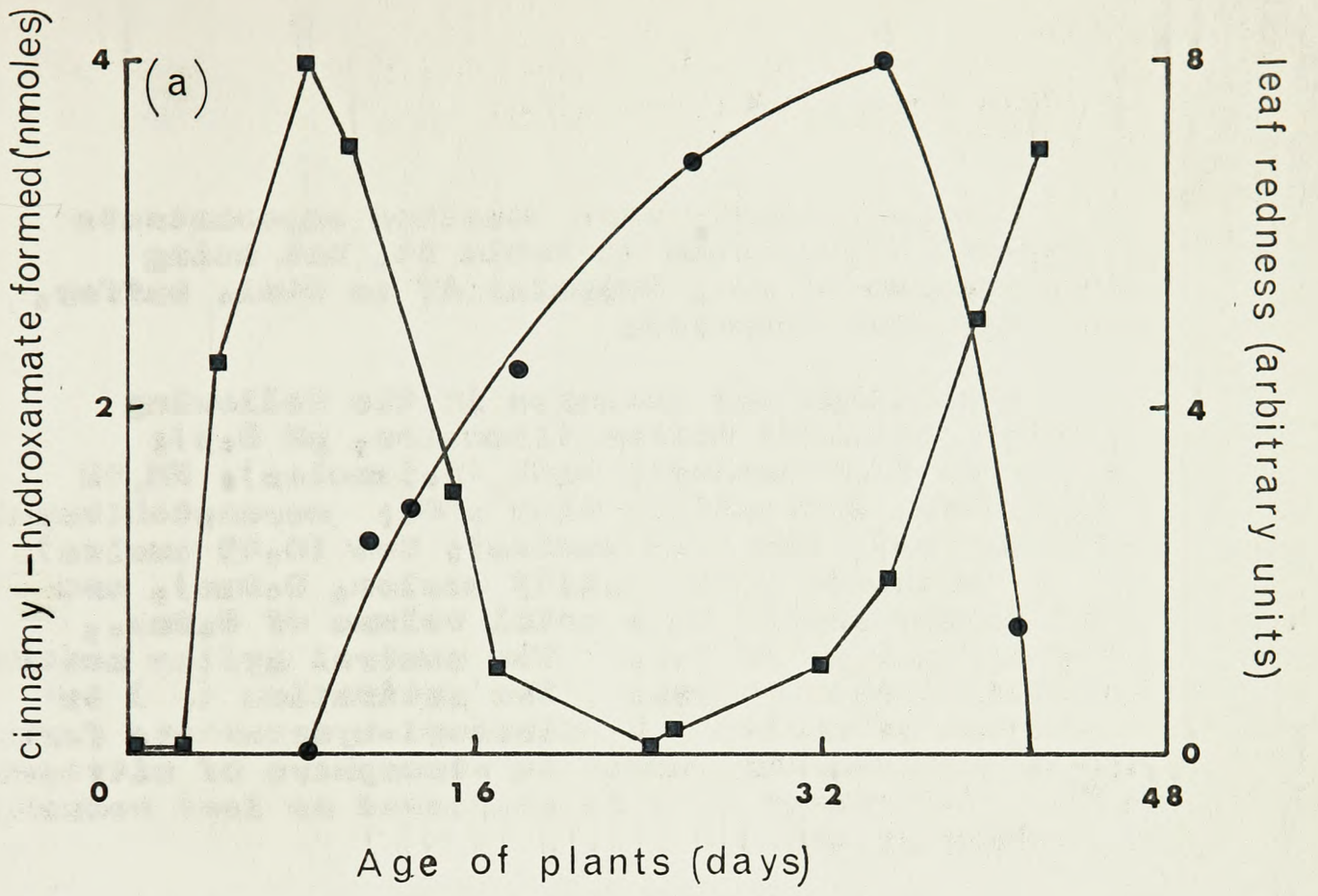
Figs.34. Relationship between age and colour of spinach beet leaves and cinnamate-activating activity.

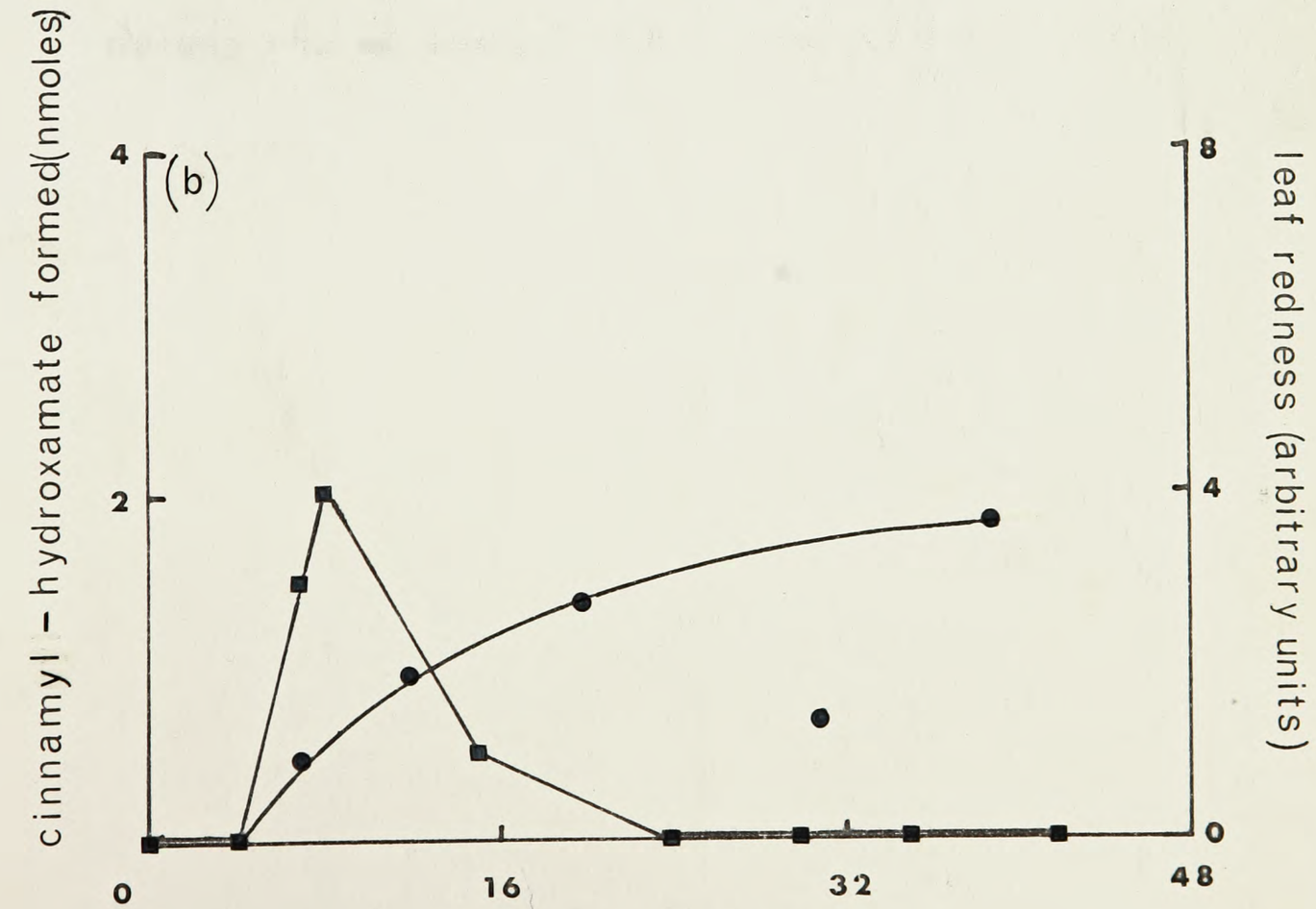
Enzyme extracts were desalted supernatants prepared as described in Table 14, but using a suspension of 10g. Polyclar AT in 60ml. buffer, and 15g. leaf material.

Activation was measured in the following system: tris-HCl buffer (13μmoles, pH 8.5); ascorbate (2.7 μmoles); $MgCl_2$ (1.3μmoles); $NH_2OH$ (20 μmoles, neutralised with KOH); mercaptoethanol (0.5 μmoles); ATP (0.5 μmoles); CoA (0.03 μmoles); [2-$^{14}$C] cinnamic acid (0.1185 μmoles, 0.2μc), and 70μl enzyme sample in a total volume of 0.2ml., with a final pH of 7.8. The control system contained 70μl. boiled enzyme. The activation ( ● ) is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of protein/4hr. under an atmosphere of nitrogen at 30°. The colour ( ■ ) is expressed as leaf redness in arbitrary units.

(a) Spinach beet plants raised in the growth cabinet.

(b) Spinach beet plants raised in the growth room.


(a)
cinnamyl-hydroxamate formed(nmoles)
leaf redness (arbitrary units)
4
2
0
8
4
0
0
16
32
48
Age of plants (days)


(b)
cinnamyl - hydroxamate formed(nmoles)
leaf redness (arbitrary units)
4
2
0
8
4
0
0
16
32
48
Age of plants (days)

Figs. 34 a and b show an inverse correlation between the intensity of the red colour in the leaves, and the activity of the cinnamate-activating enzyme. Furthermore, the activity extracted from leaves from the growth cabinet, after the primary betalain peak had passed was very much higher than that extracted from leaves from the growth room. This difference reached a maximum 35 days after planting the seeds. The activity extracted from leaves from the growth cabinet then fell very sharply when the secondary betalain peak developed, whereas the activity extracted from leaves from the growth room showed no change, and no secondary betalain peak.

ii) The characteristics of the activating system shown by this assay.

The formation of [$^{14}$C] cinnamyl-hydroxamate was dependent on CoA in most cases. In a few cases a very low formation of [$^{14}$C] hydroxamate was detected in the presence of ATP alone, and in only one case, in the absence of any added cofactors. Using a desalted enzyme preparation, the value given by a control incubation using boiled enzyme in the complete system, was generally similar to that obtained from a control incubation carried out in the absence of any added cofactors (ATP and CoA). Only in the case of a supernatant preparation which had not been desalted was the value for the boiled extract control often higher than that from the complete

Table 16. The characteristics of the cinnamate-activating system from spinach beet leaves raised in the growth cabinet.

Enzyme extracts were prepared from 26-day plants raised in the growth cabinet, as described in Fig.34. The activation catalysed by 70µl. extract both before and after desalting was measured in the system described in Fig.34. Systems without CoA, without CoA and ATP, and with boiled enzyme, were used as controls. The activation is expressed as moles $[^{14}C]$ cinnamyl-hydroxamate formed/mg. of protein /4hr. under an atmosphere of nitrogen at 30°, using the boiled enzyme system as control.

a, complete system; b, without CoA; c, without CoA and ATP; d, complete system with boiled extract.

| Enzyme preparation | System | C.P.M. | Cinnamyl-hydroxamate formed (moles) |
|---|---|---|---|
| Extract | a | 180.6 | 0 |
| | b | 138.3 | 0 |
| | c | 135.5 | 0 |
| | d | 202.2 | - |
| Desalted extract | a | 206.0 | 3.42 |
| | b | 163.4 | 1.55 |
| | c | 117.1 | 0 |
| | d | 128.2 | - |

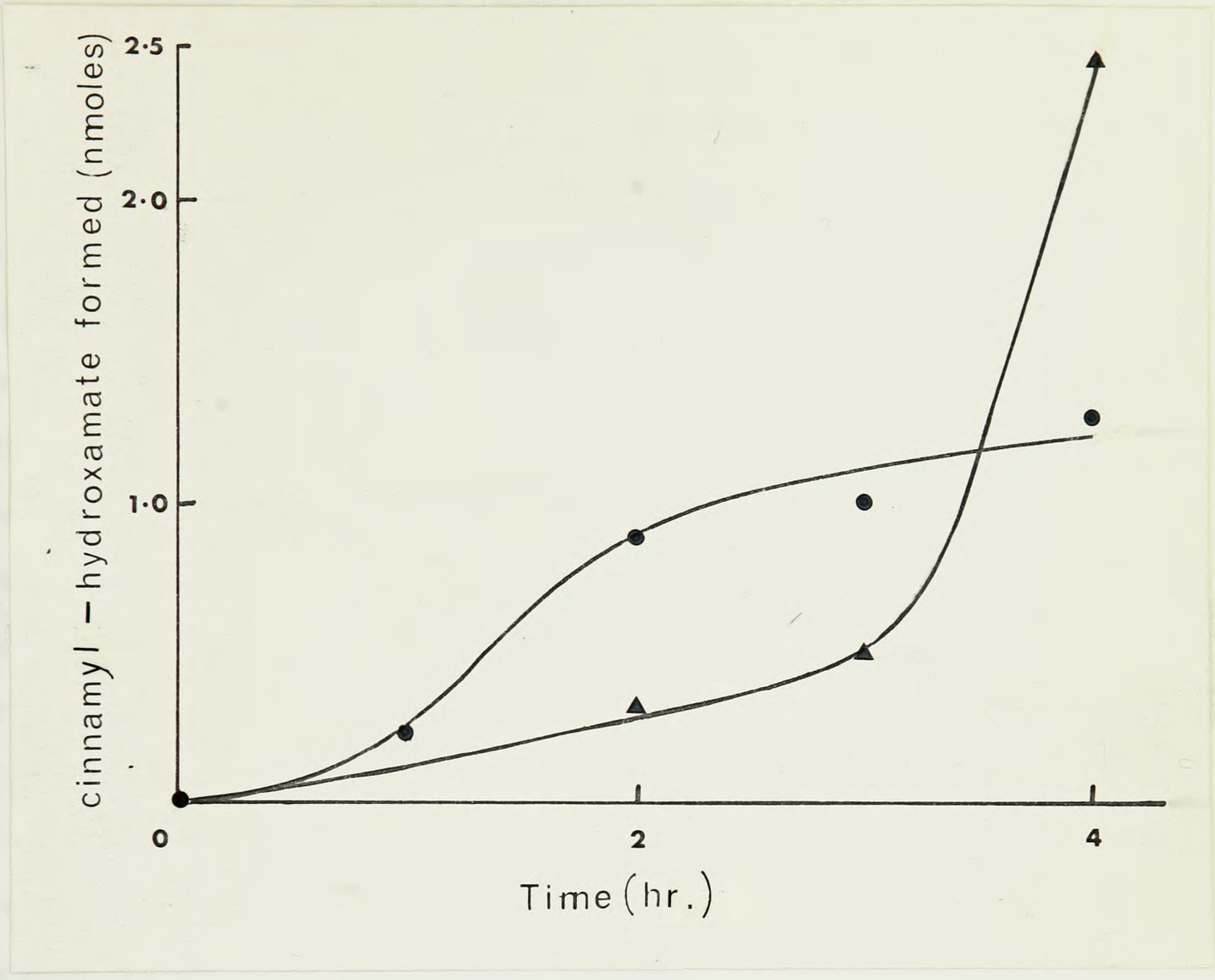



Fig. 35. Cinnamate activation in the presence and absence of bovine serum albumin. The enzyme extract was a desalted supernatant (cf.Fig.34), prepared from spinach beet leaves from 20-day plants raised in the growth room. The activation catalysed by 70µl. extract was measured in the system described in Fig.34, using the system without ATP and CoA as control. Incubations in the presence of bovine serum albumin contained 250µg. of albumin /0.2ml. The progress of the reaction is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/ mg. of extract protein under an atmosphere of nitrogen at 30°, in the presence ( ▲ ) and absence ( ● ) of bovine serum albumin.

assay system. In fact, desalting was almost invariably necessary to obtain active preparations (Table 16).

Extractions of leaves from spinach beet in the presence of various antioxidants showed that in the undesalted extract, the oxidation of betalains to browner products was controlled most effectively by 0.02M-ascorbate, 0.1% mercaptoethanol, or 25mM-thioglycollate. 10mM-thioglycollate, and 1mM-and 5mM-diethyldithiocarbamate were comparatively ineffective. 0.02M-ascorbate was used in all these experiments. The presence of betalains, and any oxidation products from them, was probably a major factor responsible for the absence of activity in most undesalted extracts, for their colour was completely separated from the reddish enzyme extract which became a pale-green during passage through the Sephadex-G 25 column. Activity was then detectable excepting in preparations from very young leaves.

When the assay of an active enzyme preparation was carried out in the presence of bovine serum albumin, added to give a final protein concentration in the assay system of more than 1mg/ml., the measurable activity was at least doubled after an incubation period of 4 hr. During shorter incubation periods the activity was less in the presence of added protein, and under either condition, a lag period in the development of activity was evident (Fig.35).

The activity of the enzyme, assayed in the presence of bovine serum albumin, was directly proportional to the

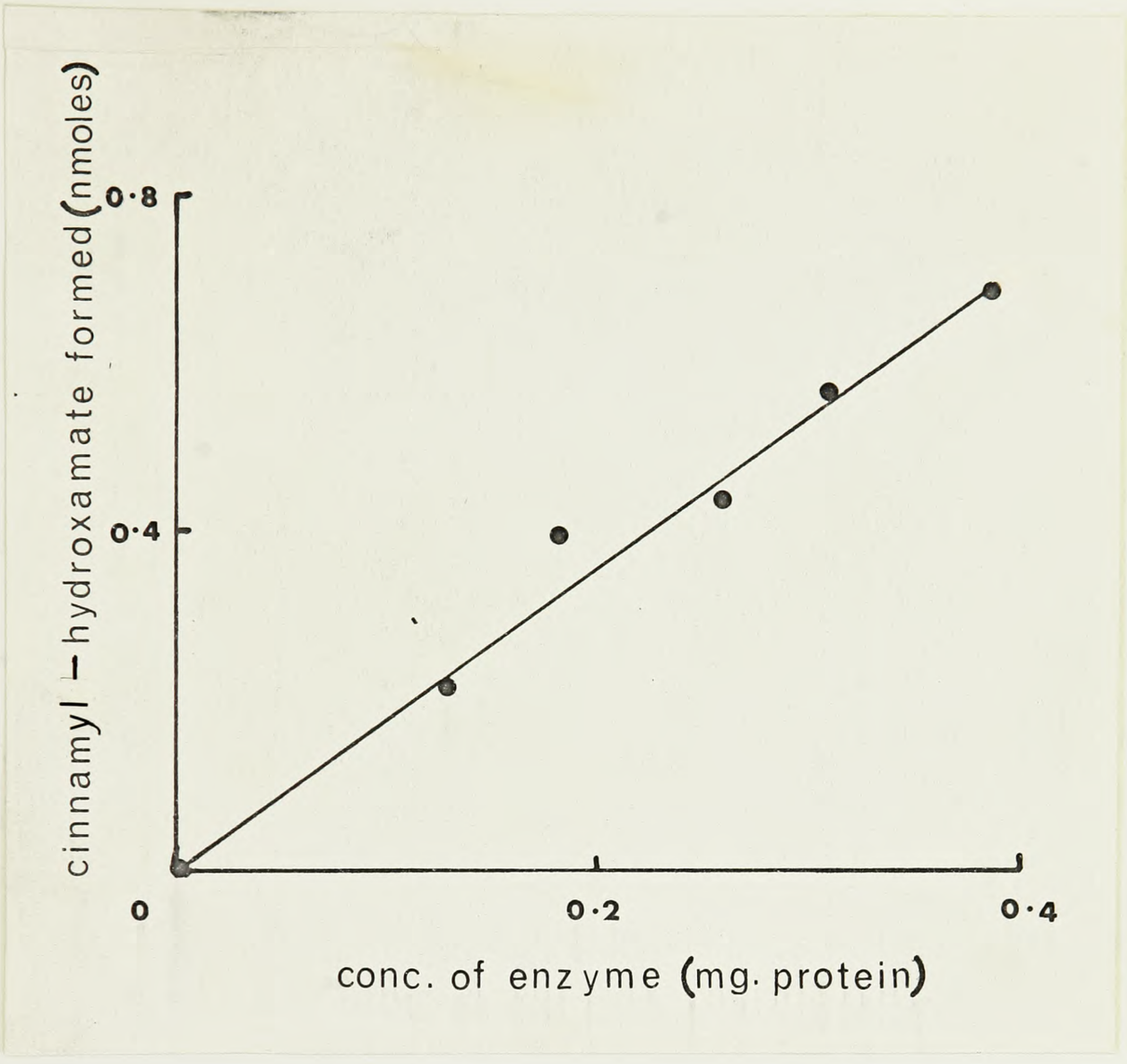



Fig. 36. Relationship between cinnamate activition and enzyme concentration. The activation catalysed by 0-150 µl. desalted supernatant (cf. Fig.34), prepared from spinach beet leaves from 31-day plants raised in the growth room, was measured in the system described in Fig.34, in the presence of 250µg. bovine serum albumin. The system without ATP and CoA was used as control. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/µg. of extract protein/4hr. under an atmosphere of nitrogen at 30°.

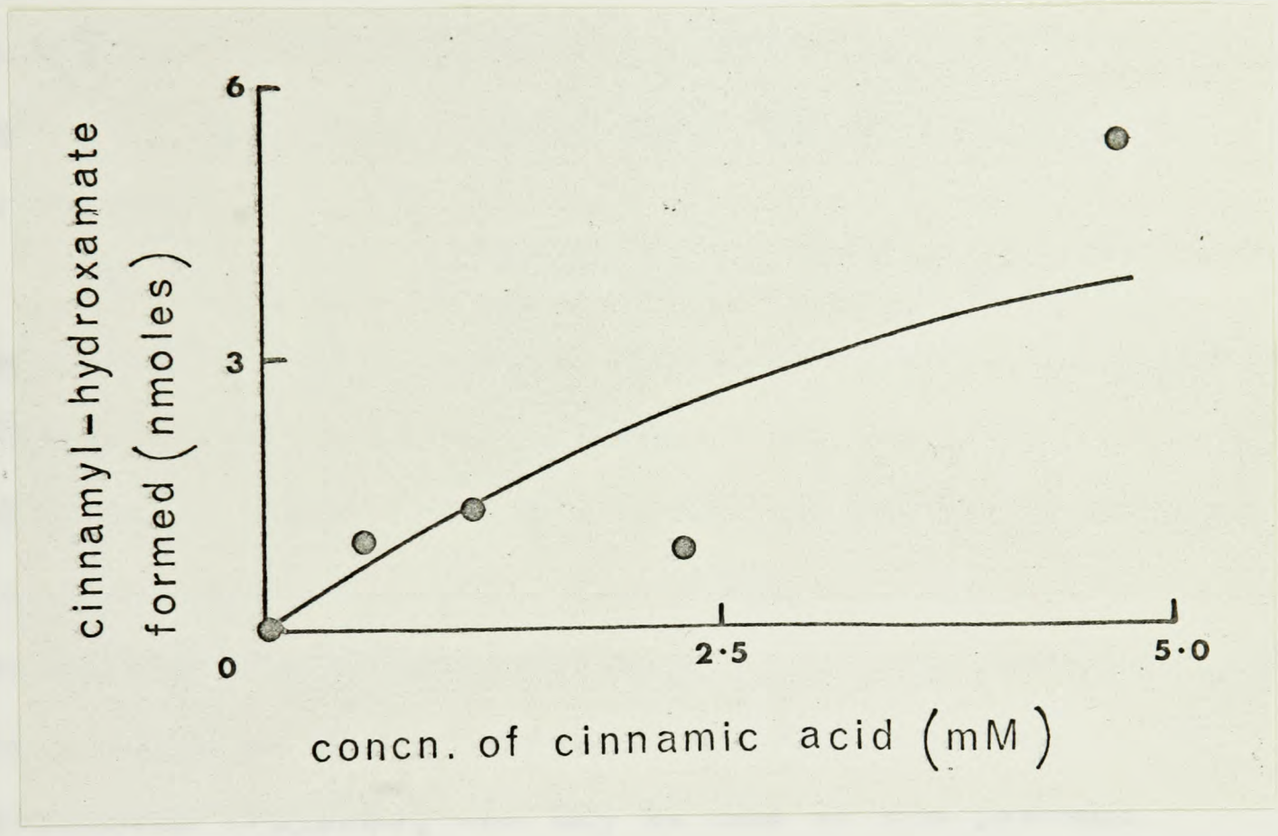



Fig.37. Relationship between cinnamate activation and cinnamate concentration. The activation catalysed by 70µl. desalted supernatant (cf.Fig.34), prepared from spinach beet leaves from 13-day plants raised in the growth room, was measured in the system described in Fig.34, using concentrations of cinnamic acid up to 4.7mM. The system without ATP and CoA was used as control. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of protein/4hr. under an atmosphere of nitrogen at 30°.

concentration of the enzyme preparation (Fig.36). The activity was also proportional to the concentration of [$^{14}$C] cinnamic acid up to 4.7mM (Fig.37). A Km value cannot strictly be calculated from this data, since this assay gives no values for the initial rate, but a Lineweaver-Burk plot for these figures gives the Km for cinnamic acid as 12.5mM.

The formation of [$^{14}$C] cinnamyl-hydroxamate was increased by increasing the concentration of hydroxylamine (Fig.38), but no optimum concentration of hydroxylamine was found up to 0.26M; the concentration of hydroxylamine normally used was 0.1M. The absence of a visible inhibitory effect over the range measured, contrasts with the results of the preliminary studies with acetate-activation (Fig.24), and may be due to the present neutralisation of hydroxylamine with KOH, for which NaOH was earlier employed.

The pH optimum of [$^{14}$C] cinnamyl-hydroxamate formation was defined at a high concentration of tris-HCl buffer (0.163M) as near 7.2, although the activity below this pH was not measured (Fig.39a). The pH/activity relationship was studied at this concentration of tris-HCl since although enzyme activity was higher at normal concentrations of tris-HCl (0.07M), the pH-dependence of [$^{14}$C] cinnamyl-hydroxamate formation here was erratic (Fig. 39b).

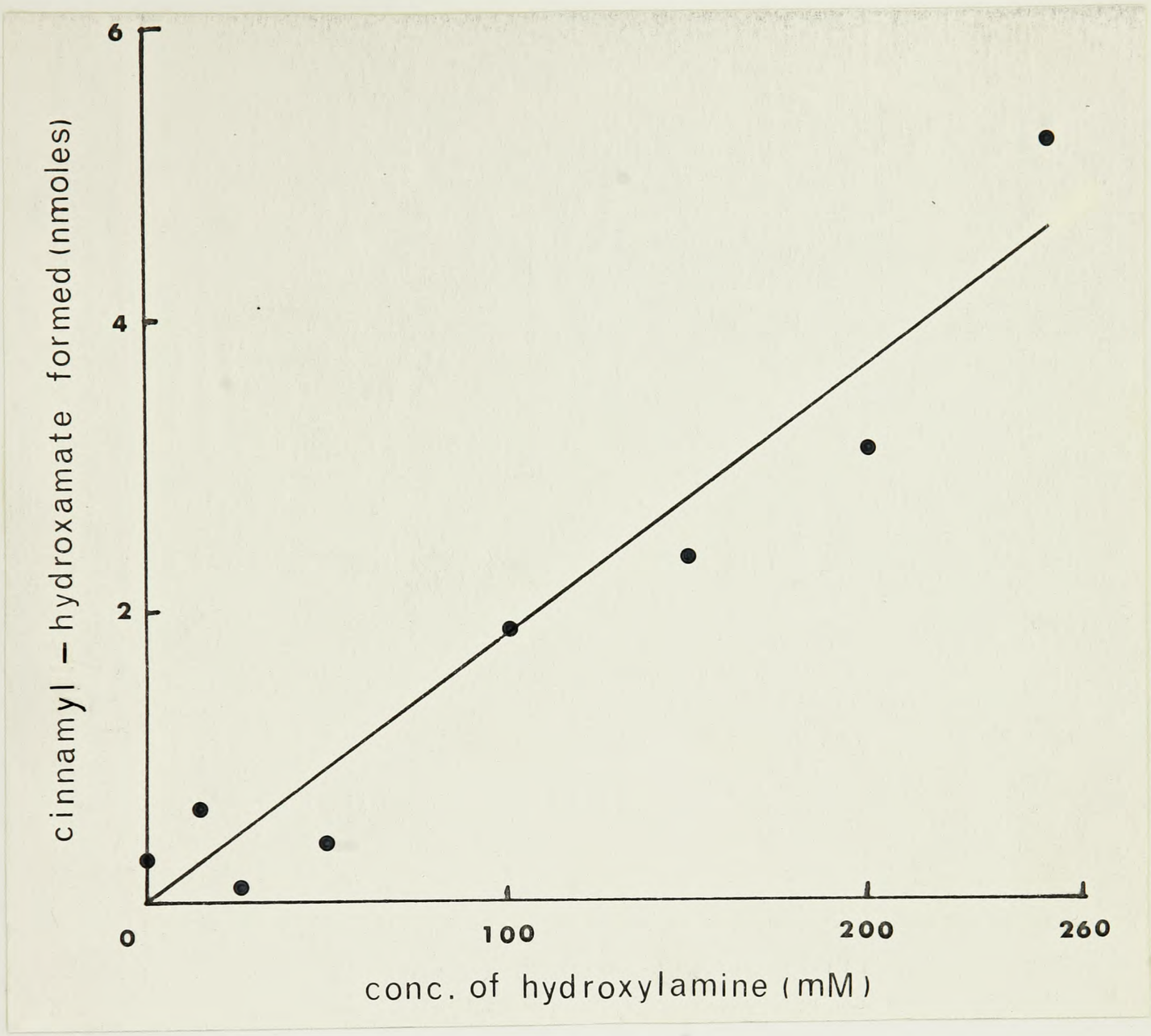
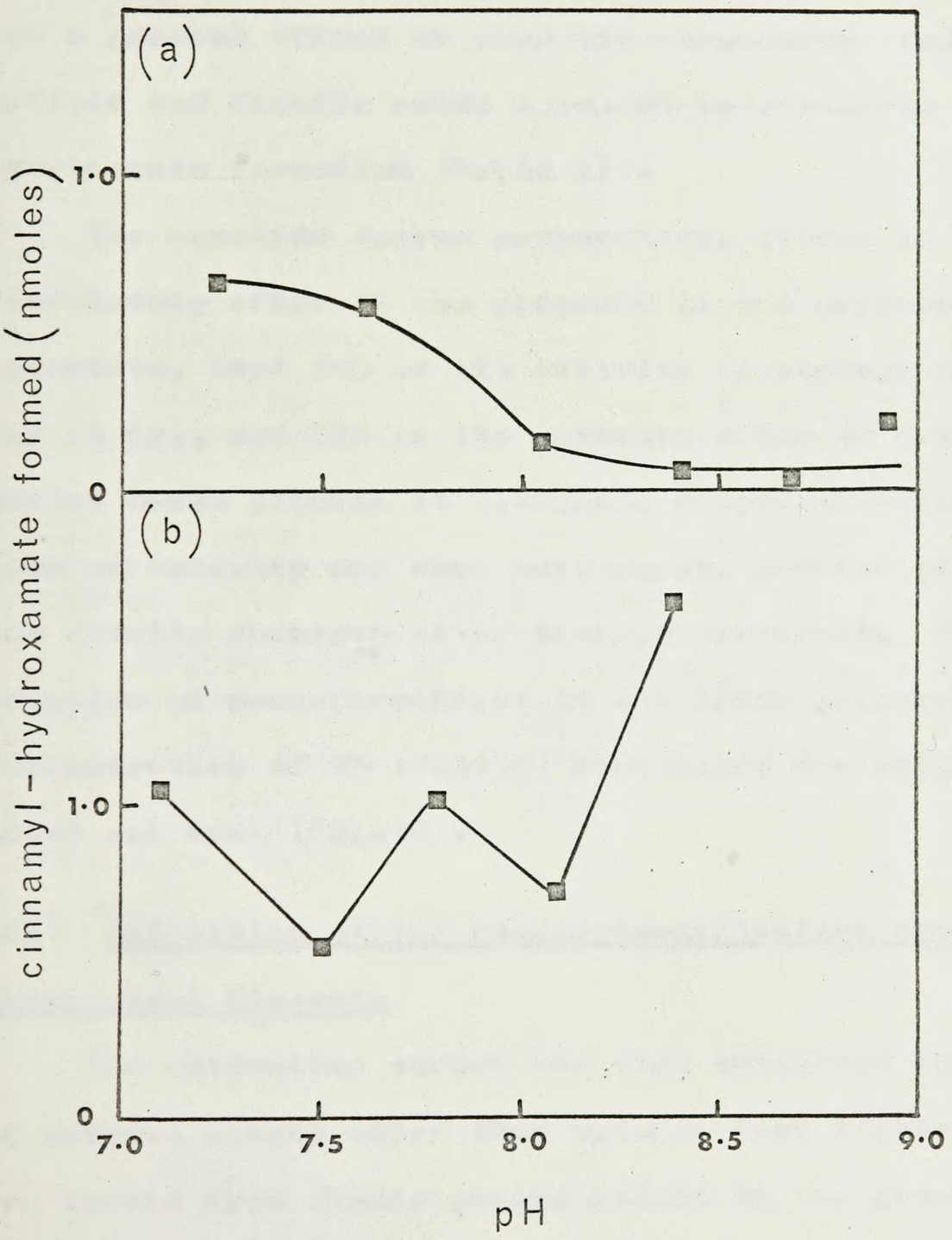



Fig.38. Relationship between formation of [$^{14}$C] cinnamyl-hydroxamate and hydroxylamine concentration. The enzyme extract was the same preparation as that used in Fig.35. The activation catalysed by 70µl of this preparation was measured in the system described in Fig.34, in the presence of 250µg. bovine serum albumin/0.2ml. and using hydroxlamine concentrations up to 260mM. The system without ATP and CoA was used as control. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of extract protein /4 hr. under an atmosphere of nitrogen at 30°.

Figs. 39. The pH-dependence of [$^{14}$C]-cinnamyl-hydroxamate formation. The enzyme extract was a desalted supernatant (cf.Fig.34), prepared from spinach beet leaves from 31-day plants raised in the growth cabinet. The data in Fig.39(b) was obtained using a fresh preparation, and that in Fig.39(a) using the preparation stored in the presence of 1% mercaptoethanol for 48hr. in the refrigerator. The stored and fresh preparations had the same enzymic activity. The activation catalysed by 70µl. extract was measured in the system described in Fig.34, except that tris-HCl buffer was 32.5 µmoles/0.2ml. and at pH values from 7.2 to 9.2 (Fig.39a), or at the normal concentration of 13 µmoles/0.2ml. and at pH values from 7.2 to 9.2 (Fig.39b). The system contained 250 µg. bovine serum albumin/0.2ml., and the control incubations were carried out in the absence of ATP and CoA. The pH of similar systems with a total volume of 2.0ml. was measured to give the pH of incubation. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of extract protein/4hr. under an atmosphere of nitrogen at 30°.


(a)
1·0
0
(b)
1·0
0
7·0
7·5
8·0
8·5
9·0
pH
cinnamyl-hydroxamate formed (nmoles)

The formation of [$^{14}$C] cinnamyl-hydroxamate appeared to be inhibited when p-coumaric acid was added to the assay system at the same concentration as [$^{14}$C] cinnamic acid. Caffeic, ferulic and sinapic acids did not inhibit the formation of [$^{14}$C] hydroxamate, so this inhibition was not a general effect of phenolic compounds; indeed, caffeic and ferulic acids appeared to stimulate [$^{14}$C] hydroxamate formation (Table 17).

The desalted enzyme preparation, stored in the refrigerator immediately after it was prepared in the presence of 0.05M-ascorbate, lost 50% of its activity on storage overnight for 24 hr., and 75% of its activity after 48 hr. With bovine serum albumin at 5.6mg/ml. enzyme preparation, the loss of activity was even more rapid, and the preparation was totally inactive after storage overnight. However, addition of mercaptoethanol to the fresh preparation, to a concentration of 1% (0.143M) stabilised the activity for at least one week (Fig.40).

iii) Extraction of the cinnamate-activating enzyme from other plant tissues.

The activating enzyme was also extracted from the leaves of several plants other than spinach beet (Table 18). Pea leaves from 20-day plants raised in the greenhouse under tungsten lamps, yielded a fairly active desalted preparation, but very little activity was detected in the

Table 17. The effect of various phenolic acids on the activation of cinnamate.

The enzyme preparation used was the desalted extract described in Fig.39. It had been stored in the presence of 1% mercaptoethanol for 24 hr. in the refrigerator.

70 µl. enzyme preparation was assayed in the system described in Fig.34, in the presence of 250 µg. bovine serum albumin. Incubations were also carried out with added p-coumaric, caffeic, ferulic or sinapic acids (0.1183 µmoles each). The control system contained no CoA and ATP. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of extract protein 14 hr. under an atmosphere of nitrogen at 30°.

| System | Cinnamyl-hydroxamate formed (nmoles) | % Inhibition | % Activation |
|---|---|---|---|
| Normal | 1.39 | - | - |
| + p-coumaric acid | 0.62 | 55.3 | - |
| + caffeic acid | 1.68 | - | 20.7 |
| + ferulic acid | 1.98 | - | 42.7 |
| + sinapic acid | 1.48 | - | 6.7 |

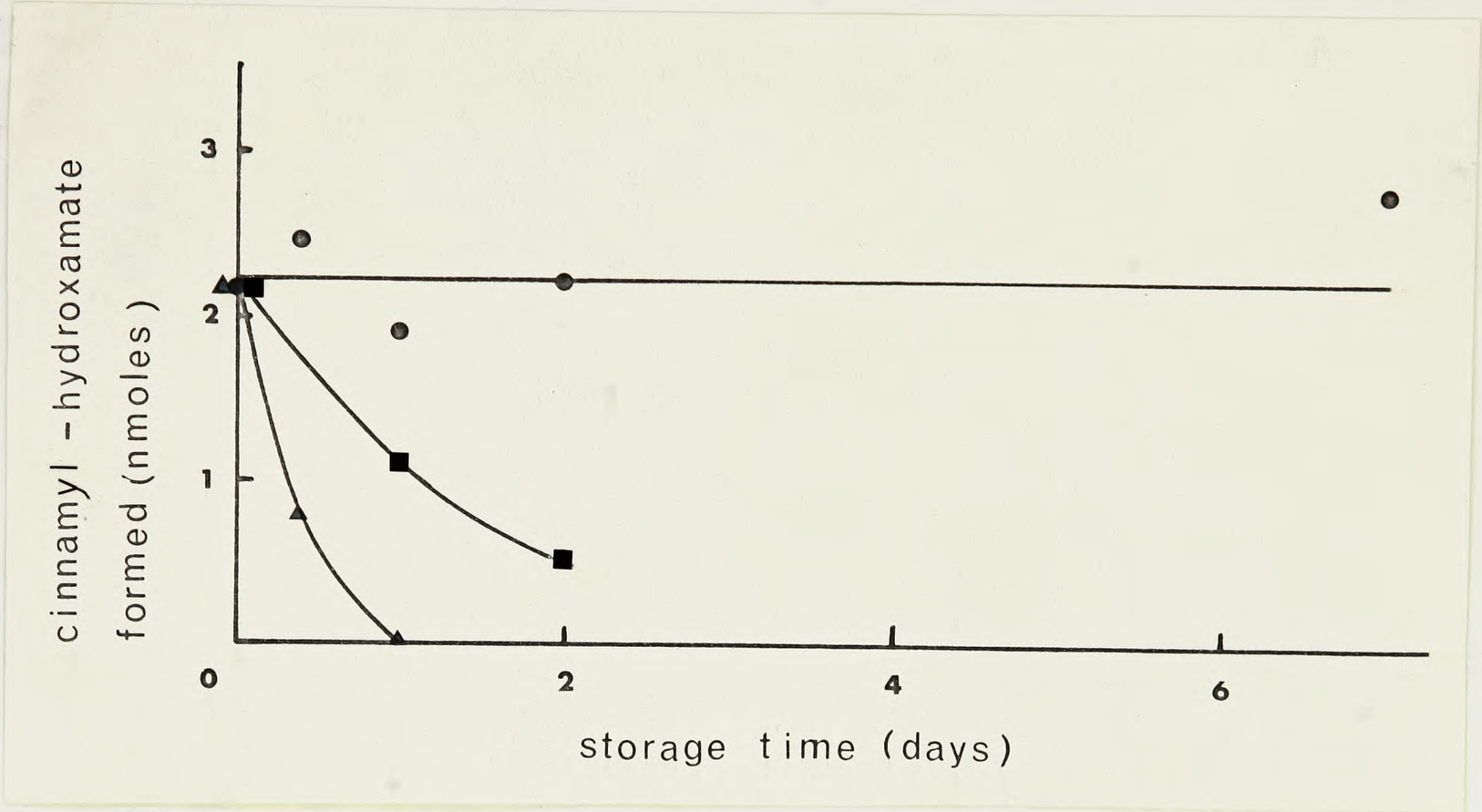



Fig.40. Stability of the cinnamate-activating system under different conditions. The enzyme extract was the same preparation as that used in Fig.35 containing 0.02M-ascorbate, ( ■ ); with the addition of 3.6mg./ml. of bovine serum albumin, ( ▲ ); with the addition of 0.143 m-moles/ml. (1%) of mercaptoethanol ( ● ); and was stored in the refrigerator. The activation catalysed by 70µl of each preparation was measured in the system described in Fig.34, adding bovine serum albumin to 250 µg/0.2ml., and mercaptoethanol to 0.5µmoles/0.2ml., only when these were not present in the enzyme preparation. The control system contained no ATP and CoA. The activation is expressed as nmoles [$^{14}$C]cinnamyl-hydroxamate formed/mg.of extract protein/4hr. under an atmosphere of nitrogen at 30°.

undesalted extract. Pea leaves from 26-day plants raised in the growth room did not give a very active preparation. Leaves from 18-day runner bean plants raised in the growth room, yielded an active desalted preparation, but no activity was detected in the undesalted extract. Spinach leaves from 24-day plants raised in the growth room showed a very low level of activity, and no activity was detected in a desalted preparation from leaves of 23-day cabbage plants raised in the growth room.

Excepting the more active preparation from pea leaves, no activation was observed in the absence of CoA.

iv) Use of the spectrophotometric hydroxamate assay on extracts shown to be active by the chromatographic method.

Enzyme extracts active when assayed by the chromatographic method in the experiments described above, showed little or no activity in the spectrophotometric hydroxamate assay. The failure of this method therefore appears to be due to its insensitivity.

2. The spectrophotometric method of assaying activation, by measuring the appearance of cinnamyl-CoA.

Several of the enzyme extracts active when assayed by the chromatographic hydroxamate method were also active using the spectrophotometric method for measuring the appearance of cinnamyl CoA. The use of this assay revealed

Table 18. The extraction of cinnamate-activating enzyme from leaves of plants other than spinach beet.

Enzyme extracts were prepared from leaves of plants as described in Fig.34 and used fresh. The activation catalysed by 70ul. extract both before and after desalting was measured in the system described in Fig.34. Systems without CoA, without CoA and ATP, and with boiled enzyme were used as controls. The activation is expressed as nmoles [$^{14}$C] cinnamyl-hydroxamate formed/mg. of protein/4hr. under an atmosphere of nitrogen at 30°, using the boiled extract system as control.

a, complete system; b, without CoA; c, without CoA and ATP.

| Plant | Conditions of growth | Age (days) | System | Cinnamyl-hydroxamate (nmoles) Crude Extract | Desalted Extract |
|---|---|---|---|---|---|
| Pea | Greenhouse under tungsten lamps | 20 | a | 0.41 | 2.25 |
| | | | b | 0 | 1.88 |
| | | | c | 0 | 0 |
| Pea | Growth room | 25 | a | 0.29 | 0.42 |
| | | | b | 0 | 0 |
| | | | c | 0 | 0 |
| Runner bean | " " | 18 | a | 0 | 3.00 |
| | | | b | 0 | 0 |
| | | | c | 0 | 0 |
| Spinach | " " | 24 | a | 0.07 | 0.22 |
| | | | b | 0 | 0 |
| | | | c | 0 | 0 |
| Cabbage | " " | 23 | a | 0 | 0 |
| | | | b | 0 | 0 |
| | | | c | 0 | 0 |

characteristics of the cinnamate-activating system which it was not possible to measure by other methods. For instance, initial rates could be measured, since the enzyme reaction started as soon as substrate was added, and had finished after seven minutes incubation or sooner (Fig.41).

The formation of cinnamoyl-CoA appeared to be directly proportional to the concentration of cinnamate over the range measured (up to 3.5mM, Fig.42). A Lineweaver-Burk plot of the data showed that the $K_m$ for cinnamate was 0.25mM.

The pH-activity relationship of the enzyme is shown in Fig.43. The enzyme had a broad pH optimum between pH 7.5 and 8.0. The relationship appears to be quite different from that measured by the chromatographic [$^{14}$C] hydroxamate method, but probably represents the pH-activity relationship of the enzyme system itself, and does not include a pH factor in the assay system.

The enzyme preparations were also tested for activity towards p-coumaric, caffeic, and ferulic acids, by the alternative assay method (p.30): the extinction at the $\lambda_{max}$ of the appropriate CoA ester was followed in a system containing CoA, but no added substrate, until no change occurred, and substrate was then added to each cuvette providing a concentration of 3.3mM, to initiate the reaction. Cinnamic acid alone showed formation of its CoA ester by this

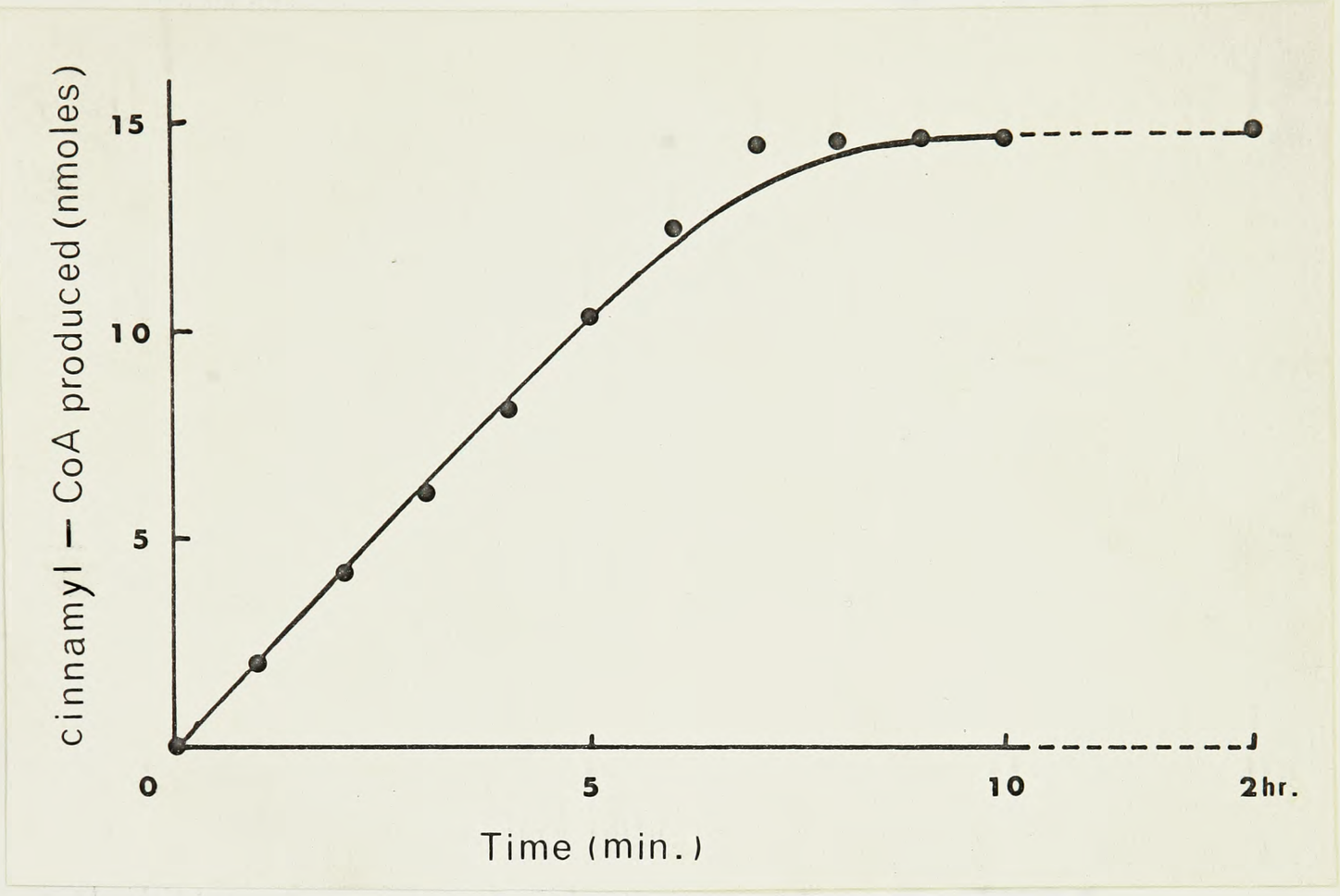



Fig.41. Activation of cinnamate measured by cinnamyl-CoA formation. The enzyme preparation was a desalted supernatant (cf. Fig.34), prepared from spinach beet leaves from 35-day plants raised in the growth cabinet, and contained 0.7mg. of protein/ml. The formation of cinnamyl-CoA was measured in the following system: tris-HCl buffer (0.22m-moles, pH 8.5); ascorbate (44 μmoles); $MgCl_2$ (10 μmoles); mercaptoethanol (10 μmoles); ATP (10 μmoles); CoA (0.6 μmoles); cinnamate (5μmoles) and 1.2ml. enzyme sample in a total volume of 3.0ml. The control system contained no cinnamic acid. The amount of cinnamyl-CoA formed (nmoles) at an average temperature of 25° under an atmosphere of nitrogen was determined.

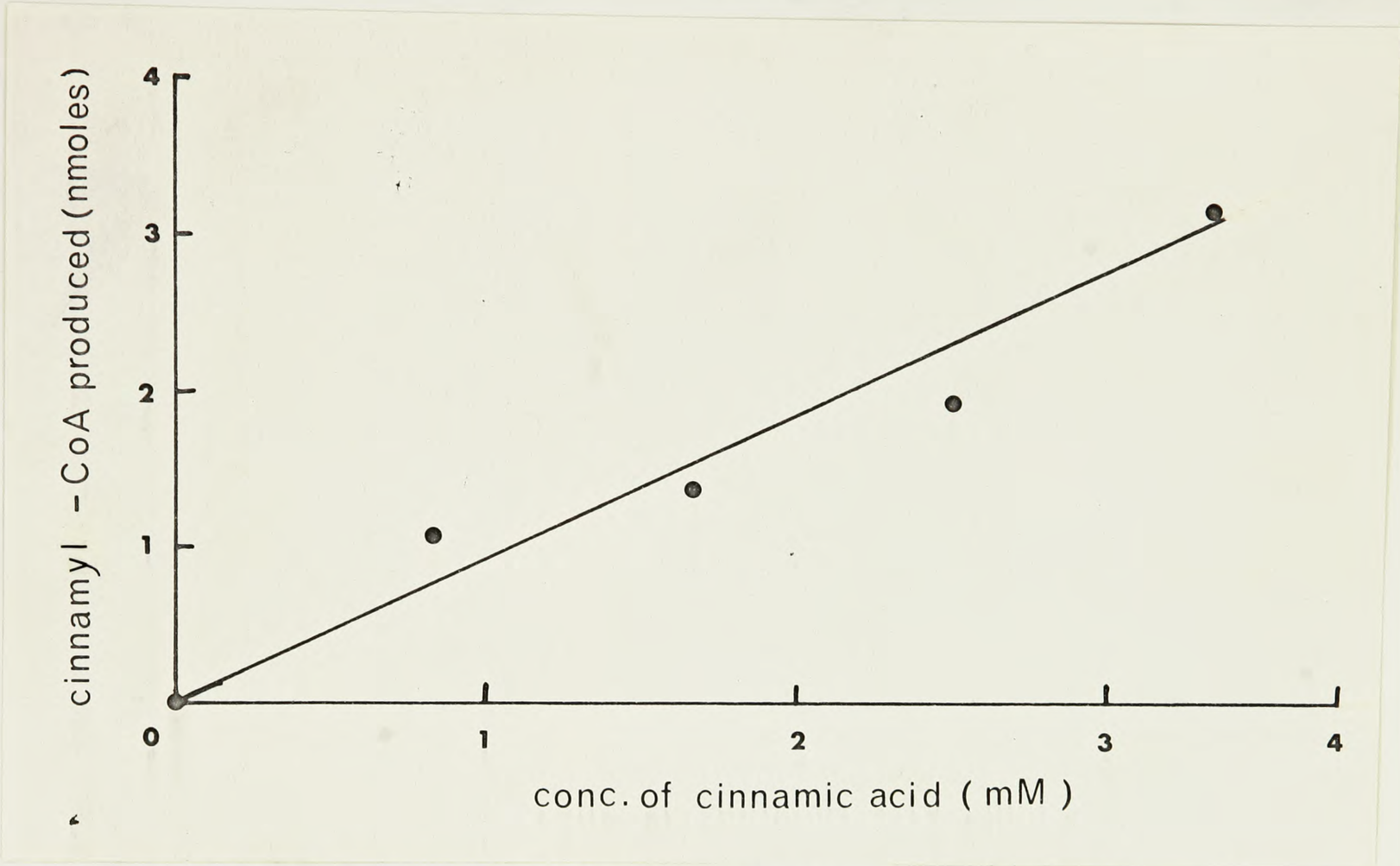



Fig.42. Relationship between enzyme activity and concentration of cinnamate. The enzyme extract was a 24hr. old desalted supernatant prepared from spinach beet leaves from 39-day plants raised in the growth room (cf.Fig.34), and contained 1.2mg. of protein/ml. The formation of cinnamyl-Co A was measured in the system described in Fig.41 using 2.5-10 μmoles cinnamate/3.0ml. The control system contained no cinnamate. The formation of cinnamyl-CoA is expressed as nmoles/min. at an average temperature of 25° under an atmosphere of nitrogen, and was measured as an initial rate.

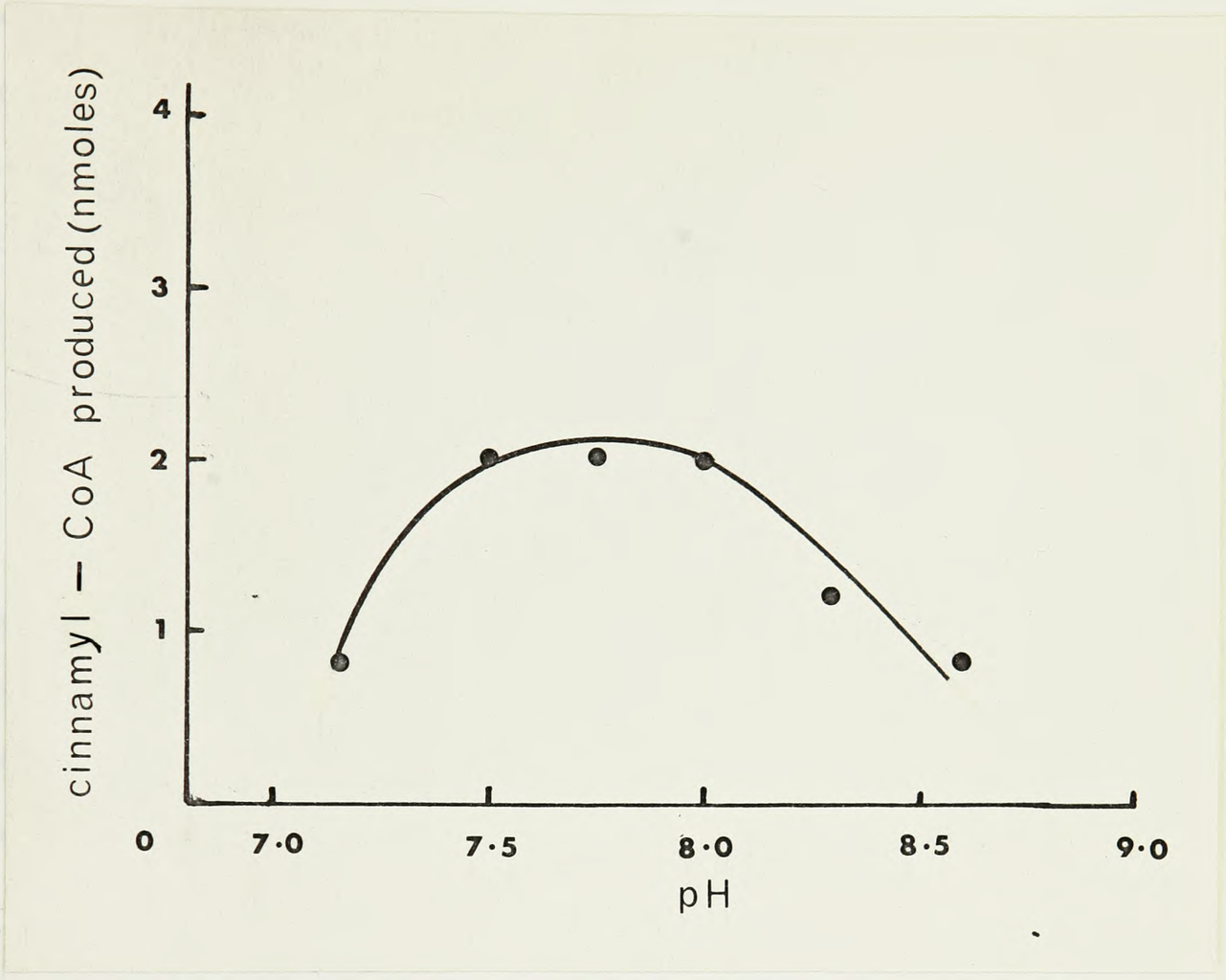



Fig.43. The pH-dependence of cinnamyl-CoA formation. The enzyme extract was the same desalted supernatant as used in Fig.39, stored in the presence of 1% mercaptoethanol for 6 days in the refrigerator. It contained 3.2mg. of protein/ml. The formation of cinnamyl-CoA was measured in the following system: tris-HCl buffer (0.1m-moles, pH 7.0 to 8.5); ascorbate (28 μmoles); $MgCl_2$ (7 μmoles); mercaptoethanol (0.1 m-moles, added with enzyme); ATP (5 μmoles); CoA (0.3 μmoles); cinnamate (5μmoles); bovine serum albumin (2.5mg.), and 0.7ml of enzyme sample in a total volume of 2.0ml. The control system contained no cinnamic acid. The formation of cinnamyl-CoA is expressed as nmoles/min. at an average temperature of 25° under an atmosphere of nitrogen, and was measured as an initial rate.

method. p-Coumaric, caffeic, and ferulic acids did not appear to be activated. The only system which showed an extinction change dependent on CoA at the appropriate $\lambda$ max, before the addition of substrate, was the system followed at 311nm., at which wavelength cinnamyl-CoA absorbs maximally. This suggested that the enzyme preparation contained endogenous cinnamate.

This assay gave satisfactory results with several enzyme preparations, but had the drawback that certain preparations active in [$^{14}$C]cinnamyl-hydroxamate formation, showed only very small spectrophotometric changes due to cinnamyl-CoA formation.

## The hydroxylating enzyme

### Section 1. Leaf disc studies

When leaf discs of spinach or spinach beet were incubated for 24hr. in the dark, p-coumaric and ferulic (or sinapic) acids, and sometimes cinnamic acid, were extracted from the discs, even when cinnamic acid was not added to the incubation medium. None of these acids could be extracted from freshly cut discs. Since p-coumaric acid was formed in the absence of added cinnamic acid, experiments using unlabelled cinnamic acid as a possible precursor of p-coumaric acid, could not furnish definitive results on the hydroxylation of cinnamic acid *in vivo*. Further experiments were therefore carried out with added [2-$^{14}$C] cinnamic acid.

When 50 leaf discs were incubated for 24hr. with 2μc [2-$^{14}$C] cinnamic acid, under illumination or in darkness, labelled cinnamic, p-coumaric and ferulic (or sinapic) acids could be extracted. A small amount of labelled caffeic acid was also tentatively identified together with an unknown labelled product. Larger amounts of p-coumaric and ferulic (or sinapic) acids were formed in darkness than when the discs were illuminated (Figs.44 and 45).

When the concentration of unlabelled cinnamic acid added to the [$^{14}$C] cinnamic acid was increased up to 5mM, only p-coumaric acid became labelled. Increasing the concentration of carrier cinnamic acid further, to 10mM or 15mM,

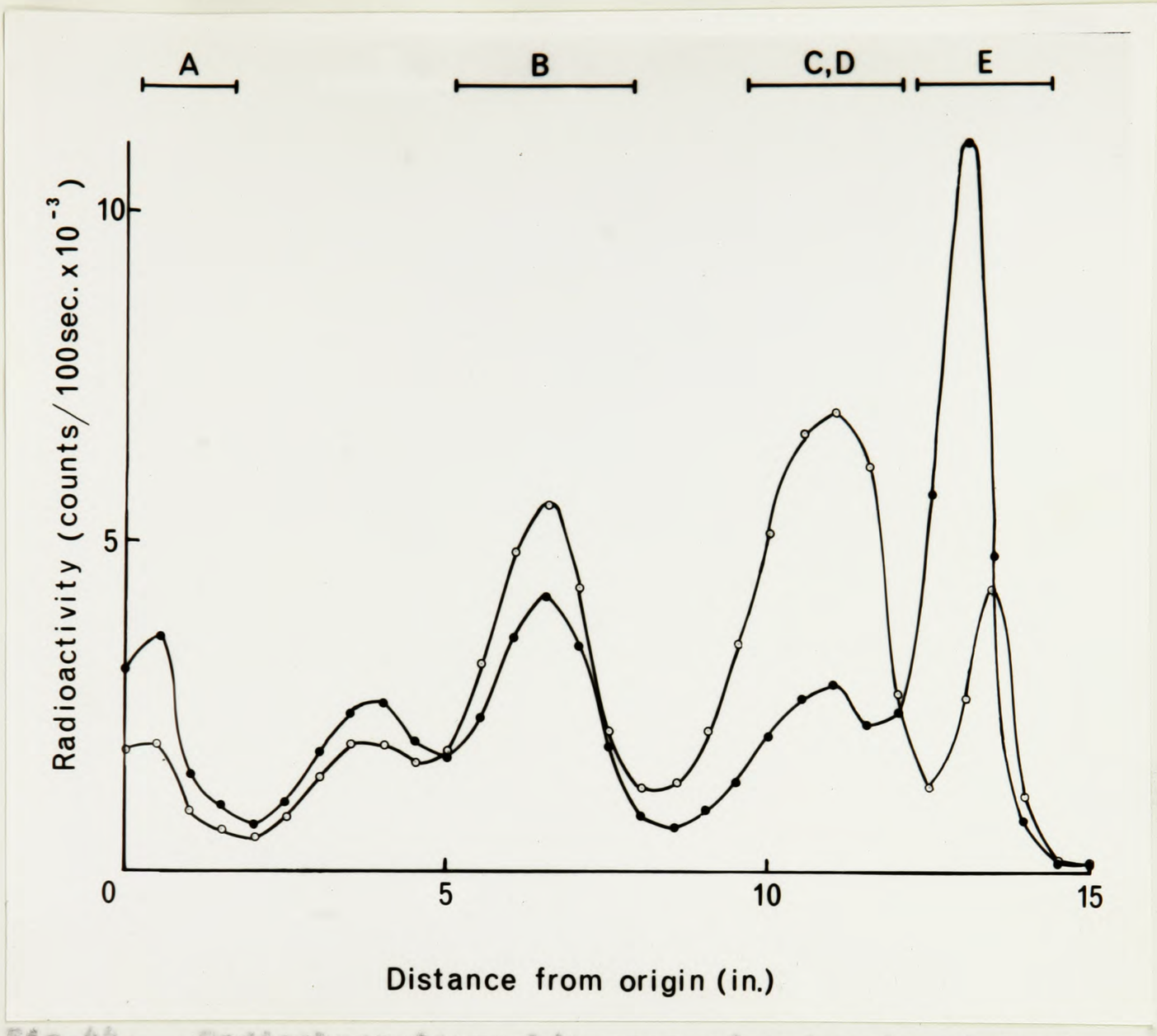



Fig.44. Radiochromatographic scan of extracts of leaf discs of spinach after treatment with [$^{14}$C] cinnamic acid. 50 x 1cm. leaf discs of Spinacia oleracea were incubated at room temperature in 7.5ml. $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.05M, pH5.2), containing 2μc [$^{14}$C] cinnamic acid (1.19 μmoles) for 24 hr. under illumination or in darkness. The discs were extracted (p.36) and the ethereal extract of the alkaline hydrolysate was chromatographed on Whatman No.1 paper in the upper phase of benzene: acetic acid: water (6:7:3 v/v) solvent, and 4 cm. strips scanned. A, B, C, D, E represent the positions of unlabelled samples of caffeic, p-coumaric, sinapic, ferulic and cinnamic acids respectively, determined in parallel strips. The scan of products formed under illumination ( ● ), and darkness ( ○ ), is shown.

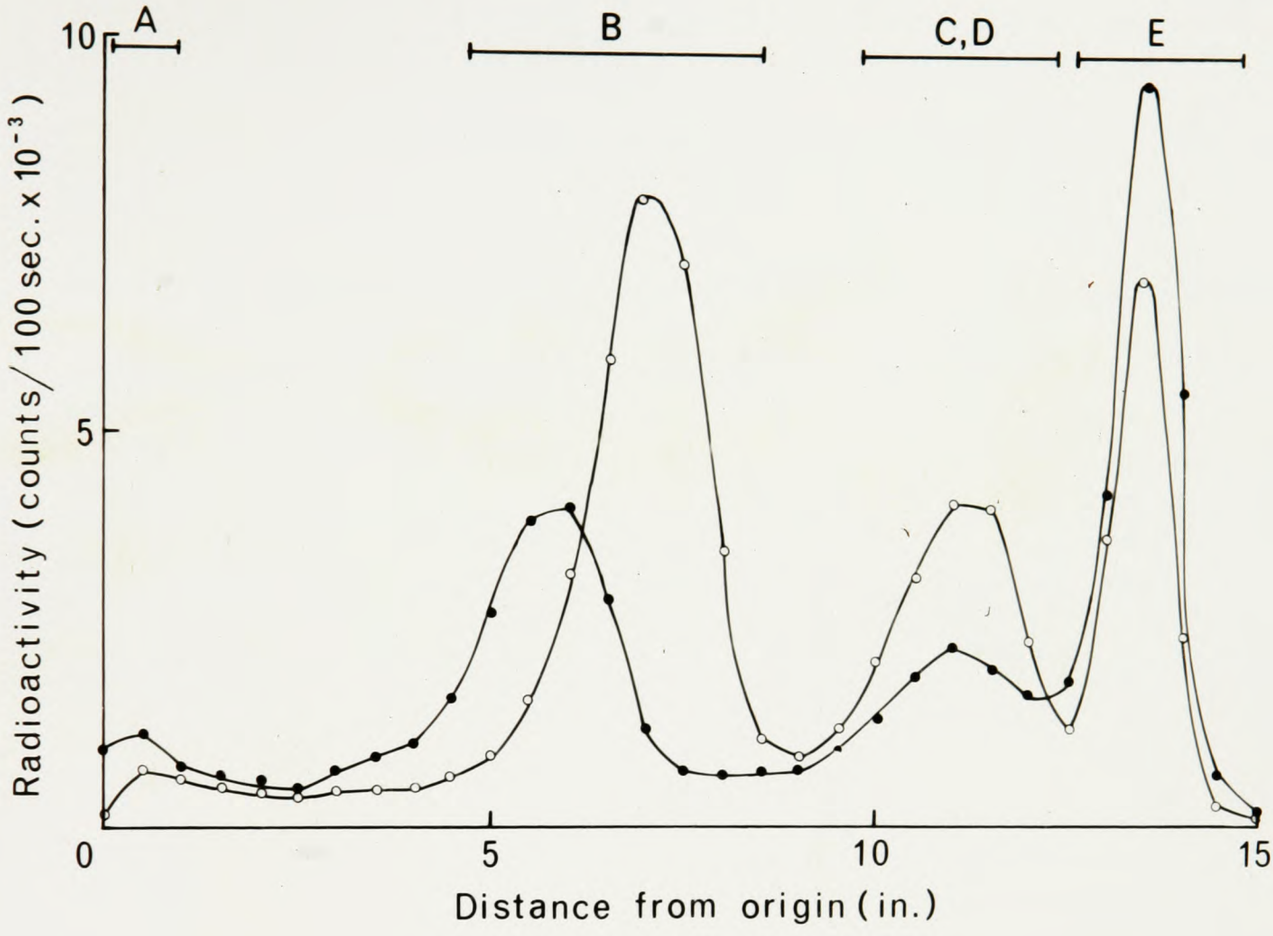



Fig.45. Radiochromatographic scan of extracts of leaf discs of spinach beet after treatment with [$^{14}$C] cinnamic acid. 50x1cm. leaf discs of Beta vulgaris were incubated and extracted as described in Fig.44, and the extracts chromatographed in the same way. A, B, C, D and E represent the positions of unlabelled samples of acids as in Fig.44. The scan of products formed under illumination (●), and darkness (○), is shown.

almost eliminated any measurable conversion of cinnamic acid (Table 19).

The main product formed from cinnamic acid therefore appears to be p-coumaric acid, followed by ferulic acid. Vaughan (1968) has shown that under comparable conditions to those used in these experiments, no sinapic acid is present in the ferulic acid spot, which the benzene: acetic acid: water system fails to resolve. Spinach and spinach beet leaf discs appear similar in their capacity to produce labelled p-coumaric acid from labelled cinnamic acid. Hence, cell-free extracts from leaves of both these plants were examined for cinnamic acid hydroxylation.

Table 19. The utilisation of [$^{14}$C] cinnamic acid by spinach leaf discs under different conditions.

50 x 1cm. leaf discs of S.oleracea were incubated for 24 hr. as described in Fig.44, under illumination (L) or in the dark (D), with [2-$^{14}$C] cinnamic acid (1.19 µmoles, 2µc), but in the presence of 0-15mM unlabelled cinnamic acid. The discs were extracted as described in Fig.44, and the extracts werechromatographed and the radioactivity in each spot measured similarly. The counts for each spot were obtained by summation of the counts for each ½ in. increment, and are given as counts/100 sec. from 2µc [$^{14}$C] cinnamic acid incubated with 50 leaf discs.

| | | Radioactivity of spot (c.p.100 sec. x $10^{-3}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concn. of unlabelled cinnamate | None | | 1mM | | 2mM | | 5mM | | 10mM | | 15mM | |
| Spot | $R_F$ | L | D | L | D | L | D | L | D | L | D | L | D |
| ? caffeic acid | .05 | 9.1 | 5.6 | - | - | - | - | - | - | - | - | - | - |
| unknown | .24 | 11.1 | 7.8 | - | - | - | - | - | - | - | - | - | - |
| p-coumaric acid | .37 | 18.1 | 24.1 | 5.1 | 6.3 | 7.4 | 6.0 | 1.2 | 2.1 | 0.4 | - | - | - |
| ferulic acid | .73 | 12.2 | 37.4 | - | - | - | - | - | - | - | - | - | - |
| cinnamic acid | .88 | 26.4 | 9.5 | 23.0 | 23.4 | 33.7 | 24.8 | 29.3 | 13.1 | 40.1 | 12.4 | 14.2 | 3.3 |

Section 2. Cinnamic acid hydroxylation in crude extracts from spinach and spinach beet leaves.

1. Estimation of p-coumaric acid by the diazotised sulphanilic acid method (Hair & Vining, 1965a).

Assay of an aqueous extract of an acetone powder of spinach beet leaf, prepared by the method of Hair and Vining (1965a), was carried out in citrate-phosphate buffer at pH 4.6 in the presence of cinnamic acid, NADH, and tetrahydrofolic acid. Control incubations contained no cinnamic acid, and varying combinations of cofactors. No p-coumaric acid formation was detected during a 20min. incubation by the diazotised sulphanilic acid method.

No p-coumaric acid formation was detected with a different preparation from spinach beet leaves, in which frozen and powdered leaves were extracted in the presence of insoluble PVP (p. 50 ) and the pH adjusted to 4.6 before centrifugation.

2. Estimation of p-coumaric acid by the modified diazotised sulphanilic acid method (p. 40 ).

A series of extracts of spinach beet leaves were prepared by a method similar to that described by Russell and Conn (1967), in which the extraction of frozen leaf material was carried out in a suspension of insoluble PVP in $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.0; containing 0.02M-

ascorbate and 0.5mM-EDTA). The assay system contained the same buffer, but without EDTA, at pH 7.5, and p-coumaric acid was estimated by the modified diazotised sulphanilic acid method. One positive result was obtained, in which p-coumaric acid was formed in the presence of cinnamic acid, NADPH or NADH, and tetrahydrofolic acid, by the 500xg supernatant from the extract, over the course of a 2hr. incubation. p-Coumaric acid was not formed in the absence of any cofactor.

However, the value of the control containing no cinnamic acid, and the initial readings, were both very high, and the time course of colour development was erratic. A more specific and reliable estimation for p-coumaric acid was therefore used in subsequent experiments.

3. Estimation of p-coumaric acid by Lugg's method.

A series of spinach beet leaf extracts were prepared similar to those described in the previous section, except that the leaves were extracted by maceration in a PVP suspension. The incubation was carried out in $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.5; containing 0.03M-ascorbate) at 30°.

In one experiment only, using a fresh growth of young spinach beet leaves, were low levels of p-coumaric acid formation detected by Lugg's method. p-Coumaric acid

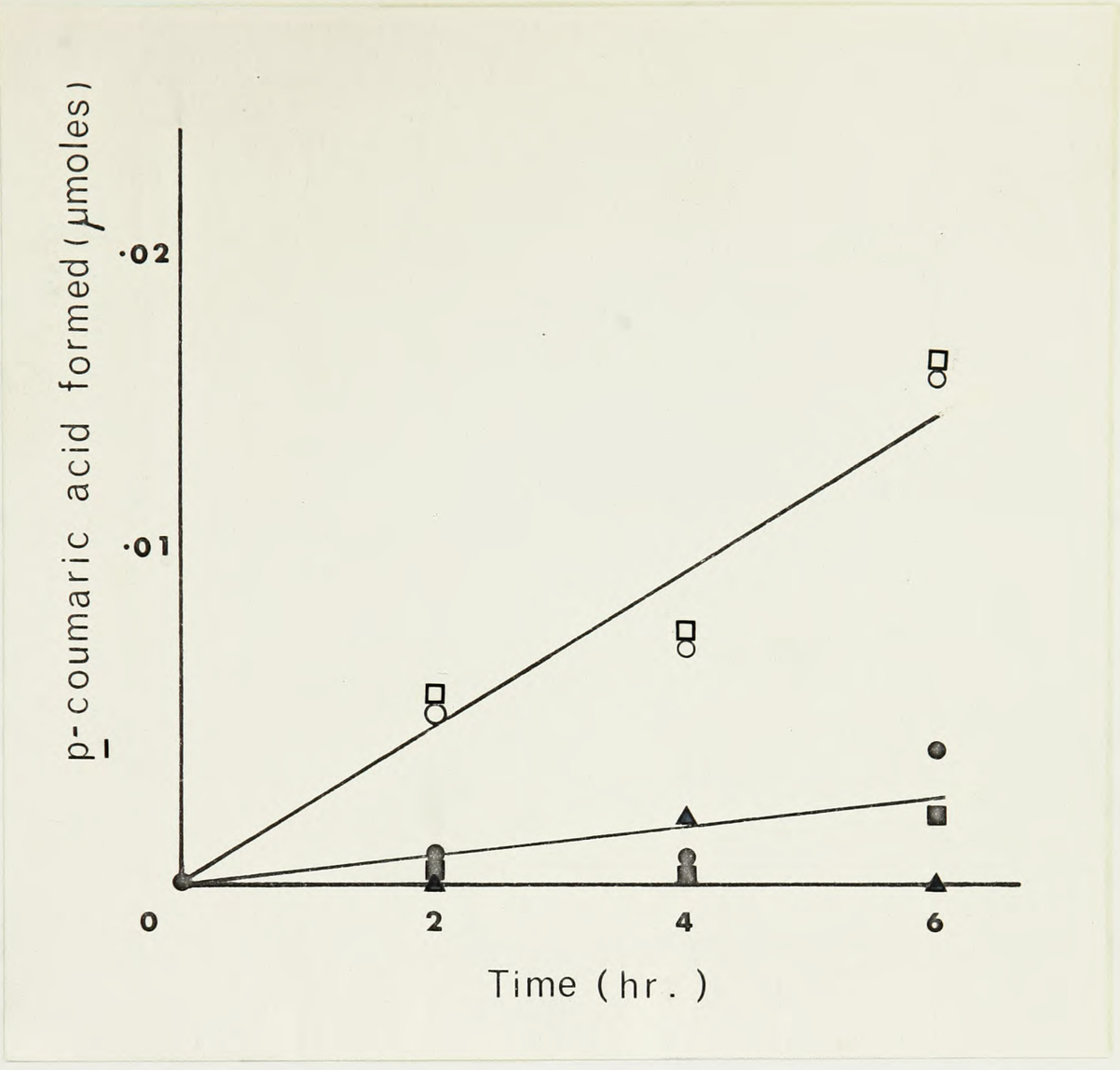



Fig.46. Formation of p-coumaric acid by a crude extract of spinach beet leaves measured by the method of Lugg. The enzyme extract was prepared by macerating 10g. leaf material in a suspension of 10g. Polyclar AT in 80ml. $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.0; containing 0.02M-ascorbate and 0.5mM-EDTA). This extract was filtered, and the pH of the filtrate adjusted to 7.0 with NaOH. The supernatant from centrifugation at 500xg was used directly. The formation of p-coumaric acid was measured in the following system: $Na_2HPO_4$-$NaH_2PO_4$ buffer (225 μmoles, pH 7.5); ascorbate (50 μmoles); cinnamate (5 μmoles); NADH or NADPH (2 μmoles); tetrahydrofolic acid (0.6 μmoles), and 0.5ml. enzyme sample. Control incubations contained no cinnamate. The formation of p-coumaric acid (μmoles) is shown in the absence of cofactors ( ▲ ), with NADH alone ( ■ ), with NADPH alone ( ● ), with NADH and tetrahydrofolic acid ( □ ), and with NADPH and tetrahydrofolic acid ( ○ ).

was formed in the presence of cinnamic acid, NADPH or NADH, and tetrahydrofolic acid. Omission of tetrahydrofolic acid reduced p-coumaric acid formation to a barely measurable level, while omission of all cofactors abolished even this small quantity (Fig.46).

When this experiment was repeated with similar spinach beet leaves no productionof p-coumaric acid was observed, nor was any obtained from an extract of spinach beet leaves raised in the growth room at 25° with a 16hr.-day. An assay carried out in the presence of 1mM-diethyldithiocarbamate, to inhibit the hydroxylation of p-coumaric acid to caffeic acid, did not show p-coumaric acid formation. Further tests on the activity of extracts from spinach beet leaves grown under controlled conditions, and assayed over a pH range from 5 to 10 (using Teorell & Stenhagen's citrate-phosphate-borate buffer), and with a range of cinnamic acid concentrations up to 12.5mM, gave only negative results.

4. Estimation of p-coumaric acid by a radioactive method.

A series of extracts of spinach and spinach beet leaves were prepared by macerating the leaves in a suspension of insoluble PVP in $Na_2HPO_4$-$NaH_2PO_4$ buffer (0.1M, pH 7.0; containing 0.02M-ascorbate and 0.5mM-EDTA). The supernatant from centrifugation of the filtrate of the macerate at 500xg was assayed for cinnamic acid hydroxylase in

$Na_2HPO_4$-$NaH_2PO_4$ buffer (0.15M, pH 7.5; containing 0.02M-ascorbate), in the presence of [2-$^{14}$C] cinnamic acid, NADPH or NADH, and tetrahydrofolic acid or dimethyltetra-hydropteridine. [$^{14}$C] p-Coumaric acid was estimated chromatographically.

Active extracts were prepared from both spinach and spinach beet leaves, grown outside in winter, spring and summer, and inside in the growth room (Table 20). As well as catalysing the formation of p-coumaric acid, the extracts appeared to give a compound, probably caffeic acid, which did not move far from the origin on paper chromatograms run in the upper layer of the benzene: acetic acid: water solvent (6:7:3 v/v)-Table 2). Tetrahydrofolic acid was a much more effective cofactor for cinnamic acid hydroxylation than dimethyltetrahydropteridine, which was almost entirely ineffective. With spinach extracts, NADH appeared to be more effective than NADPH as a hydroxylation cofactor, but with spinach beet extracts, NADPH was more effective. When the volume of enzyme extract from spinach beet was doubled, hydroxylation in the presence of NADH, but not NADPH, was increased.

When the filtrate of the crude extract was centrifuged at 10,000xg instead of 500xg, and both supernatant and residue assayed for cinnamic acid hydroxylation, the activity

Table 20. The formation of [$^{14}$C] p-coumaric acid by crude extracts of spinach and spinach beet leaves.

The enzyme extract was a 500xg supernatant prepared in the presence of insoluble PVP as described in Fig.46. The formation of [$^{14}$C] p-coumaric acid in the following system was measured: $Na_2HPO_4$-$NaH_2PO_4$ buffer (75 μmoles, pH 7.5); ascorbate (22-33 μmoles); NADPH or NADH (4 μmoles); tetrahydrofolic acid (THFA, 1.2 μmoles) or dimethyltetrahydropteridine (DMTP, 4 μmoles); [2-$^{14}$C] cinnamic acid (1.19 μmoles, 2μc) and the enzyme sample. The total volume was 4.0ml. Cinnamic acids were extracted and estimated as described on p. 41, using the radiochromatographic scanner. The radioactivity is given as counts/100 secs. in each acid peak formed in 4hr. at 30°.

| Plant | Vol. of extract used(ml.) | Incubation conditions | Radioactivity of spot (c.p.100sec. x10$^{-3}$) Near origin ? caffeic acid | p-Coumaric acid | Cinnamic acid |
|---|---|---|---|---|---|
| Spinach | 2.50 | NADPH, THFA | 1.1 | 1.7 | 125.7 |
| | " | NADH, DMTP | 0.3 | - | 56.1 |
| | " | NADH, THFA | 2.8 | 3.9 | 132.9 |
| | " | NADH, DMTP | 1.8 | 0.7 | 206.2 |
| Spinach beet | 1.25 | NADPH, THFA | 2.3 | 3.7 | 181.6 |
| | 2.50 | " " | 2.4 | 3.7 | 127.1 |
| | 1.25 | NADH, THFA | 1.7 | 1.3 | 164.2 |
| | 2.50 | " " | 1.3 | 2.1 | 144.2 |

was found to be present in the supernatant only.

The counts measured in the estimation of [$^{14}$C] p-coumaric acid by the radiochromatographic scanning method employed in the above experiments, were low. Insubsequent experiments therefore [$^{14}$C] p-coumaric acid was estimated by cutting out the p-coumaric acid spot observed under the ultra-violet, and counting it by a scintillation method. The counts obtained were then very much higher (Table 21.). The results obtained with spinach beet extracts again showed that NADPH gave rise to a larger quantity of p-coumaric acid than NADH.

The formation of p-coumaric acid was measured over a range of cinnamic acid concentrations, using again a 300 xg supernatant from a spinach beet extract (Fig.47). The formation of p-coumaric acid increased linearly with cinnamic acid concentration up to 1.9mM, above which it fell sharply, suggesting that the substrate exerted an inhibitory effect. Although again, a Km value cannot strictly be calculated from these results, since no initial rates were measured, the figures correspond to a Km of about 2mM for cinnamic acid.

The major drawback encounted in this work lay in the high counts obtained in the p-coumaric acid spot from control incubations using boiled extract. These radio-activities were always higher than those obtained in any

Table 21. The formation of [$^{14}$C] p-coumaric acid by a crude extract of spinach beet leaves.

The enzyme extract was a 500 xg supernatant prepared in the presence of insoluble PVP as described in Fig.46. The formation of [$^{14}$C] p-coumaric acid was measured as described in Table 20, but the radioactivity was counted in a scintillation counter. The radioactivity in p-coumaric acid is given as counts/100 sec. formed in 4 hr. at 30°.

| Vol. of extract used (ml.) | Incubation conditions | Radioactivity in p-coumaric acid (c.p.100 sec.x$10^{-3}$) |
|---|---|---|
| 1.25 | NADPH, THFA | 14.5 |
| 2.50 | NADPH, THFA | 14.6 |
| 1.25 | NADH, THFA | 11.4 |

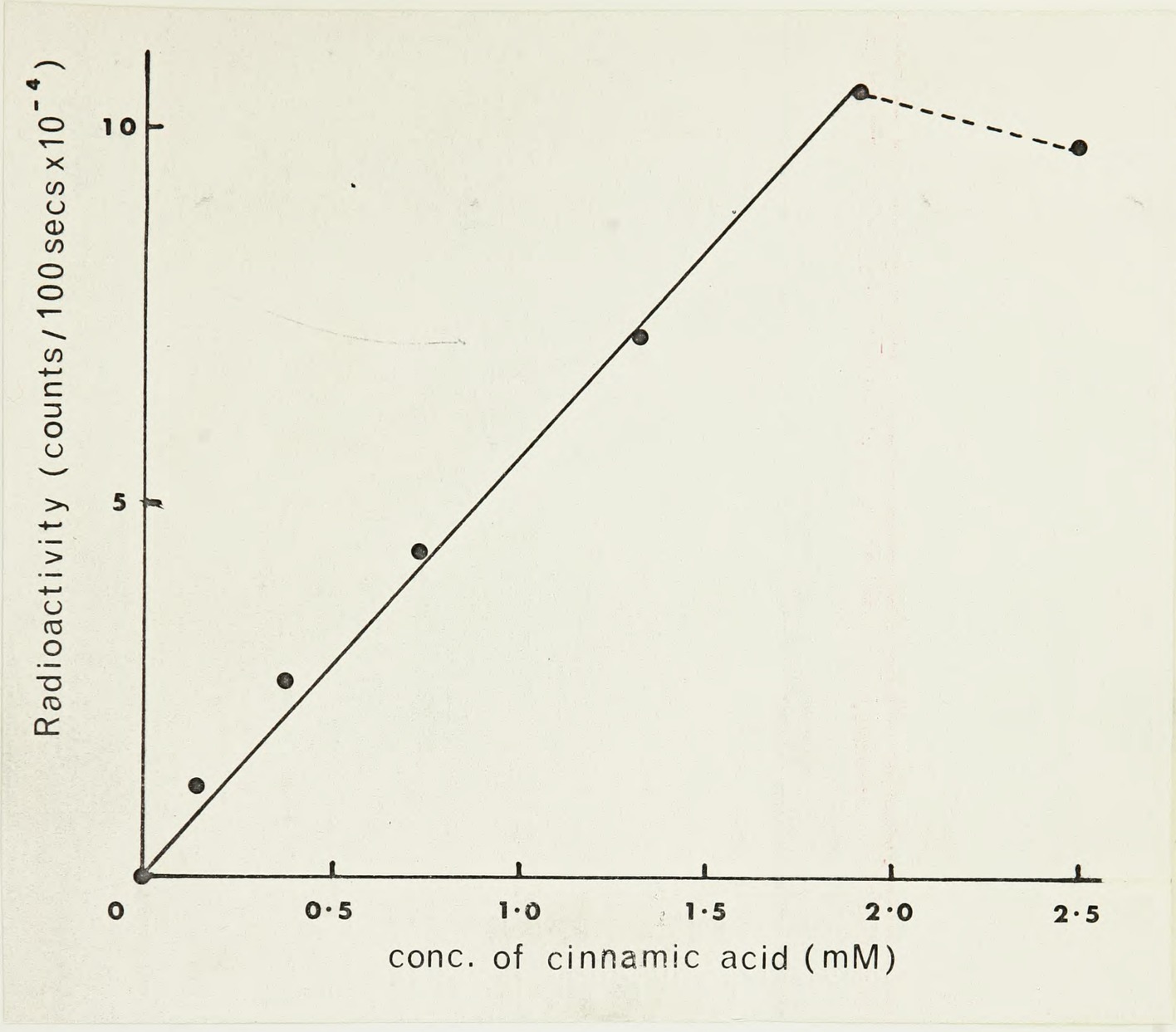



Fig.47. Relationship between concentration of cinnamic acid and p-coumaric acid formation measured by a radioactive method. The formation of p-coumaric acid catalysed by 1.25ml. 900xg$_4$ supernatant, prepared as described in Fig. 46, was measured in the system described in Table 20 using NADPH and tetrahydrofolic acid as cofactors, and unlabelled cinnamic acid (0-10 μmoles) in addition to [$^{14}$C] cinnamic acid. Radioactivity in p-coumaric acid was measured by a scintillation method. The formation of p-coumaric acid in 4hr. at 30°, is expressed as counts/100 sec. proportionated to express the increased quantity of unlabelled cinnamic acid at higher concentrations.

of the experimental incubations. The reason for this is unknown, but the results suggest that the boiled extract interfered seriously with the development of the chromatogram, leading to selective contamination of the p-coumaric acid spot with labelled cinnamic acid; or that there was non-enzymic hydroxylation in the presence of boiled extract, catalysed by bound components which were released from the extract by boiling.

# DISCUSSION

The work described in this thesis has shown that it is possible to obtain an enzyme preparation from the leaves of several higher plants, which is capable of catalysing the activation of cinnamic acid. The presence of an enzyme catalysing the hydroxylation of cinnamic acid to p-coumaric acid has also been established, although much less rigorously.

## THE ACTIVATING ENZYME

### A. The evidence for a cinnamate-activating enzyme distinct from the enzyme catalysing acetate activation.

The evidence in favour of an enzyme which could catalyse the activation of cinnamate, and which was distinct from that activating acetate was gained using only the spectrophotometric assay for hydroxamate formation. Two major differences between the cinnamate-and acetate-activations catalysed by highly active extracts prepared from spinach leaves in the presence of insoluble polyvinylpyrrolidone were distinguished (1 and 2). Preliminary studies on activation systems, carried out

before the optimum conditions of extraction and assay of the cinnamate-activating enzyme had been established, furnished other evidence for the separate identity of acetate- and cinnamate- activation (3-6).

1. The activation of cinnamate was very sensitive to oxygen, whereas the activation of acetate was indifferent. The maximum level of cinnamate activation was increased from 0.1 to 0.6 μmoles in 4hr. by using an atmosphere of ~~air~~ nitrogen instead of ~~nitrogen~~ air. The maximum level of acetate-activation approached 2.0 μmoles in 4hr. irrespective of the atmosphere.
2. The activation of cinnamate in an anaerobic atmosphere was sensitive not only to the presence, but also to the type, of thiol reagent employed in the assay system, whereas the activation of acetate was insensitive. In the absence of a thiol reagent, the level of cinnamate-activation occurring in an anaerobic atmosphere was reduced to that occurring in an aerobic atmosphere in the presence of a thiol reagent.

Since anaerobiosis and the presence of thiol reagents each stimulated the activation of cinnamate, but did not affect acetate-activation, and since both enzymic reactions are dependent on CoA, it is unlikely that the reducing conditions were acting by controlling oxidation of CoA. They probably affected the activity of the

cinnamate-activating system by preventing the oxidation of essential groups on the enzyme. This view is supported by the results obtained with cinnamate-activating enzyme prepared in later studies, which was stabilised by the addition of large quantities of mercaptoethanol.

3. Extracts prepared from various plant organs contained high activity towards acetate and other aliphatic acids, but showed very low or no activity towards cinnamate. Similar patterns of activity towards aliphatic acids have been found in enzyme preparations from wheat germ (Hatch & Stumpf, 1962), spinach (Millerd & Bonner, 1954), and germinating peanut cotyledons (Hebeis et al, 1964, 1965a, 1965b). The only report of cinnamate-activation, with partially purified preparations from parsley and chick pea (Grisebach et al, 1966), also showed very low activity towards cinnamate, but high activity towards a range of aliphatic acids. This work was however insufficient to distinguish more than one enzyme in the extract.

4. Where cinnamate-activation could be measured it could be often detected only in one ammonium sulphate fraction, whereas activity towards acetate showed a much wider distribution among the fractions. The ratio of the activities towards acetate and cinnamate respectively thus varied widely in the different fractions.

5. Activity towards cinnamate was usually lost overnight, whereas activity towards acetate always survived, although it may have fallen by at least half.
6. The pH-dependence of the two activations was different, with maxima close to 7.0 for acetate, but near 7.7 for cinnamate.

B. The properties of the cinnamate-activating system.

Work on the activating system was carried out sequentially, so that it is not possible to compare directly the characteristics shown by each assay system, as they were usually carried out under different conditions, and using different types of enzyme extracts. It has not been considered worthwhile to repeat assays used earlier with later methods of extraction, or later assays with extraction methods found to give extracts with a low activity in the preliminary studies. Nevertheless, certain broad conclusions on the characteristics of the cinnamate-activating system can be drawn from the various preparations and assays.

1. Characteristics of the enzyme which are affected by the method of assay.

Despite difficulties of comparison, certain features are clearly distinguishable between the results obtained using different assay procedures: (i) different assay methods give maximum activation at different pH optima; (ii) different assay methods measure different affinities of the enzyme for cinnamate; and (iii) different assay methods measure different amounts of activation in a given time, and may show a clear lag before the activation reaches its maximum rate.

The pH-activity relationship measured by the cinnamyl-CoA assay would be expected to measure the pH optimum of the overall enzyme reaction, since the assay measures directly the appearance of end-product and is not carried out in the presence of foreign agents (such as hydroxylamine) or excessive concentrations of intermediate products (such as pyrophosphate). The pH optimum measured by this method was broad and fell between 7.5 and 8.0. By the spectrophotometric hydroxamate method, the optimum lay near 7.7, but by the chromatographic hydroxamate method it was either near 7.2 at inhibitory concentrations of tris, or undefinable at the normal concentrations of tris used.

The measurement of pH-dependence by a hydroxamate assay is affected not only by the pH-activity relationship of the enzyme, but also by the reactivity and stability of hydroxylamine at different acidities. At low levels of activating enzyme, the greater stability of hydroxylamine at a pH near neutrality may give an effectively higher concentration of hydroxylamine at a low pH than at a higher one, and may therefore become a determining factor in the definition of the pH optimum for the system. This factor may only become apparent at high buffer concentrations which maintain the pH of the system constant throughout the assay. The pyrophosphate exchange method may also give an erroneous value for the pH-optimum of an activating enzyme, since only the first partial reaction of the system is being studied.

Stulberg and Novelli (1962) and Peterson (1967) have given several examples of different pH optima measured for amino-acid-activating enzymes by different assay methods. Webster (1962) has shown that for the acetate-activating enzyme from bovine heart mitochondria, the pH optima for acetyl-adenylate formation from acetate, and for acetyl-CoA formation from the adenylate, are different. The pH optimum measured by the cinnamyl-CoA assay must therefore be considered the true value for the enzyme, since this measurement is either the resultant of the optimal values

for the two partial reactions, or the optimal value for the rate-limiting partial reaction.

For reasons similar to those discussed for the pH-activity relationship, the $K_m$ value for cinnamate measured by the cinnamyl-CoA assay should be given the greatest weight, especially since this method alone measures the initial rates necessary for this type of study. This $K_m$ value was $4.25 \times 10^{-3}$M, which is higher than the $K_m$ values measured for other activating systems, especially those for amino acids. The $K_m$ value calculated from measurements obtained using the chromatographic hydroxamate method has only limited validity, since initial rates could not be measured. However this $K_m$ was considerably higher than the value given by the cinnamyl-CoA assay, which may indicate that hydroxylamine reduced the affinity of the enzyme for cinnamate by some action on its structure or activity.

The most noteworthy difference between the assay methods was that they each measured the time course and the extent of activation differently. This was particularly important in making comparisons between measurements made in a fixed period of time. In similar preparations, the total activation measured by the cinnamyl-CoA assay was higher than that measured by the chromatographic hydroxamate assay, in a very much shorter period of

incubation. The initial rate measured by the cinnamyl-CoA assay was higher than that measured by the pyrophosphate exchange assay, although the total activation measured by the latter assay was greater over a prolonged period. The activity of preparations assayed by the above three methods was very low when measured with the spectrophotometric hydroxamate method. The cinnamyl-CoA assay showed no lag in the development of enzyme activity, and exhibited a maximal rate over the first two minutes, or more, of the reaction. The pyrophosphate exchange assay did not show a lag period of negligible activity, but nevertheless the course of activation was prolonged and increased slowly to a maximum value at 2hr. or longer after starting the reaction. Both methods of assay involving the formation of hydroxamate showed a lag over the first 2 hr. of incubation, and activation then occurred for the subsequent 2hr. or more.

The slow development of activity measured in the hydroxamate and exchange assays was, at an early stage of the work, attributed to a necessary reduction of essential enzyme groups occurring in an anaerobic atmosphere and in the presence of thiol reagents. However, if necessary, this reduction process appears to be immediate, as shown by the absence of a lag in the cinnamyl-CoA assay. Slow development of activity must then be ascribed to conditions

inherent in particular assay systems.

For the hydroxamate assay system, there are several explanations available for low levels of activation and a lag period:

a) A number of studies (Davie et al, 1956; Hoagland et al, 1956; Schweet & Allen, 1958) have shown that pyrophosphate exerts a potent inhibitory effect on hydroxamate formation in several amino acid-activating systems, by competing with hydroxylamine for the activated intermediate.

b) The ineffectiveness of hydroxylamine as a reagent for the detection of activated groups has been shown in this work and by others. A linear increase in the amount of [$^{14}$C] cinnamyl-hydroxamate formation was found with increasing concentrations of hydroxylamine up to 0.26M, and other work has shown that high concentrations of hydroxylamine are necessary for maximum hydroxamate formation (Attwood & Cocking, 1965; Davie et al, 1956). This ineffectiveness of hydroxylamine could manifest itself in two ways: it may cause a lag period during which the concentration of activated intermediates increases to a critical level at which hydroxylamine begins to act, or it may simply not detect all the activated groups present.

c) Hydroxylamine may lower the activity of the enzyme by some inhibitory action on its structure or mechanism, and it may cause a lag period after which activity develops as the effective concentration of hydroxylamine is lowered by decomposition and by interaction with the plant extract, or as the enzyme recovers from its deleterious effects. The even longer lag found in the presence of bovine serum albumin may be attributed to effectively lower concentrations of thiol reagents at the higher protein concentration, so that any reactivation of the enzyme would be slower.

The slow development of activity in the exchange assay is also subject to a number of explanations:

a) The presence of CoA may be necessary for the fully active conformation of the enzyme. Attwood and Cocking (1965) found that t-RNA increased the rate of hydroxamate formation during alanine activation, and Hele and Barth (1966) found that aged t-RNA preparations acquired the property of stimulating the isoleucine-dependent pyrophosphate exchange rate. Both these results suggest that t-RNA may be necessary for the proper function of an amino acid-activating enzyme, although t-RNA, like CoA in these experiments and in results reported by Jones et al (1953) and Berg (1956), usually inhibits substrate-dependent pyrophosphate exchange

CoA may therefore, like t-RNA, be necessary for the proper function of CoA-dependent activating enzymes.

b) Many activating enzymes have a high affinity for their substrates, and therefore retain them in bound forms after extraction and isolation (Stulberg & Novelli, 1962). The cinnamic acids in particular can be isolated in metabolically-active forms (El-Basyouni & Neish, 1966). The addition of cinnamic acids to activating enzyme extracts already containing these acids in a bound form, could lead to a slow development of exchange, or fail to initiate it altogether. The amounts of bound cinnamic acids occurring in a plant extract would depend on the physiological state of the plant organ at the time it was harvested. It would then be impossible to predict whether an exchange assay would give a positive result; this difficulty was found both in these experiments, and in those described by Grisebach et al (1966). With several preperations, no exchange promotion was stimulated by cinnamic acids, but instead, a partial inhibition of background exchange occurred.

2. Properties of the enzyme established by all methods of assay.

Certain properties of the cinnamate-activating system are the same with all assay methods: (i) the enzyme is

dependent on CoA; (ii) the enzyme is probably specific for cinnamic acids, and may be restricted to cinnamic acid itself, although p-coumaric acid may also be activated; (iii) the enzyme probably enjoys a wide distribution in higher plant tissues; and (iv) the presence of the enzyme is dependent upon the physiological state of the plant.

The measurement of cinnamate-activation by the assay for cinnamyl-CoA must require CoA. The spectrophotometric hydroxamate assay indicated CoA-dependence of the enzyme, although some activation of endogenous substrate, as well as of cinnamate, appeared to take place in the presence of CoA. With the chromatographic hydroxamate assay there was, in some experiments, formation of [$^{14}$C] cinnamyl-hydroxamate in the presence of ATP alone, but this was greatly increased by the addition of CoA. Cinnamate-dependent exchange was completely inhibited by CoA.

Millerd and Bonner (1954), and other workers, have demonstrated the dependence of activating enzymes upon CoA by the hydroxamate method. These results indicate that CoA has a greater affinity than hydroxylamine for the acyl-adenylate intermediate, that CoA is more accessible than hydroxylamine to the intermediate, or that CoA is necessary for the activity of the enzyme system. In many activating systems, hydroxylamine has been demonstrated to have a very low affinity for the primary reaction product (Kingdon et al,

1958), which is tightly bound and inaccessible to external reagents. The possibility that CoA is necessary for the fully active conformation of the enzyme, as well as causing an inhibition of substrate-dependent pyrophosphate exchange, has already been discussed.

The specificity of the activating enzyme for cinnamic acid was indicated by the cinnamyl-CoA assay, in which the substituted cinnamic acids were not activated. However the measurement of the activation of these substituted cinnamic acids required their presence in both test and control cuvettes, and this appeared to reduce the sensitivity of the spectrophotometer. These results should therefore be regarded as less reliable than those obtained for unsubstituted cinnamic acid. p-Coumaric acid partially inhibited [$^{14}$C] cinnamyl-hydroxamate formation, and may have acted by competition as an alternative substrate, especially since caffeic and ferulic acids did not cause this inhibition, indicating that the inhibition by p-coumaric acid was not of a type caused generally by phenolics. However, with enzyme preparations which were inactive in promoting cinnamate-dependent pyrophosphate exchange, a partial inhibition of the background exchange rate occurred not only with cinnamic acid, but also with p-coumaric and ferulic acids. If these acids are not activated, such a suppression must be attributed to a non-specific inhibition.

The distribution of the activating enzyme among higher plants has been studied mainly by the [$^{14}$C] cinnamyl-hydroxamate method. Using this assay, the enzyme has been measured in extracts from leaves of spinach beet, spinach, runner bean and pea. Preliminary experiments using the spectrophotometric hydroxamate method under aerobic conditions measured low levels of the enzyme in extracts from spinach beet, broad bean and pea leaves, maize roots, pea epicotyls and cauliflower florets.

Dependence of enzyme levels on the physiological state of the plant was indicated first by the spectrophotometric hydroxamate assay. This assay, when carried out under anaerobic conditions, showed the presence of high levels of cinnamate-activating enzyme only in leaves from spinach beet plants raised outside in a hot and dry summer. The failure of the assay to show comparably high levels of the enzyme later, demonstrated that they occurred only when optimal conditions of temperature, illumination and humidity coincided. These conditions could not be imitated artificially with the controlled conditions of growth available.

The [$^{14}$C] cinnamyl-hydroxamate assay showed that the presence of the cinnamate-activating enzyme from spinach beet leaves depended on the age of the plants from which the leaves were harvested. The enzyme was absent from very

young leaves, and its presence was inversely related to the presence of betalains in the leaves. When the betalain level in the leaves was high so that they developed a deep red coloration, the level of cinnamate-activating enzyme was low.

The betalain pigments are found only in plants of the order Centrospermae (Mabry, 1966), and in these plants they fulfil the role which the anthocyanins play in other orders. The two types of compound are mutually exclusive, although the Centrospermae form flavonoid pigments other than anthocyanins. The mechanism of betalain synthesis has not been studied in detail, but Mabry described the incorporation of [2-$^{14}$C], 3, 4-dihydroxyphenylalanine into betanin in beet (Beta vulgaris) seedlings. It is therefore unlikely that this group of phenolic compounds is synthesised by the normal phenolic synthetic pathway, which commences with the deamination of phenylalanine, and is followed by activation of the side-chain and hydroxylation of the benzene ring. The appearance of the cinnamate-activating enzyme after betalain synthesis has started and when the betalain level in the leaves is falling, is in accord with this suggestion, and it would initiate the synthesis of more typical phenolic compounds. Other associated enzymic activities should follow a similar pattern. Thus Stafford (1969) has shown that polyphenolase activity in Sorghum seedlings increases

rapidly in green shoots 4 to 5 days after germination, at a rate similar to that of the accumulation of caffeic acid esters.

c. The variation of cinnamate-activating enzyme levels with physiological conditions.

The control of phenolic synthesis by light, and more specifically by phytochrome, has been implicated in both physiological (Siegelman, 1964; Furuya, 1968) and enzymic studies. The enzymic studies have concerned phenylalanine deaminase (Zucker, 1965; Engelsma, 1967a, 1967b; Durst & Mohr, 1966; Scherf & Zenk, 1967), and cinnamic hydroxylase (Engelsma, 1965, 1966; Russell & Conn, 1967; Amrhein & Zenk, 1969). The phenylalanine deaminase studies indicate that control is exerted at the beginning of the phenolic synthetic pathway, analogous to the point at which feedback repression and inducible enzyme synthesis operate in a number of well-characterized microbiological systems.

Although cinnamic hydroxylase has been shown to be controlled by light, the levels of this enzyme do not appear to vary as widely as those of the cinnamate-activating system; cinnamic hydroxylase was extractable from plant leaves in

winter and spring when the activating enzyme was either undetectable or only feebly active.

The relative activities of the cinnamate-activating enzyme and the hydroxylation system may well determine the pattern of complex phenolic synthesis in plants. The studies which were carried out on cinnamate-activating enzyme levels indicated that the levels of this enzyme were very dependent on the physiological state of the plant, and especially on light. When grown under different conditions of illumination, the growth rates of different spinach beet plants varied greatly. The rate of growth, and the development of cinnamate-activating enzyme, were greater when illumination was higher, and also contained a greater proportion of red light (as in the growth cabinet, under "natural" fluorescent lighting.) Very high levels of the enzyme were also extracted from spinach beet plants grown under conditions of high illumination outside. The relative energy distribution in the spectrum of direct sunlight is similar to that of "natural" fluorescent lighting. From the experiments which have been carried out it is impossible to state whether the high light intensity, or the greater proportion of red light was the primary factor responsible for more rapid growth and higher levels of the cinnamate-activating enzyme.

Siegelman and Hendricks (1958) showed that $C_2$ compounds, which may be considered alternative products to anthocyanins, are formed by apple skins in darkness. The experiments of Swain (1960) also suggested that the effect of light on flavonoid synthesis is concerned with the portion of the molecule derived from acetate; this light could act in promoting the linkage of $C_6$ and $C_9$ moieties of the flavonoid by activating the $C_9$ (cinnamic acid) moiety. The synthesis of chlorogenic acid from quinic and caffeic acids (an esterification reaction) in tobacco leaves and potato tubers is also stimulated by light (Zucker et al, 1958, 1959; Levy et al, 1960). Mensball and Goodwin (1964) found that the total amino-acid activating level in 4-day etiolated pea seedlings was increased by exposure to red light, and that this effect was reversed by far-red light, demonstrating the role of phytochrome. The red light also acted by stimulating the growth of the seedling. It therefore seems quite possible that both growth-rate and cinnamate-activating enzyme levels are under the control of red light.

However, when the betalain synthesising system was active in spinach beet leaves, the levels of cinnamate-activating-enzyme were low. Wohlpart and Mabry (1968) have given evidence that betalain synthesis is dependent on light, and it must therefore be concluded that any light-

dependence of the cinnamate-activating system must be different from that of the betalain synthesising system.

Control at the cinnamate-activation step may be important not only in the regulation of the relative levels of cinnamate-activation and hydroxylation, which may well determine the pattern of complex phenolic synthesis, but also in determining whether free phenolic acids accumulate, or are metabolised to more complex phenolic compounds. The accumulation of free phenolic acids may have far-reaching effects on further phenolic synthesis, since substrate-induction or end-product repression mechanisms may play a part in the sequence of events which could occur. Hess (1968) has produced evidence for substrate induction by cinnamic acids as a physiological mechanism in the initiation of anthocyanin synthesis in flower development in Petunia. Ingelsma (1968) has demonstrated end-product repression of phenylalanine deaminase induction in hypocotyl segments of gherkin seedlings by cinnamic and p-coumaric acids. The accumulation of free cinnamic acids could therefore have important effects on the biochemistry, physiology and development of the plant by alteration or prevention of the synthesis of complex phenolic compounds.

It would be interesting to investigate whether the cinnamate-activating enzyme is susceptible not only to light control but also to substrate induction. Hagemen and

Flesher (1960) have demonstrated that such a dual control mechanism can exist in the induction of nitrate reductase activity in corn (*Zea mays*) seedlings.

## CINNAMIC ACID HYDROXYLASE

### A. Leaf disc studies

The leaf disc studies carried out on material from spinach and spinach beet plants demonstrated three interesting features:

1. The supply of exogenous cinnamic acid was not necessary for the formation of p-coumaric and ferulic acids on incubation of the leaf discs. Detectable amounts of these acids were not found on extraction of freshly cut leaf discs. It is therefore suggested that the hydroxycinnamic acids were formed during incubation either from phenylalanine arising from the breakdown of protein after excision of the discs, or from the release of bound forms of phenolic acids, such as described by El-Basyouni and Neish (1966).

p-Coumaric acid is formed from phenylalanine by the successive actions of phenylalanine deaminase and cinnamic acid hydroxylase. The induction of phenylalanine deaminase has been observed on excision of various plant tissues, for example discs of potato tuber (Zucker, 1965), and has since been described by Engelsma (1968) in gherkin hypocotyl segments and by Creasy (1968) in strawberry leaf discs. The enzyme can be photoinduced both before and after excision and it is repressed by cinnamic and p-coumaric acids. There is also evidence for the induction of cinnamic acid hydroxylase

by both excision and light in gherkin hypocotyl segments (Engelsma, 1965, 1966). Maximum induction of the enzyme occurs when the effects of excision and photoinduction are combined by irradiating the whole plant at least 1 hr. before excision. There is a striking correlation between cinnamic acid hydroxylase activity and the accumulation of hydroxycinnamic acids in the gherkin hypocotyl, indicating that the development of this enzyme is the rate-limiting factor in determining phenylalanine conversion.

The probable formation of p-coumaric acid from phenylalanine arising from protein degradation in unfed leaf discs, may therefore be primarily due to the induction of cinnamic acid hydroxylase in these discs. The development of cinnamic acid hydroxylase activity is likely to have been high since the leaves were in effect irradiated before excision, having been gathered from plants in daylight. Also the end-product repression of phenylalanine deaminase by cinnamic and p-coumaric acids, and the inhibition of cinnamic acid hydroxylase by p-coumaric acid (Russell & Conn, 1968) may not occur in excised tissues to the same extent as it does in intact tissues because it is possible for these acids to diffuse outwards from excised tissues (Engelsma, 1968).

2. The main conversion products of exogenous [$^{14}$C]cinnamic acid were p-coumaric and ferulic acids. Only a low level of

labelled caffeic acid was detected. The further conversion of p-coumaric acid therefore appeared to be on a relatively small scale, and any caffeic acid formed was apparently rapidly methylated to ferulic acid.

3. The elimination of all products from [$^{14}$C] cinnamic acid except [$^{14}$C] p-coumaric acid, when unlabelled cinnamic acid was added to the incubation medium, was probably due to the formation of a large pool of cinnamic acid in the leaf discs. Ferulic acid appeared to be formed only when the pool size was small i.e. when no exogenous cinnamic acid was supplied, or when the labelled cinnamic acid was undiluted. The fact that cinnamic acid hydroxylation was detected at concentrations of unlabelled cinnamic acid up to 10mM, may suggest the presence of a highly active cinnamic hydroxylase system, in comparison with the activity of the enzymes catalysing caffeic acid and ferulic acid formation.

B. Studies with crude extracts

Within the limitations placed by the measurement of high control values with boiled enzyme preparations, cinnamic acid hydroxylase was detected in crude extracts from spinach

and spinach beet leaves, prepared and assayed under the pH and buffer conditions described by Russell and Conn (1967). The enzyme was not found when extracted and assayed under the conditions described by Nair and Vining (1965a).

Whereas Russell and Conn found the cinnamate hydroxylase from pea buds to be specific for NADPH, and entirely inactive in the presence of NADH, the results described in this thesis indicated that the pyridine nucleotide specificity depended upon the plant extract, and was not absolute. With the spinach beet extracts, NADPH was preferred, and with the spinach extracts, NADH was preferred. These cofactor requirements are similar to those of Nair and Vining, who found NADPH and NADH to be equally effective for cinnamic acid hydroxylation with an aqueous extract of an acetone powder from spinach leaves. Dimethyltetrahydropteridine, which has been shown to act as a cofactor for p-coumaric acid hydroxylation (Vaughan, 1968), was almost ineffective in comparison with tetrahydrofolic acid, in the presence of either NADPH or NADH.

# BIBLIOGRAPHY


Amrhein, N. & Zenk, M.H. (1969) Phytochem.8, 107-113. Das auftreten des "NIH-shifts" bei der in vivo und in vitro bildung hydroxylierter zimtsäurederivate in Buchweizenhypokotylen (Fagopyrum esculentum).

Anderson, J.W. (1968). Phytochem. 7, 1973-1988. Extraction of enzymes and subcellular organelles from plant tissues.

Attwood, M.M. & Cocking, E.C. (1963). Biochem. J. 96, 616-625. The purification and properties of the alanyl-transfer ribonucleic acid synthetase of tomato roots.

Berg, P. (1956). J.Biol. Chem. 222, 991-1013. Acyl adenylates: an enzymatic mechanism of acetate activation.

Brown, S.A. (1967). Bioscience, 19, 115-121. Biochemistry of lignin formation.

Campagnari, F. & Webster, L.T. (1963). J.Biol.Chem. 238, 1628-1633. Purification and properties of acetyl Coenzyme A synthetase from bovine heart mitochondria.

Canham, A.E. (1966). Artificial Light in Horticulture, pp.42-90. Eindhoven: Centrex Publishing Co., Chapter 3. Lamps.

Creasy, L.L. (1968). Phytochem. 7. 441-446. The increase in phenylalanine ammonia-lyase activity in strawberry leaf discs and its correlation with flavonoid synthesis.

Cruickshank, I.A.M. & Perrin, D.R. (1964). Biochemistry of Phenolic Compounds, pp.511-544. Ed.Harborne, J.B., London: Academic Press. Chapter 13. Pathological function of phenolic compounds in plants.

Davie, E.W., Koningsberger, V.V. & Lipmann, F. (1956). Arch.Biochem.Biophys. 65, 21-38. The isolation of a tryptophan-activating enzyme from pancreas.

Davis, J.N. & Novelli, G.D. (1958). Arch.Biochem.Biophys. 75, 299-308. The activation of amino acids in extracts of pea seedlings.

Dixon, M. & Webb, E.C. (1961). Adv. Prot. Chem. 16, 197-219. Enzyme fractionation by salting-out: a theoretical note.

Durst, F. & Mohr, H. (1966). Naturwiss. 53, 531-532. Phytochrome-mediated induction of enzyme synthesis in mustard seedlings. (Sinapis alba L.)

El-Basyouni, S.Z., Chen, D., Ibrahim, R.K., Neish, A.C. & Towers, G.H.N. (1964) Phytochem. 3, 485-492. The biosynthesis of hydroxybenzoic acids in higher plants.

El-Basyouni, S.Z. & Neish A.C. (1966). Phytochem. 5, 683-691. Occurrence of metabolically-active bound forms of cinnamic acid and its phenolic derivatives in acetone powders of wheat and barley plants.

Engelsma, G. (1965). Nature, Lond. 208, 1117-1119. Photo-induced hydroxylation of cinnamic acid in gherkin hypocotyls.

Engelsma, G. (1966). Acta. Bot. Neerl. 15, 394-405. The influence of light of different spectral regions on the synthesis of phenolic compounds in gherkin seedlings in relation to photomorphogenesis III. Hydroxylation of cinnamic acid.

Engelsma, G. (1967a). Planta (Berl.) 75, 207-219. Photoinduction of phenylalanine deaminase in gherkin seedlings. I. Effect of blue light.

Engelsma, G. (1967b). Planta (Berl.) 77, 49-57. Photoinduction of phenylalanine deaminase in gherkin seedlings. II. Effect of red and far-red light.

Engelsma, G. (1968). (Planta Berl) 82, 355-368. Photoinduction of phenylalanine deaminase in gherkin seedlings. III. Effects of excision and irradiation on enzyme development in hypocotyl segments.

Furuya, M. (1968). Progress in Phytochemistry 1, 347-405. Biochemistry and Physiology of Phytochrome.

Green, D.E. & Silman, I. (1967). Ann. Rev. Pl. Physiol. 18, 147-178. Structure of the mitochondrial electron transfer chain.

Grisebach, H. & Brandner, G. (1962). Experientia [Basel] 18, 400-401. Einbau des 2', 4, 4', 6'- tetrahydroxy-chalkon-2'-glucosid-[β-$^{14}$C] in isoflavone.

Grisebach, H., Barz, W., Mahlbrock, K., Kellner, S. & Patschke, L.(1966). Proc. 2nd Meeting Fed.Eur.Biochem. Soc., Vienna. 2, 25-36. Biosynthesis of Aromatic Compounds. Ed.Billek, G. Pergamon Press. Chapter 3. Recent investigations on the biosynthesis of flavonoids.

Gross, G.G. & Zenk, M.H. (1966). Z.Naturforschg. 21b, 683-690. Darstellung und Eigenschaften von Coenzym A - thiolestern substituierter zimtsäuren.

Gross, G.G., Bolkart, K.H. & Zenk, M.H. (1968). Biochem. Biophys. Res.Comm. 32, 173-178. The reduction of cinnamic acid to cinnamaldehyde and alcohol.

Grunert, R.R. & Phillips, P.H. (1951). Arch. Biochem. Biophys. 30, 217-225. A modification of the nitroprusside method of analysis for glutathione.

Hageman, R.H. & Flesher, D. (1960). Pl. Physiol. 35, 700-708. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media.

Harborne, J.B. (1964). Biochemistry of Phenolic Compounds, pp.129-170. Ed. Harborne, J.B., London: Academic Press. Chapter 4. Phenolic glycosides and their natural distribution.

Hatch, M.D. & Stumpf, P.K.(1962). Pl.Physiol. 37, 121-126. Fat metabolism in higher plants. XVII. Metabolism of malonic acid and its α-substituted derivatives in plants.

Hauser, C.R. & Renfrow, W.B. (1959). Organic Syntheses. Coll. Vol.2. p.67-68. Ed.Blatt, A.H. New York: John Wiley & Sons. Synthesis of benzohydroxamic acid.

Hele, P. & Barth, P.T. (1966). Biochim. Biophys. Acta. 114, 149-157. Possible "allosteric" effects controlling the kinetic behaviour of amino acid-dependent pyrophosphate exchange reactions.

Henshall, J.D. & Goodwin, T.W. (1964). Phytochem. 3, 677-691. Amino-acid activating enzymes in germinating pea seedlings.

Hess, D. (1967). Z. Pflanzenphysiol. 56, 13-19. Die wirkung von zimtsäuren auf die anthocyansynthese in isolierten petalen von Petunia hydrida.

Hess, D. (1968). Z.Pflanzenphysiol. 59, 295-296. Substratinduktion durch zimtsäuren als physiologischer mechanismus bei der einleitung der anthocyansynthese.

Hiatt, A.J. & Evans, H.J. (1960) Pl.Physiol. 35, 673-677. Influence of certain cations on the activity of acetic thiokinase from spinach leaves.

Hiatt, A.J. (1964). Pl. Physiol. 39, 475-479. Further studies on the activation of acetic thiokinase by magnesium and univalent cations.

Hoagland, M.B. (1955). Biochim. Biophys. Acta. 16, 288-289. An enzymic mechanism for amino acid activation in animal tissues.

Hoagland, M.B., Keller, E.B. & Zamecnik, P.C. (1956). J.Biol.Chem. 218, 345-358. Enzymatic carboxyl activation of amino-acids.

Jones, M.E., Lipmann, F., Hilz, H. & Lynen, F. (1953). J.Amer.Chem.Soc. 75, 3285-3286. On the enzymatic mechanism of Coenzyme A acetylation with adenosine triphosphate and acetate.

Kertesz, D. & Zito, R. (1962). Oxygenases. pp.307-354 Ed.Hayaishi, O. New York: Academic Press. Chapter 8. Phenolase.

Kingdon, H.S., Webster, L.T. & Davie, E.W. (1958). Proc. Nat.Acad.Sci. 44, 757-765. Enzymatic formation of adenyl tryptophan: isolation and identification.

Layne, E. (1957). Methods in Enzymology. (1st Edition). Volume III. pp.447-454. Ed. Colowick, S.P. & Kaplan, N.O. New York: Academic Press Inc. Chapter 73. Spectrophotometric and turbidometric methods for measuring proteins.

Leggett Bailey, J. (1962). Techniques in Protein Chemistry. (2nd Edition) pp.340-352. Amsterdam: Elsevier Publishing Co., Chapter 11. Miscellaneous analytical methods.

Levy, C.C. & Zucker, M. (1960). J.Biol. Chem. 235, 2418-2425. Cinnamyl and p-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid.

Lipmann, F. & Tuttle, L.C. (1945). J.Biol.Chem. 159, 21-28. A specific micromethod for the determination of acyl phosphates.

Loomis, W.D. & Battaile, J. (1966). Phytochem. 5, 423-438. Plant phenolic compounds and the isolation of plant enzymes.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). J.Biol.Chem. 193, 265-275. Protein measurement with the Folin phenol reagent.

Lugg, J.W.H. (1937). Biochem. J. 31, 1422-1433. On the use of mercuric salts and nitrous acid in the colorimetric determination of tyrosine and tryptophan present in solution.

Mabry, T.J. (1966). Comparative Phytochemistry. pp.231-244. Ed. Swain, T., London: Academic Press. Chapter 14. The Betacyanins and Betaxanthins.

Mahler, H.R., Wakil, S.J. & Bock, R.M. (1953). J.Biol.Chem. 204, 453-468. Studies on fatty acid oxidation. I. Enzymatic activation of fatty acids.

Mason, H.S. (1957). Adv. Enzymol. 19, 79-233. Mechanisms of oxygen metabolism.

Millerd, A. & Bonner, J. (1954). Arch. Biochem. Biophys. 49, 343-355. Acetate activation and acetoacetate formation in plant systems.

Mudd, J.B. (1967). Ann.Rev.Pl.Physiol. 18, 329-352. Fat metabolism in plants.

Nair, P.M. & Vining, L.C. (1965a). Phytochem. 4, 161-168. Cinnamic acid hydroxylase in spinach.

Nair, P.M. & Vining, L.C. (1965b). Phytochem. 4, 401-411. Phenylalanine hydroxylase from spinach leaves.

Nason, A. (1955). Methods in Enzymology. (1st Edition). Volume I. pp.62-65. Ed.Colowick, S.P. & Kaplan, N.O. New York: Academic Press Inc. Chapter 8. Extraction of soluble enzymes from higher plants.

Nelsh, A.C. (1964). Biochemistry of Phenolic Compounds, pp.295-360. Ed.Harborne, J.B., London: Academic Press. Chapter 8. Major pathways of biosynthesis of phenols.

Peterson, P.J. & Fowden, L. (1963). Nature, Lond. 200, 148-151. Different specificities of proline-activating enzymes from some plant species.

Peterson, P.J. (1967). Biol. Rev. 42, 552-613. Amino acid selection in protein biosynthesis.

Porter, J.W. & Anderson, D.G. (1967). Ann. Rev. Pl.Physiol. 18, 197-228. Biosynthesis of carotenes.

Rasche, I.D. & Bové, J. (1960). Experientia 16, 195-196. Notes on the hydroxamate assay for amino-acid activating enzymes.

Robinowitch, E.I. (1951) Photosynthesis and Related Processes. Volume II. Part 1. Spectroscopy and Fluorescence of Photosynthetic Pigments; Kinetics of Photosynthesis. pp.730-759. New York: Interscience Publishers, Inc. Chapter 22, Appendix: Natural Light Fields.

Rebeiz, C.A. & Castelfranco, P. (1964). Pl.Physiol. 39, 938-938. An extra-mitochondrial enzyme system from peanuts catalysing $\beta$-oxidation of fatty acids.

Rebeiz, C.A., Castelfranco, P. & Engelbrecht, A.H. (1965a). Pl.Physiol. 40, 281-286. Fractionation and properties of an extra-mitochondrial enzyme system from peanuts catalysing $\beta$-oxidation of palmitic acid.

Rebeiz, C.A., Castelfranco, P. & Breidenbech, P.W. (1965b) Pl.Physiol. 40, 286-289. Activation and oxidation of acetic acid-1-$C^{14}$ by cell-free homogenates of germinating peanut cotyledons.

Russell, D.W. & Conn, E.E. (1967). Arch. Biochem.Biophys. 122, 256-258. The cinnamic acid 4-hydroxylase of pea seedlings.

Russell, D.W. & Conn, E.E. (1968). Pl.Physiol. 43, Suppl. (Abstracts). S.39. Studies on the cinnamic acid 4-hydroxylase of pea seedlings.

Scherf, H. & Zenk, M.H. (1967). Z.Pflanzenphysiol. 56, 203-206. Induction of anthocyanin and phenylalanine ammonia-lyase formation by a high energy light reaction and its control through the phytochrome system.

Schwoet, M.S., Holley, R.W. & Allen, E.H. (1957). Arch. Biochem. Biophys. 71, 311-325. Amino-acid activation in hog pancreas.

Schwoet, M.S. & Allen, E.H. (1958). J.Biol.Chem. 233, 1104-1108. Purification and properties of tyrosine-activating enzyme of hog pancreas.

Siegelman, H.W. & Hendricks, S.B. (1958). Pl.Physiol. 33, 409-413. Photocontrol of alcohol, aldehyde, and anthocyanin production in apple skin.

Siegelman, H.W. (1964). Biochemistry of Phenolic Compounds. pp.437-456. Ed.Harborne, J.B., London: Academic Press. Chapter 11. Physiological studies on phenolic biosynthesis.

Smith, I.K. & Fowden, L. (1968). Phytochem. 7, 1065-1076. Studies on the specificities of the phenylalanyl - and tyrosyl - SRNA synthetases from plants.

Stafford, H.A. (1967). Pl.Physiol. 42, 450-455. Biosynthesis of phenolic compounds in first internodes of Sorghum: lignin and related products.

Stafford, H.A. (1969). Phytochem. 8, 743-752. Changes in phenolic compounds and related enzymes in young plants of Sorghum.

Stiller, M. (1962). Ann. Rev. Pl. Physiol. 13, 151-170. The path of carbon in photosynthesis.

Stulberg, M.P. & Novelli, G.D. (1962). The Enzymes. (2nd Edition). pp. 401.-432. Ed.Boyer, P.D., Lardy, H. & Myrback, K. London: Academic Press. Vol.VI. Chapter 25. Amino-acid activation.

Stumpf, P.K. & Bradbeer, C. (1959). Ann. Rev.Pl.Physiol. 10. 197-222. Fat metabolism in higher plants.

Swain, T. (1953). Biochem. J. 53, 200-208. The identification of coumarins and related compounds by filter-paper chromatography.

Swain, T. (1960). Phenolics in Plants in Health and Disease. pp.45-55. Ed.Pridham, J.B., Oxford: Pergamon Press. Section II. Some interrelationships between leuco-anthocyanins and lignin in plants.

Towers, G.H.N. (1964). Biochemistry of Phenolic Compounds, pp.249-294. Ed.Harborne, J.B., London: Academic Press. Chapter 7. Metabolism of phenolics in higher plants and micro-organisms.

Van Sumere, C.F. (1960). Phenolics in Plants in Health and Disease, pp.25-34. Ed.Pridham, J.B. Oxford: Pergamon Press. Section I. Germination inhibitors in plant material.

Vaughan, P.F.T. (1968). D. Phil. Thesis. Botany School. University of Oxford. Aromatic hydroxylation in plants.

Warburg, O. & Christian, W. (1941). Biochem. Z. 310. 384-421. Isolierung und Kristallisation des Gärungsferments Enolase.

Webster, L.T. & Campagnari, F. (1962). J.Biol.Chem. 237. 1050-1055. The biosynthesis of acetyl and butyryl adenylates.

Webster, L.T. (1965). J.Biol.Chem. 240. 4164-4169. Studies of the acetyl Coenzyme A synthetase reaction. III. Evidence of a double requirement for divalent cations.

Webster, L.T. (1966). J.Biol.Chem. 241. 5504-5510. Studies of the acetyl Coenzyme A synthetase reaction. IV The requirement for monovalent cations.

Wohlpart, A. & Mabry, T.J. (1968). Pl.Physiol. 43 457-459. On the light requirement for betalain biogenesis.

Zenk, M.H. & Gross, G.G. (1965). Z.Pflanzenphysiol. 53 356-362. Reduktion von veratrumsäure zu veratrylaldehyd und veratrylalkohol durch zellfreie extrakte von Polystictus versicolor, L.

Zenk, M.H. (1966). Proc.2nd Meeting Fed.Eur.Biochem Soc., Vienna. 2. 45-60. Biosynthesis of Aromatic Compounds. Ed.Wilick, G. Pergamon Press. Chapter 5. Biosynthesis of $C_6$-$C_1$ compounds.

Zucker, M. & Abrens, J.F. (1958). Pl.Physiol. 33, 246-249. Quantitative assay of chlorogenic acid and its pattern of distribution within tobacco leaves.

Zucker, M. & Levy, C.C. (1959). Pl.Physiol. 34, 108-112. Some factors which affect the synthesis of chlorogenic acid in disks of potato tuber.

Zucker, M. (1965). Pl. Physiol. 40, 779-784. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue.