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THE DEMONSTRATION OF CINNAMYL-CoA SYNTHETASE ACTIVITY IN LEAF EXTRACTS

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Abstract—Cinnamyl-CoA synthetase activity has been demonstrated in extracts of leaves of spinach beet (*Beta vulgaris* L. ssp. *vulgaris*) by direct spectrophotometric assay and by CoA-dependent formation of cinnamyl hydroxamate. These extracts also promoted a cinnamate-dependent incorporation of pyrophosphate into nucleotide phosphate, which was inhibited by CoA. The activity of extracts was affected by the age of the plants and leaves and by their growth conditions; it showed an inverse correlation with the accumulation of red pigment in the leaves. Measurable activity was also found in leaves from young plants of pea, runner bean and spinach.

INTRODUCTION

THE SYNTHESIS of many phenolic compounds in plants includes oxidation, reduction and condensation stages, in which the side-chain of cinnamic acid or its hydroxylated derivatives is involved. These stages are presumed to require activation of the carboxylic group, probably by formation of the CoA ester.[1] The activation of cinnamic acid and *p*-coumaric acid in the presence of CoA has already been reported with extracts of chickpea (*Cicer arietinum*) and parsley (*Apium petroselinum*),[2] but acetic, succinic and other aliphatic acids were also readily activated. More recently, extracts from leaves of spinach beet (*Beta vulgaris* ssp. *vulgaris*) have been prepared, which activated cinnamate and acetate providing that CoA was added.[3] A much higher rate of cinnamate activation relative to acetate was found, and the cinnamate activation was distinguished by its sensitivity to oxidizing conditions and its greater lability on storage. This system was found only in extracts of young leaves during periods of rapid growth, when measured by a spectrophotometric estimation of hydroxamate formation.[4]

In order to detect the low activities of this enzyme in many plant extracts, more sensitive methods of assay have been developed. By analogy with acetyl-CoA synthetase (acetate: CoA ligase (AMP), E.C. 6.2.1.1.), the activation of acetate can be assumed to proceed in two stages:

$$\text{Cinnamate} + \text{ATP} \leftrightharpoons \text{Cinnamyl-AMP} + \text{PP}_i \quad (1)$$

$$\text{Cinnamyl-AMP} + \text{CoASH} \leftrightharpoons \text{Cinnamyl-SCoA} + \text{AMP} \quad (2)$$

Since hydroxylamine may react with the products of each stage, the assay of cinnamyl-CoA synthetase using hydroxylamine depends on an enhanced production of hydroxamate in the

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[1] A. C. NEISH, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 295, Academic Press, London (1964).

[2] H. GRISEBACH, W. BARZ, K. HAHLBROCK, S. KELLNER and L. PATSCHKE, *Proc. 2nd Meeting Fed. Eur. Biochem. Soc., Vienna*, **3**, 25 (1966).

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presence of CoA. Alternatively, the production of cinnamyl-CoA can be followed spectrophotometrically. Stage (1) can be followed in isolation by the incorporation of pyrophosphate into nucleotide triphosphate. Each of these methods has been employed in sensitive procedures to measure the enzymic activities of various leaf extracts and their changes during growth, and to demonstrate further the requirement for CoA in the reaction.

RESULTS

Formation of [2-^{14}C] *Cinnamyl Hydroxamate*

The activity of extracts from leaves of spinach beet was successfully determined by measuring the radioactivity of [2-^{14}C] cinnamyl hydroxamate on chromatograms after incubating the extracts with [2-^{14}C] cinnamate in nitrogen. The extracts were passed through columns of Sephadex G-25 before assay to remove interfering substances which, when present, gave abnormally high values in the control tubes with boiled extracts. The formation of hydroxamate required ATP, and with all extracts, the addition of CoA gave

TABLE 1. REQUIREMENT FOR ATP AND CoA IN CINNAMYL HYDROXAMATE FORMATION

Extract	Cinnamyl hydroxamate formed (nmole/mg protein)					
	A	B	C	D	E	F
Complete system	1·03	10·3	2·21	4·02	0·963	0·698
Complete system—CoA	0·69	7·86	0	2·28	0	0
Complete system—CoA—ATP	0·54	0	0	0	0	0

Reaction conditions in Experimental, using a crude extract (A) and Sephadex eluate of the extract (B) from leaves of 12-day plants in growth cabinet, and Sephadex eluates of leaf extracts of 18-day (C) and 35-day (D) plants in growth cabinet and 12-day (E) and 31-day (F) plants in growth room. Incubation period, 4 hr. Complete system with boiled extract was used as control.

maximum values (Table 1). The reaction course showed a lag during the first 2 hr. In the presence of bovine serum albumin (1 mg/ml), more hydroxamate was formed after 4 hr, but the lag was very severe during the first 3 hr before the reaction rate increased rapidly (Fig. 1).

Despite the reaction course, the amount of hydroxamate formed in 4 hr incubation was directly proportional to the amount of extract used over a five-fold range, and increased with the concentration of cinnamic acid at least up to 4·7 mM. It was also proportional to hydroxylamine concentration up to 0·26 M (Fig. 2), but for normal assay procedure, 0·1 M hydroxylamine was used. The incorporation of label from cinnamic acid into its hydroxamate under these conditions was severely inhibited by an equal concentration of *p*-coumaric acid, but the same concentration of sinapic acid did not affect the reaction, while it was stimulated by both caffeic and ferulic acids (Table 2).

The activity of extracts stored overnight in the presence of 0·02 M ascorbate in the refrigerator fell by 50 per cent in 24 hr, and by 75 per cent in 48 hr. The inactivation was accelerated with bovine serum albumin, but the addition of 1% mercaptoethanol to a fresh preparation stabilized its activity without loss for at least 1 week.

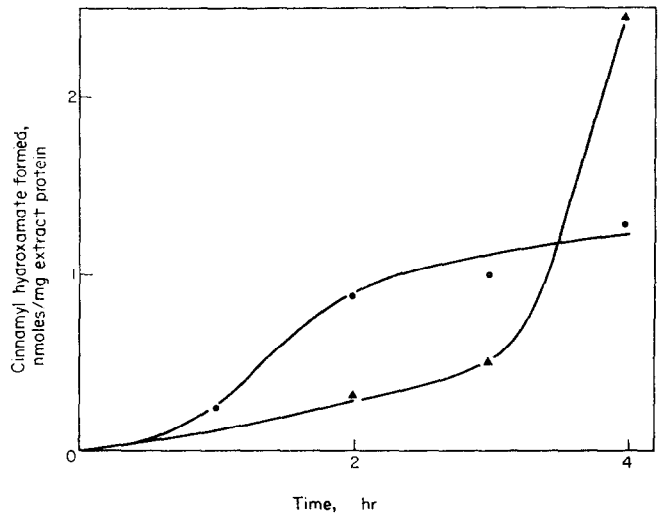

FIG. 1. TIME COURSE OF CINNAMYL HYDROXAMATE FORMATION IN THE ABSENCE (●) AND PRESENCE (▲) OF 250 μg BOVINE SERUM ALBUMIN.
Reaction conditions in Experimental, using extracts from leaves of 20-day plants. System without ATP and CoA used as control.

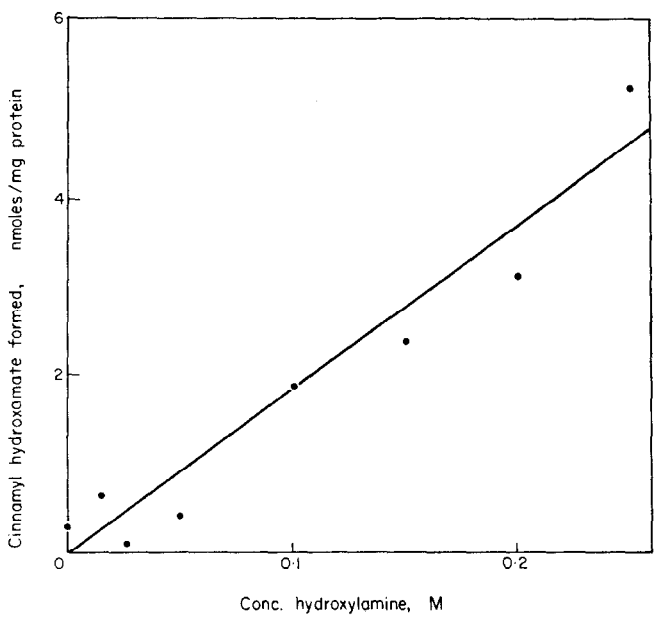

FIG. 2. RELATIONSHIP BETWEEN CINNAMYL HYDROXAMATE FORMATION AND HYDROXYLAMINE CONCENTRATION.
Reaction conditions in Experimental, with 250 μg bovine serum albumin added. Incubation period, 4 hr. System without ATP and CoA used as control.

TABLE 2. EFFECT OF SUBSTITUTED CINNAMIC ACIDS ON CINNAMYL HYDROXAMATE FORMATION

Additions	Cinnamyl hydroxamate formed (nmoles)	% Control
None	1·39	100
p-Coumaric acid	0·62	55
Caffeic acid	1·68	121
Ferulic acid	1·98	143
Sinapic acid	1·48	105

Reaction conditions in Experimental, using 24 hr-stored extract from leaves of 31-day plants, in presence of 250 μg bovine serum albumin. 0·119 μmoles substituted cinnamic acid added. Incubation period, 4 hr. System without ATP and CoA used as control.

Demonstration of [^{32}P]-*Pyrophosphate Incorporation*

Extracts, similar to those which effected the formation of cinnamyl hydroxamate, were found to promote an incorporation of isotope from [^{32}P]-pyrophosphate into nucleotide phosphate under an atmosphere of nitrogen. Some incorporation occurred without cinnamate, but this was clearly stimulated by its addition (Table 3). It was further increased by

TABLE 3. CHARACTERISTICS OF PYROPHOSPHATE EXCHANGE PROMOTED BY CINNAMIC ACID

Incubation period (hr)	[^{32}P] ATP formed (μmoles)			
	1	2	3	4
Reaction system	0·17	0·20	0·14	0·10
Reaction system + CoA	0	0	0	0
Reaction system + KF	0·10	0·18	0·27	0
Reaction system + KF + CoA	0	0	0	0

Enzyme extracts were prepared from 10 g leaf material. Reaction conditions in Experimental, adding 0·15 μmoles CoA and 10 μmoles KF where indicated. System without cinnamic acid used as control.

the addition of fluoride, but both in the presence and absence of fluoride, CoA completely abolished the cinnamate-dependent incorporation.

This method could not be applied to the assay of enzymic activity, because it frequently failed to give measurable activities. In these cases, the activation measured could not be stimulated by the addition of thiol reagents to the extract, by variation of the reagent concentrations, nor by altering the pH of the reaction mixture.

Spectrophotometric Estimation of Cinnamyl-CoA Formation

A direct assay of cinnamyl-CoA synthetase is afforded by measurement of the characteristic extinction of cinnamyl-CoA at 311 nm.[5] Extracts active in the formation of cinnamyl

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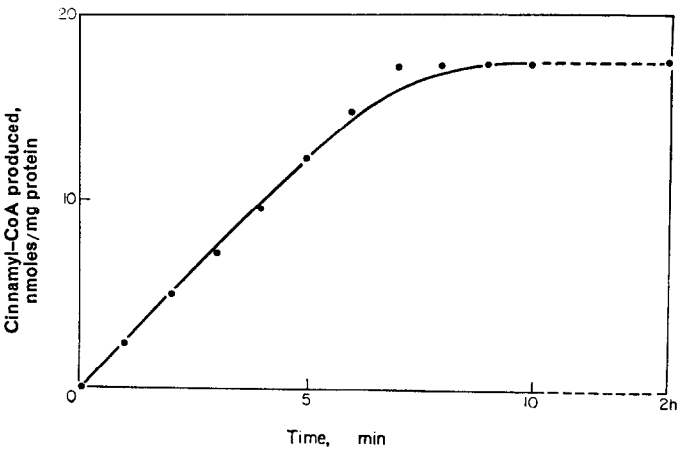

FIG. 3. TIME COURSE OF CINNAMYL-CoA FORMATION MEASURED SPECTROPHOTOMETRICALLY. Reaction conditions in Experimental, using extracts from leaves of 35-day plants. System without cinnamic acid used as control.

hydroxamate were active in this assay, which was rapid and showed a linear time-course for up to 10 min (Fig. 3). The reaction rate increased with cinnamate concentration more or less linearly up to 3·5 mM cinnamate (Fig. 4).

The pH-activity relationship for the enzyme (Fig. 5) showed a broad maximum between 7·5 and 8·0, and activity fell sharply at pH values below 7·5. Other methods of assay which have been employed gave more complex pH relationships.

Enzyme preparations active in the formation of cinnamyl-CoA were inactive towards *p*-coumaric, caffeic and ferulic acids under the conditions detailed in Experimental. Absorbance by the leaf extract did not allow a useful spectral identification of the cinnamyl-CoA ester.

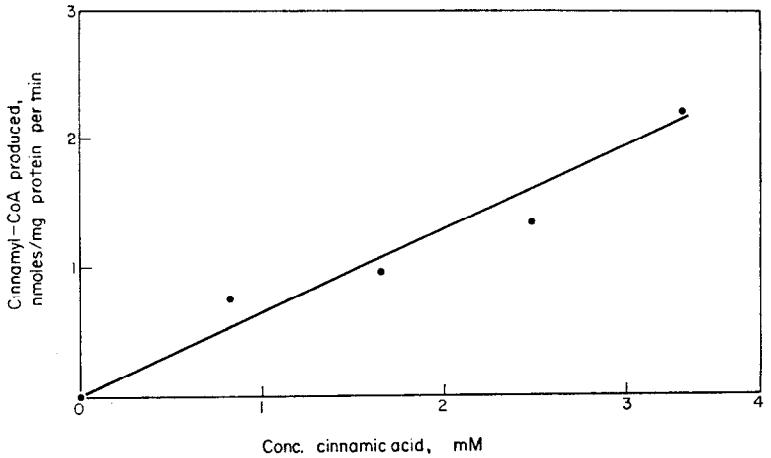

FIG. 4. RELATIONSHIP BETWEEN RATE OF CINNAMYL-CoA FORMATION AND CINNAMIC ACID CONCENTRATION.
The initial rate was measured (Fig. 3) using a 24-hr-stored extract from leaves of 39-day plants.

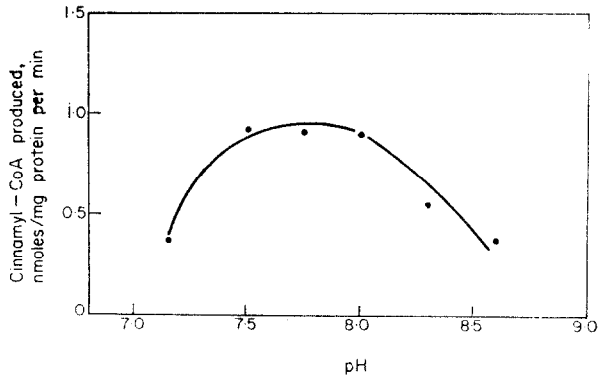

FIG. 5. pH-DEPENDENCE OF CINNAMYL-CoA FORMATION.
The initial rate (Fig. 3) was measured in tris HCl buffers, using an extract from leaves of 31-day plants, which had been stored in the refrigerator for 6 days in 1% 2-mercaptoethanol.

This assay method was satisfactory with many preparations but was more sensitive to extraction and storage conditions, so that extracts active in the formation of [^{14}C] cinnamyl hydroxamate sometimes showed surprisingly weak activity measured spectrophotometrically.

Cinnamyl-CoA Synthetase Activity in Various Plant Extracts

The activity of extracts from leaves of spinach beet seedlings, measured by the formation of [^{14}C] cinnamyl hydroxamate in nitrogen, showed marked changes as the seedlings developed (Fig. 6). These changes appeared to be inversely related to the quantity of red pigments, presumably betalains, in the leaves. The coloration was measured only in arbitrary units because its estimation in extracts was made difficult by the variable spectral characteristics of the preparations, while the pigment was also unevenly distributed in leaves at different development stages.

The coloration was first observed only on the under side of the young leaves. It reached a maximum after about 8 days, but seedlings grown in the more intense light of the growth cabinet were much more heavily pigmented. Subsequently, the colour subsided until none

TABLE 4. ACTIVITIES OF LEAF EXTRACTS FROM VARIOUS PLANTS

	Age of plant (days)	Cinnamyl hydroxamate formed (nmoles/mg protein)
Pea*	20	2·25
Pea	25	0·42
Runner bean	18	3·00
Spinach	24	0·22
Spinach beet	31	0·70
Cabbage	23	0

* All plants were from the growth room, excepting pea plants, which were grown in the greenhouse under tungsten lamps. Reaction conditions as Table 1.

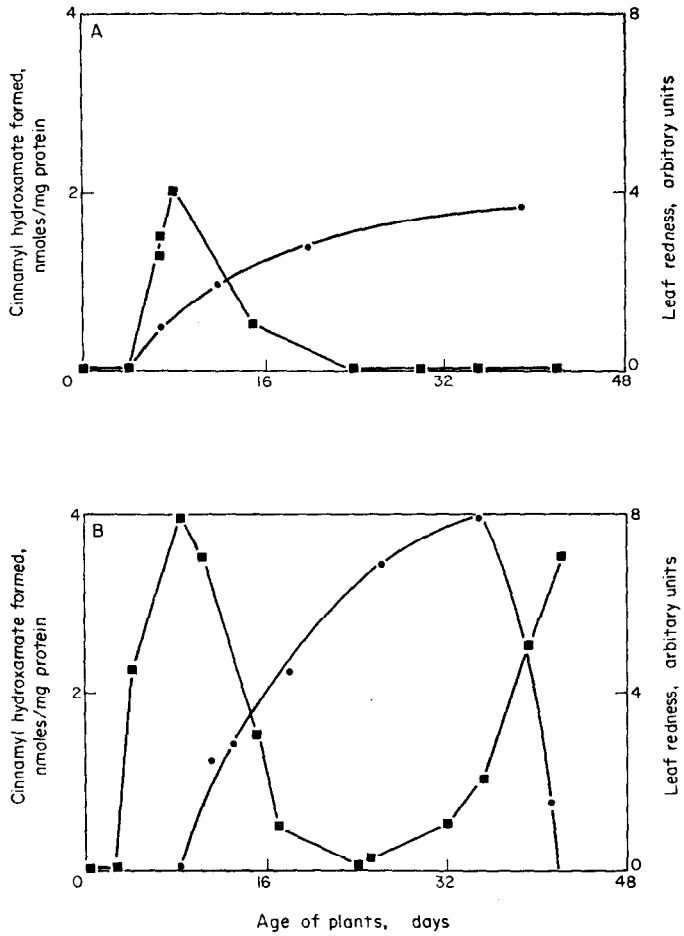

FIG. 6. CHANGE IN CINNAMYL HYDROXAMATE FORMATION WITH AGE AND COLOUR OF SPINACH BEET LEAVES.

Reaction conditions as in Fig. 1, with addition of 250 μg bovine serum albumin. Complete system with boiled extract was used as control. Incubation period, 4 hr. Leaves from seedlings in (A) growth room, (B) growth cabinet. Leaf colour (■); cinnamyl hydroxamate formed (●).

could be observed in 24-day plants, whether grown in the cabinet or growth room. However, in the cabinet, the pigment again developed almost at once; both surfaces of the leaves were affected as it spread from the edges (perhaps due to dehydration) across the leaves.

The activity of the cinnamyl-CoA synthetase developed as the red coloration disappeared, to reach a maximum 35 day after planting the seed. The enzyme was much more active from plants in the growth cabinet, but disappeared as the coloration again developed. By contrast, the lower activity in plants from the growth room was subsequently maintained, while red pigments were not again produced.

The leaves of young pea, runner bean and cabbage plants were examined for activity (Table 4). High activities were obtained from pea and bean leaves, but the considerable difference in the activities of extracts from pea plants grown under different illumination conditions further emphasizes the effect of growth conditions on enzyme activity.

DISCUSSION

Collectively, studies on the formation of cinnamyl hydroxamate, cinnamyl-CoA synthesis and pyrophosphate incorporation establish cinnamyl-CoA synthetase activity in the extracts from leaves of spinach beet. Cinnamyl hydroxamate was usually formed only when CoA was included in the reaction mixture, although significant amounts were sometimes found when only ATP had been added. The hydroxamate can be formed by the reaction of hydroxylamine with either the adenylate intermediate or the CoA ester, and the activity of the extract or the conditions used may have allowed appreciable reaction with the former. The marked stimulation with CoA, however, suggests that hydroxylamine reacted preferentially with the CoA ester. The inhibition of pyrophosphate exchange by CoA further indicates that the adenylate readily reacts with CoA.

Despite the general similarity of the assay methods in indicating the CoA-dependence, substrate specificity and distribution of the activating enzyme, important differences exist between them. Most important of these are the different time courses of hydroxamate and cinnamyl-CoA formation and [^{32}P] pyrophosphate exchange, the different activities with these assays when measured with the same extract, and the different pH values used to obtain optimum rates.

The rapid linear time course for cinnamyl-CoA synthesis probably represents the normal progress of the reaction. Hydroxylamine appeared to induce a severe lag during the first 2 hr, and this method measured less activation than any other. This may have been due to a direct inhibition of the enzyme by hydroxylamine which may be partially relieved by its utilization or destruction in the crude preparation, to the ineffectiveness of hydroxylamine as an agent for detecting activated groups,[6,7] or to a competition between pyrophosphate and hydroxylamine for the adenylate intermediate.[6,8] A similar time course for hydroxamate formation was observed when the reaction was followed spectrophotometrically by forming the ferric complex of the hydroxamate.[3] The slow development of the pyrophosphate incorporation might be due to the gradual release of substrate bound to the extracted enzyme[9] (cf. amino-acid activating enzymes)[10] or to the necessity for CoA for the full development of enzyme activity (cf. the requirement for tRNA in amino-acid activation).[7,11]

The different pH optima measured for hydroxamate and cinnamyl-CoA formation are probably due to the operation of two additional factors in the former method: the formation of hydroxamate at the adenylate and CoA ester stages may have different pH-relationships,[12] and the reactivity and stability of hydroxylamine may itself be affected by pH. The pH-optimum measured by the spectrophotometric assay for cinnamyl-CoA is probably the true value for the overall reaction. The crudeness of the preparations, the complexity of the enzymic reaction and the different principles on which the methods are based are probably together responsible for the variations found.

The activation system seems virtually specific for cinnamic acid. No formation of the CoA esters of *p*-coumaric, caffeic, ferulic or sinapic acids could be observed, although the inaccuracy of the spectrophotometric method for these acids may have disguised this. Only *p*-coumaric acid interfered with the formation of [^{14}C] cinnamyl hydroxamate, but this may

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be no more than enzyme inhibition. Although they can be used in the biogenesis of flavonoids,[13] the activation of these substituted cinnamic acids may be exceptional, for there is evidence that their interconversion may take place in bound[9] or esterified[14] forms. At present, however, it is only supposition that these forms are derived from CoA ester precursors.

The control of the levels of enzymes concerned with the synthesis of plant phenolics, especially phenylalanine ammonia-lyase, by light and other environmental conditions has been studied extensively.[15–17] With the limited evidence described here, it is not possible to correlate the activity of cinnamyl-CoA synthetase with the synthesis of flavonoids, but its control by cultural conditions conforms with these earlier studies. Some early experiments suggest a role for acetate activation in the control of flavonoid synthesis;[18,19] this is no longer accepted, but the activation of cinnamic acid and its hydroxylated derivatives necessarily follows the phenylalanine ammonia-lyase reaction. The production of betalains appears to correlate with a low level of enzyme activity for the activation of cinnamate, and suggests they are synthesized by a pathway diverging at an early stage from the conventional pathway for plant phenolics.

EXPERIMENTAL

Materials

Seeds of spinach beet (*Beta vulgaris*), spinach (*Spinacia oleracea*), runner bean (*Phaseolus multiflorus*, var Prizewinner), pea (*Pisum sativum*, var. Alaska) and cabbage (*Brassica oleracea*, var Primo) were germinated in sterilized or dressed soil, and the seedlings grown at 25° under a 16 hr light–8 hr dark regime either in a large growth room or a small growth cabinet. The growth room was illuminated with 'daylight' fluorescent tubes, giving a white light of average intensity of 11,840 lx at soil level; the air circulation was poor and the atmosphere humid. In the growth cabinet, illumination was provided by 'natural' fluorescent tubes, which gave a higher proportion of red light and an average intensity of 15,070 lx at soil level; the atmosphere was dry due to the continual passage of a rapid air-stream.

Chemicals

When possible, all chemicals were purchased as analytical grade from commercial sources. Polyvinylpyrrolidone was obtained as its insoluble form, Polyclar AT, and washed by the procedure of Loomis and Battaile.[20] Cinnamic acid was purchased as microanalytical reagent and used without further purification. *p*-Coumaric, caffeic, ferulic and sinapic acids were each recrystallized from 50% aq.-ethanol until colourless. Cinnamyl hydroxamate was prepared from ethyl cinnamate by the method of Gross and Zenk,[5] and purified by repeated recrystallization from chloroform. [2-^{14}C] Cinnamic acid was obtained from Tracerlab Division Laboratory for Electronics Inc., Waltham, Mass., U.S.A. and [^{32}P] pyrophosphate as its tetrasodium salt from the Radiochemical Centre, Amersham.

Preparation of Extracts

15 g washed young leaves, from which the midribs and main veins had been removed, were macerated in a suspension of Polyclar AT (10 g) in 60 ml KH_2PO_4–KOH buffer (0·1M, pH 8·0), which also contained 0·25 M sucrose, 1 mM EDTA and 0·02 M ascorbate, at 0°. The macerate was stirred for a few min and then filtered through muslin. After the pH had been adjusted to 8·0, the filtrate was centrifuged at 28,000 *g* for 15 min at 0°. The pale green supernatant, which had low ATP-ase activity, contained 3–7 mg protein/ml.

The preparations were further purified by passage through a column of Sephadex G–25, which was equilibrated and then eluted with KH_2PO_4–KOH buffer (0·1 M; pH 8·0, containing 0·02 M ascorbate). In

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some experiments, the preparations were first fractionated with $(NH_4)_2 SO_4$ into fractions corresponding with 0–25%, 25–50% and 50–80% saturation, which were suspended in the elution buffer and passed through columns of Sephadex G-25.

Protein Estimation

The protein content of extracts was determined by the Folin-Ciocalteau method, modified by Bailey,[21] using bovine serum albumin as standard.

Assay of preparations for Cinnamate Activation

(a) *Formation of* [^{14}C] *cinnamyl hydroxamate.* Preparations were incubated in sealed plastic Eppendorf tubes in N_2, shaken in a water-bath at 30°. Each tube contained 13 μmoles tris HCl buffer (pH 8·5), 2·7 μmoles ascorbate, 1·3 μmoles $MgCl_2$, 20 μmoles hydroxylamine, 0·5 μmole 2-mercaptoethanol, 0·5 μmole ATP, 0·03 μmole CoA, 0·1185 μmole [2-^{14}C] cinnamic acid (containing 0·2 μC) and the enzyme preparation (70 μl, suspended in 0·1 M KH_2PO_4–KOH buffer, pH 8·0). Total vol. 0·2 ml, with final pH 7·8. With each batch of samples, control tubes, from which ATP and CoA had been omitted, or which contained a boiled extract, were included. Hydroxylamine was prepared freshly by neutralizing its hydrochloride with KOH,[22] and the cinnamic acid was dissolved in 1% $KHCO_3$.

After incubation, the reaction was terminated by adding 0·2 ml of ethanol. 50 μl of this solution together with 50 nmoles each of unlabelled cinnamic acid and cinnamyl hydroxamate were spotted on DEAE-cellulose paper, and developed by descending chromatography using 0·001 M NaCl as solvent. After 21 hr, cinnamic acid had moved about 2 cm from the origin, while the hydroxamate had moved 6 cm. The spots were distinguished by spraying the paper with an ethanolic $FeCl_3$, but were normally detected by u.v. absorbance. The hydroxamate spot was cut out, immersed in 4 ml CIBA-1 scintillation fluid and counted in a Beckmann LS-200B scintillation counter. The radioactivity of cinnamic acid in a 50 μl sample taken before incubation was similarly measured after spotting onto a circle of DEAE–cellulose paper.

(b) *Spectrophotometric estimation of cinnamyl-CoA.*[5] Two cuvettes were prepared each containing 130 μmoles tris-HCl buffer (pH 8·5), 20 μmoles ascorbate, 5 μmoles $MgCl_2$, 5 μmoles 2-mercaptoethanol, 5 μmoles ATP, 0·3 μmoles CoA and the enzyme preparation suspended in 0·1 M KH_2PO_4–KOH buffer (pH 8·0). Total volume, 2·0 ml, with final pH 8·5. The cuvettes were flushed with N_2 and stoppered. The reaction was followed in a recording spectrophotometer after addition of 5 μmoles cinnamic acid (dissolved in 1% $KHCO_3$) to one cuvette, the increase in extinction being measured at 311 nm relative to that in the second cuvette.

When measuring the activation of substituted cinnamic acids, it was necessary to compare cuvettes with controls from which CoA had been omitted. Both cuvettes were required to contain the cinnamic acid under test, since it absorbed appreciably at the λ_{max} of its CoA ester. The cinnamic acid was added to each cuvette after any CoA-dependent extinction in the test cuvette had ceased to increase. Either method was suitable for the unsubstituted cinnamic acid, but the first was preferred because high concentrations of the cinnamic acid in both cuvettes reduced the accuracy of measurements. The amounts of cinnamyl-CoA formed were calculated from the extinction coefficients given by Gross and Zenk.[5]

Incorporation of [^{32}P] *Pyrophosphate*[23,24]

Incubations were carried out in sealed flasks, adapted for sampling in N_2, which were shaken in a water-bath at 30° for 4 hr. Each flask contained 55 μmoles tris HCl buffer (pH 8·5), 10 μmoles ascorbate, 2·5 μmoles $MgCl_2$, 2·5 μmoles 2-mercaptoethanol, 2·5 μmoles ATP, 2·5 μmoles cinnamic acid, 2·5 μmoles pyrophosphate (containing 0·83 μC ^{32}P) and 0·33 ml enzyme preparation in 0·1 M KH_2PO_4–KOH buffer, pH 8·0. Total vol. 1·0 ml. Flasks were also prepared (i) without cinnamic acid, (ii) with addition of 10 μmoles KF, (iii) with addition of 0·15 μmole CoA and (iv) with addition of 10 μmoles KF and 0·15 μmole CoA.

1·0 ml samples were transferred at intervals to 0·5 ml 12% trichloracetic acid and insoluble material removed by centrifugation. 50 mg activated charcoal, suspended in 1 ml 0·1 M pyrophosphate (adjusted to pH 8·0 with H_3PO_4), was added to the supernatant. The mixture was shaken at intervals for 1 hr and then allowed to stand overnight. The charcoal was filtered on filter-paper discs, washed with 0·1 M pyrophosphate buffer (pH 8·0) and dried slowly. The radioactivity of the charcoal disc was measured using an end-window G.M. tube.

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