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THE CONVERSION BY PHENOLASE OF *p*-COUMARIC ACID TO CAFFEIC ACID WITH SPECIAL REFERENCE TO THE ROLE OF ASCORBIC ACID*

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Abstract—Caffeic acid was isolated as a product of *p*-coumaric acid oxidation catalysed by phenolase. Time course of *p*-coumaric acid conversion was studied in the absence and presence of ascorbic acid, and caffeic acid accumulation was found to be correlated with *p*-coumaric acid disappearance in the ascorbic acid-containing system. In the above reaction, *p*-coumaric acid undergoes a coupled oxidation with ascorbic acid as follows: *p*-coumaric acid + ascorbic acid + O_2 → caffeic acid. The effect on the initial reaction stage (lag phase) of concentrations of enzyme, monohydric phenol and ascorbic acid was studied and favourable conditions for obtaining caffeic acid in quantity are discussed.

INTRODUCTION

PHENOLASE (*o*-diphenol: O_2 oxidoreductase) is known to oxidize monohydric phenols to the corresponding *o*-dihydric phenols and this oxidation has been studied with various monohydric phenols, especially with tyrosine.[1–11] However, the possible conversion by this enzyme of *p*-coumaric acid, a monohydric phenol similar to tyrosine, to caffeic acid has by no means been studied, although these phenolic acids are regarded as important intermediates of plant secondary metabolism.[12]

In a previous paper, the present author reported that the chloroplasts of some plants are able to oxidize *p*-coumaric acid to caffeic acid by an enzyme of the phenolase type.[13] The present paper describes the isolation of caffeic acid as a product of this oxidation from an ascorbic acid-containing reaction system and the role of ascorbic acid in this reaction.

RESULTS

Isolation of Caffeic Acid as an Oxidation Product of p-*Coumaric Acid*

From a reaction mixture consisting of *p*-coumaric acid, ascorbic acid and mushroom phenolase, caffeic acid was isolated as a product of *p*-coumaric acid oxidation catalysed by

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[10] S. OSAKI, *Acta Biophys. Biochim.* **100**, 378 (1963).
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phenolase. Its u.v. and i.r. spectra were found to be identical with those of authentic caffeic acid.

Time Course of p-*Coumaric Acid Conversion*

Chromatograms of the time course of the substances derived from *p*-coumaric acid by the action of phenolase in the presence and absence of ascorbic acid are shown in Fig. 1. Using radioactive *p*-coumaric acid, these substances were further estimated (Fig. 2), and the oxygen uptake in the above reactions was also measured (Fig. 3).

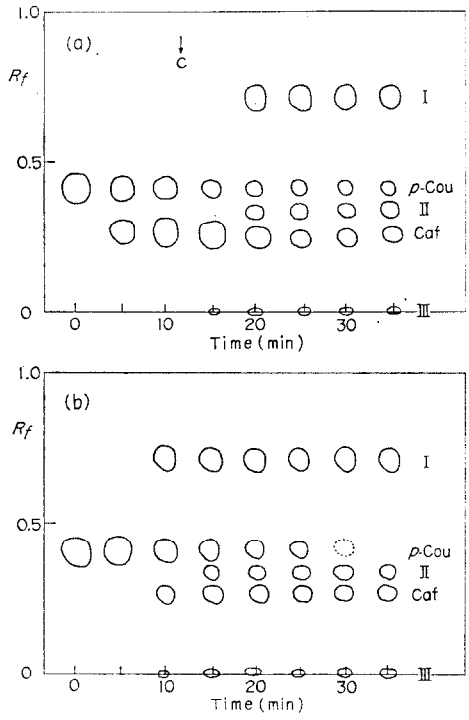


FIG. 1. CHROMATOGRAMS SHOWING THE TIME COURSE OF *p*-COUMARIC ACID OXIDATION CATALYSED BY PHENOLASE IN THE PRESENCE (*a*) AND ABSENCE (*b*) OF ASCORBIC ACID.

Standard reaction mixture was used. Point C represents the time of the coloration. Chromatograms were developed in 5% acetic acid and then viewed in u.v. light.

The early stage of the reaction in the absence of ascorbic acid was characterized by the presence of a lag phase before the decrease of *p*-coumaric acid or oxygen uptake started, and this lag was eliminated by adding ascorbic acid to the reaction system. In the absence of ascorbic acid, *p*-coumaric acid decreased continuously following the lag phase, but its oxidation product, caffeic acid, was scarcely detectable in the reaction mixture. On the other hand, when ascorbic acid was present, the formation of the *o*-dihydric phenol was correlated with the decrease of the monohydric phenol so long as the reductant existed (the point C in Figs. 1 and 2 represents the time of coloration, i.e. the time of complete utilization of the reductant). In the absence of ascorbic acid, the rate of *p*-coumaric acid decrease was approximately constant for several minutes after the lag phase, while in the presence of this reductant a gradual decrease of this rate was observed. This may be accounted for by a fact that the

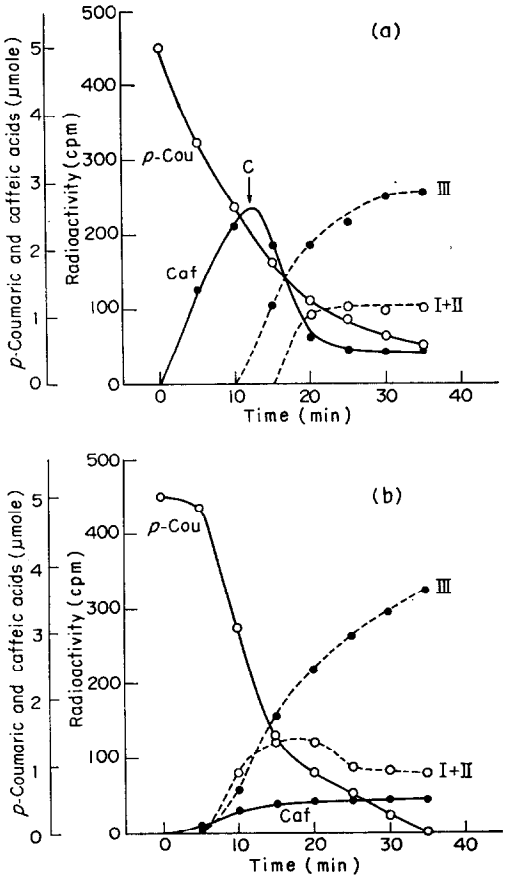


FIG. 2. CHANGING PATTERN OF RADIOACTIVITY OF *p*-COUMARIC ACID IN THE REACTIONS IN FIG. 1.

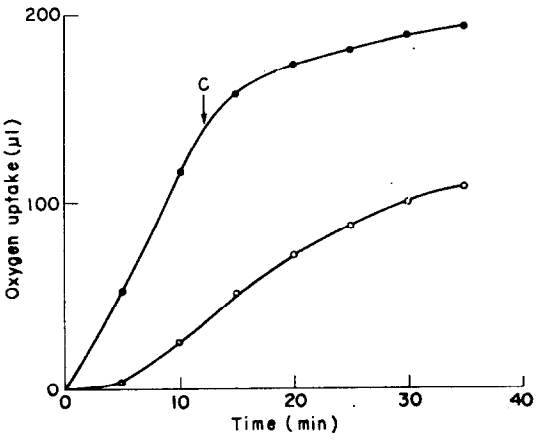


FIG. 3. OXYGEN UPTAKE IN THE REACTIONS IN FIG. 1.

accumulation of caffeic acid in the reaction mixture in turn inhibited its further formation (Table 1).

TABLE 1. EFFECT OF CAFFEIC ACID ON THE *p*-COUMARIC ACID CONVERSION

Caffeic acid added (μmoles)	*p*-Coumaric acid converted (μmoles)
0·00	2·50
0·63	2·16
1·25	1·88
1·88	1·60
2·50	1·52

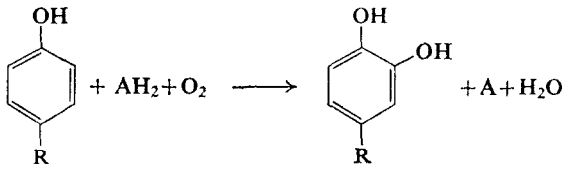
To 1 ml of the standard reaction mixture were added varied amounts of caffeic acid, and the reaction mixture was incubated for 8 min (no coloration took place within this period).

As mentioned above, the amount of caffeic acid in the absence of ascorbic acid remained at a low level. In the system to which ascorbic acid was added, the accumulation of caffeic acid ceased just at the point C, and the product decreased first rapidly and then gradually. The decrease of caffeic acid can be ascribed to its being further oxidized into a series of secondary products (I, II and III), and this seemed to proceed via the *o*-quinone, because no secondary products could be found in the presence of ascorbic acid. Substances I and II fluoresced green and the substance(s) III yellow under u.v. lamp, but their chemical nature remains unknown.

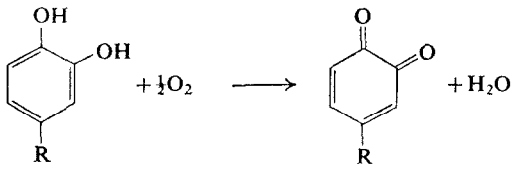
Coupled Oxidation of p-*Coumaric Acid with Ascorbic Acid*

As can be seen in Fig. 3, the oxygen consumption was distinctly higher in the presence of ascorbic acid than in its absence, and this suggested that oxygen might be consumed also for oxidizing ascorbic acid. From Figs. 2 and 3, it can be approximately estimated that 6·2 μmoles (139 μl) of oxygen and 2·4 μmoles of *p*-coumaric acid are transformed until point C, where the originally supplied ascorbic acid (10 μmoles) is all consumed, i.e. the sum of *p*-coumaric acid (x) and ascorbic acid (a) corresponds to two times oxygen (y).

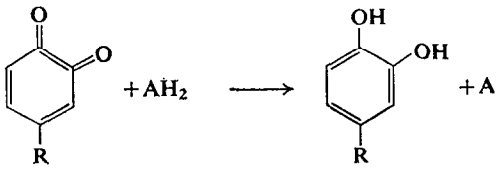
This may be explained by assuming that only the following reactions are involved so long as ascorbic acid is present in the system:



(1)



(2)



(3)

According to this scheme, where AH_2, A and R represent ascorbic acid, dehydroascorbic acid and residue —CH=CH—COOH, respectively, the amounts (on molar basis) of oxygen, ascorbic acid and *p*-coumaric acid which are transformed in step 1 are the same. If the amount of *p*-coumaric acid is experimentally determined (x), then the amounts of oxygen and ascorbic acid which are used in the above step can be calculated. The rest of ascorbic acid ($a-x$) is expected to be used for the reduction of *o*-quinone in step 3. Since *o*-quinone formed in step 2 is almost instantaneously reduced and no quinone is present in the reaction system,[14] one molecule of ascorbic acid used in step 3 should correspond to one-half molecule of oxygen consumed in step 2 irrespective of the amount of accumulated caffeic acid, namely the amount of oxygen used in step 2 is equivalent to $(a-x)/2$. The total oxygen consumed in steps 1 and 2 will therefore be $x+(a-x)/2$ or $2y=x+a$. Under the conditions where the amount of ascorbic acid is varied, this equation is certainly followed (Table 2).

The Effect of Various Factors on the Lag Phase

This was studied for the purpose of finding favourable reaction conditions for obtaining caffeic acid.

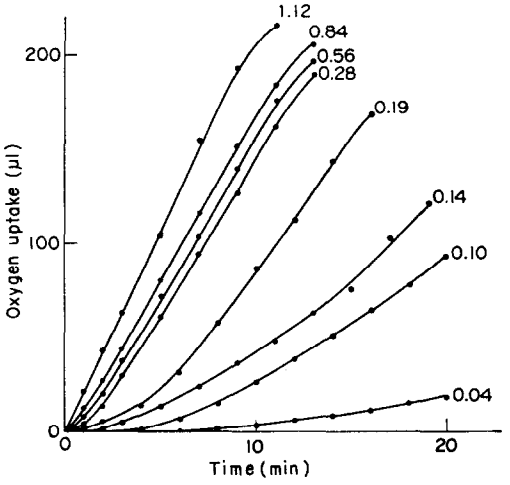


FIG. 4. EFFECT OF ENZYME CONCENTRATIONS ON TIME LAG.

Reaction mixture contained for each 1 ml 10 µmoles of *p*-coumaric acid, standard amount of ascorbic acid and buffer, and varied amounts of enzyme as illustrated.

Enzyme concentration. The results in Fig. 4 clearly show that the time lag was shortened with increase of enzyme concentration (this experiment was carried out at a substrate concentration 10^{-2} M where the lag is conspicuous, see below).

[14] W. H. MILLER and C. R. DAWSON, *J. Am. Chem. Soc.* **63**, 3375 (1941).

Monohydric phenol concentration. From Fig. 5, it seems that the higher the concentration of monohydric phenol, the more the time lag is extended, although at lower concentration only a decrease of the initial rate can be observed.

Ascorbic acid concentration. The lag-eliminating action of ascorbic acid was noticed at concentrations over 5×10^{-4} M (Fig. 6); this ability, however, is dependent upon the con-

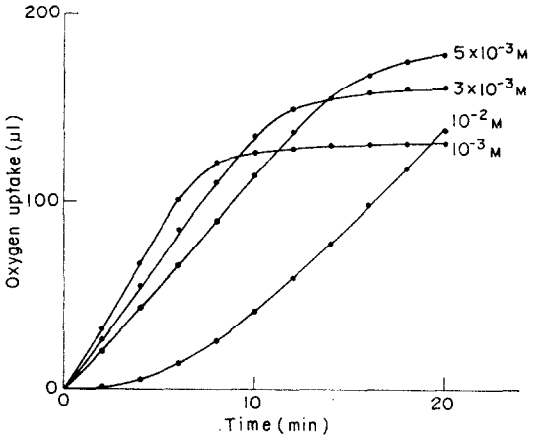


FIG. 5. EFFECT ON TIME LAG OF *p*-COUMARIC ACID CONCENTRATION.
Standard reaction mixture was used except the concentration of the monohydric phenol was varied.

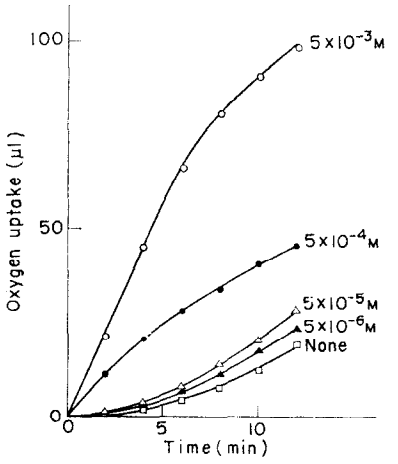


FIG. 6. EFFECT ON TIME LAG OF ASCORBIC ACID CONCENTRATION.
Standard reaction mixture was employed except the concentration of ascorbic acid was varied.

centration of monohydric phenol. As can be seen from Fig. 7, when the concentration of *p*-coumaric acid is 10^{-3} and 3×10^{-3} M, no lag can be found at any ascorbic acid concentrations, but at a higher substrate concentration (10^{-2} M) ascorbic acid tends to prolong the lag with increase of its concentration.

The role of caffeic acid. Since it has been reported that a trace amount of catechol-type substance formed exponentially in the early stage of the reaction should play a catalytic role

in the oxidation of cresol-type compound and thus eliminate the lag,[5–6, 11, 15] the effect of caffeic acid on the lag of *p*-coumaric acid oxidation was further examined. From the results of Fig. 8, where the oxygen uptake in the reaction systems containing caffeic acid instead of ascorbic acid was measured, it appeared that caffeic acid was effective in abolishing the lag. It is doubtful, however, whether the oxygen was indeed consumed for oxidizing *p*-coumaric acid itself, since caffeic acid produced also served as substrate for phenolase to cause further

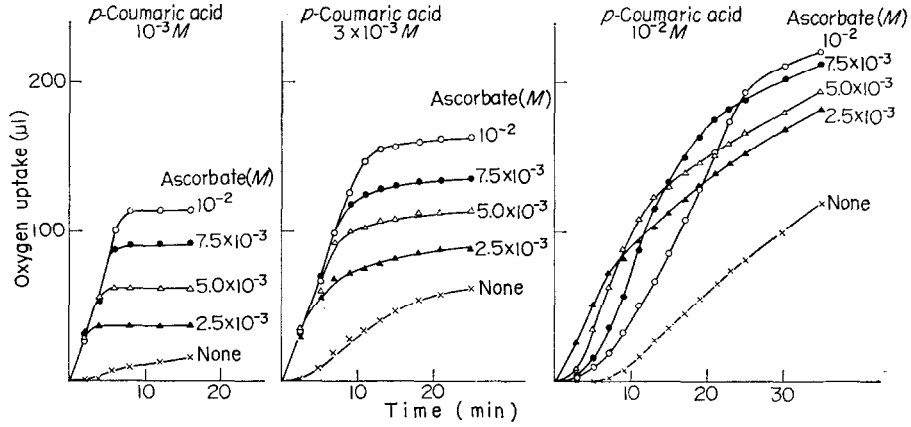


FIG. 7. EFFECT ON TIME LAG OF HIGH CONCENTRATIONS OF ASCORBIC ACID AT DIFFERENT CONCENTRATIONS OF *p*-COUMARIC ACID.

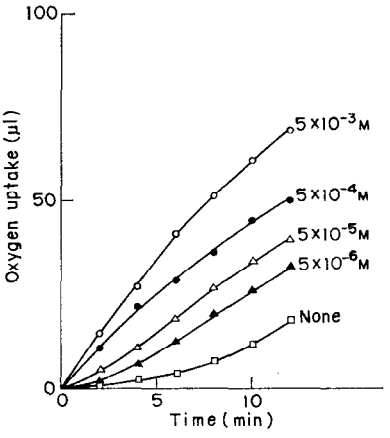


FIG. 8. EFFECT OF CAFFEIC ACID ON THE OXYGEN CONSUMPTION.
Ascorbic acid in the standard reaction mixture was replaced by varied amounts of caffeic acid as illustrated.

oxygen uptake. In order to clarify this point, the net disappearance of *p*-coumaric acid, not the consumption of oxygen, was examined in the reaction mixtures containing a large or a trace amount of caffeic acid. It can be seen from Fig. 9 that the time course of the monohydric phenol conversion does not differ from that in the control system (phenolase-*p*-coumaric acid system), although the oxygen consumption was observed to start immediately after the reaction began.

[15] J. NEUMANN, G. LEGRAND, G. LEHONGRE and J. LAVOLLAY, *Compt. Rend. Acad. Sci.* **256**, 309 (1963).

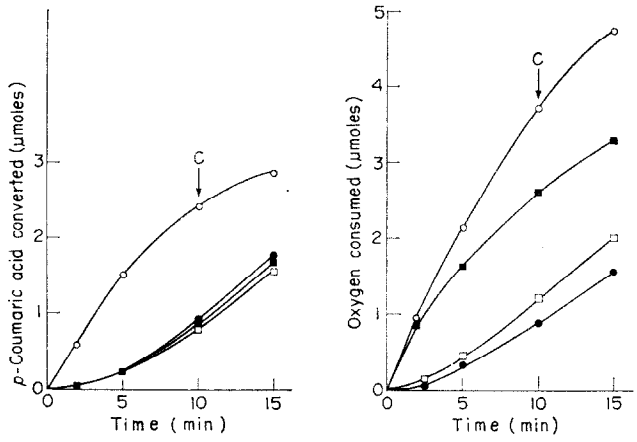


FIG. 9. CONVERSION OF *p*-COUMARIC ACID AND OXYGEN IN THE REACTION MIXTURES CONTAINING FOR EACH 1 ml, BESIDES STANDARD AMOUNTS OF *p*-COUMARIC ACID AND ENZYME, 5 μmoles OF ASCORBIC ACID (—○—), 5 μmoles OF CAFFEIC ACID (—■—), 5 mμmoles OF CAFFEIC ACID (—□—), OR NEITHER ACID (—●—).

DISCUSSION

From the results shown above, it is clear that phenolase is able to convert *p*-coumaric acid to caffeic acid. The first products of oxidation of monohydric phenols are now believed to be the corresponding *o*-dihydric phenols, but *o*-dihydric phenols have only been isolated in the cases of oxidation of tyrosine,[1] thyronine[16] and 3,4-dimethylphenol.[17] In the present experiment caffeic acid, a product of *p*-coumaric acid oxidation catalysed by phenolase, was isolated from a reaction mixture which contained a large amount of ascorbic acid.

As shown in Fig. 2, caffeic acid is not accumulated in the absence of ascorbic acid, while in its presence a parallelity is achieved between the caffeic acid accumulation and *p*-coumaric acid disappearance. This can be ascribed to an ability of ascorbic acid to prevent caffeic acid from being further oxidized. Ascorbic acid also takes part in the above reaction as substrate of phenolase. By analysing the amounts of *p*-coumaric acid and oxygen converted until a definite amount of ascorbic acid was used up, it was shown that the oxidation proceeds according to the scheme, whereby one molecule of oxygen is consumed for a coupled oxidation of one molecule each of *p*-coumaric and ascorbic acids. A similar scheme has recently been proposed by Vaughan and Butt[18] who found that one molecule of ascorbic acid is utilized for the hydroxylation of *p*-coumaric acid catalysed by spinach enzyme. It is worth noting that the reaction of this type has been suggested in the studies where monohydric phenols are regarded as being electron donor in the phenolase-catalysed oxidation of ascorbic acid and other reducing substances.[15, 19–22]

Another role of ascorbic acid is in its lag-eliminating action. It has been known that in the oxidation of monohydric phenols a lag phase (induction period) is present before the

[16] S. LISSITZKY, S. BOUCHILLOUX and D. KERTESZ, *Bull. Soc. Chim. Biol.* **38**, 821 (1956).
[17] H. S. MASON, W. L. FAWLKS and E. PETERSON, *J. Am. Chem. Soc.* **77**, 2914 (1955).
[18] P. F. T. VAUGHAN and V. S. BUTT, *Biochem. J.* **104**, 65P (1967).
[19] D. KERTESZ and O. AZZOPARDI, *Bull. Soc. Chim. Biol.* **42**, 945 (1960).
[20] J. NEUMANN, G. LEHONGRE, G. LEGRAND and J. LAVOLLAY, *Compt. Rend. Acad. Sci.* **247**, 1508 (1958).
[21] J. LAVOLLAY, G. LEGRAND, G. LEHONGRE and J. NEUMANN, *Bull. Soc. Chim. Biol.* **44**, 379 (1962).
[22] G. LEGRAND, *Ann. Sci. Université Besançon, Bot.* **2**, 1 (1965).

reaction starts,[2–4] and this can be shortened or removed by some reducing agents, for example, by ascorbic acid.[7–9] An induction period was also observed in *p*-coumaric acid oxidation and this can be eliminated with a comparatively large amount of ascorbic acid. This ability is dependent on the concentration of *p*-coumaric acid; at substrate concentration in the order of 10^{-2} M (Fig. 7), the higher the concentration of ascorbic acid, the more the lag is prolonged, whereas with ascorbic acid concentration less than $2{\cdot}5 \times 10^{-3}$ M the lag is removed. It is therefore advisable to lower the initial concentration of ascorbic acid in a reaction mixture containing a large amount of *p*-coumaric acid, in order to isolate caffeic acid. The lag-prolonging effect of monohydric phenol at higher concentrations has already been described,[7,10] but no studies have been carried out on the relationship between the concentrations of monohydric phenol and ascorbic acid.

EXPERIMENTAL

Preparation of Mushroom Phenolase

One kg of common mushroom (*Agaricus campestris*), purchased in spring and previously frozen at −20°, was homogenized with 5 l. of cold acetone at −20°. The homogenate was filtered and the residue recrushed with 2 l. of cold acetone. The wet cake was homogenized in 1 l. of 0·05 M NaCl and, after standing overnight, the homogenate was centrifuged at 12,000 *g* for 20 min. Extraction was further carried out with 1 l. of 0·1 M NaCl. The combined extracts were made up to 75 per cent saturation with $(NH_4)_2SO_4$, and after 2 hr the precipitate collected by centrifugation. It was dissolved in 20 ml of cold water and 2 ml of 10% calcium acetate were added. The precipitated materials were discarded by centrifugation, and the supernatant was dialysed for 2 days against two changes of 0·005 M phosphate buffer, pH 6·8. After removing insoluble substances by centrifugation, the supernatant was applied to a column of DEAE cellulose (30 × 80 mm) which had been previously equilibrated with 0·01 M of the above buffer. The discharged column was first washed with 250 ml of the same buffer and the enzyme then extracted with 0·1 M phosphate buffer. The colourless extract (120 ml) was concentrated by precipitating the protein with $(NH_4)_2SO_4$, the precipitate (35–75 per cent saturation) collected and dissolved in a minimum volume of water. The enzyme solution was dialysed against 0·005 M of phosphate buffer, pH 6·8, overnight, and the dialysate used as enzyme solution (11 ml). If one unit of enzyme is defined, according to the recommendation of I.U.B., as the amount of enzyme which catalyses the conversion of 1 μmole of *p*-coumaric acid in 1 min under the standard condition, then 1 ml of this enzyme solution contained 14 units of enzyme.

Isolation of Caffeic Acid as a Product of p-*Coumaric Acid Oxidation*

One mmole of *p*-coumaric acid was dissolved in 40 ml of 0·1 M phosphate buffer, pH 6·8, and to this solution 0·5 ml of 0·5 M ascorbic acid and 0·5 ml of enzyme solution were added in this order and the reaction mixture was vigorously stirred. Just as a coloration was observed after 5 min incubation (the colour was first green, then changed rapidly to a yellowish brown via a transient violet), 2 ml of the ascorbic acid solution were rapidly added and in this way 40 ml of this solution were used. After 1·5 hr, 20 ml of 0·1 M HCl were added to stop the reaction. The phenolic acids were extracted with ether (× 5), and the combined extracts concentrated to dryness. On paper chromatography, the residue was found to contain a large amount of caffeic acid and a small amount of unreacted *p*-coumaric acid. To isolate caffeic acid, the alumina method for separating DOPA from tyrosine[23] was modified as follows. The above residue was dissolved in 30 ml of 0·2 M sodium acetate, and to this solution 0·5 ml of 0·2 M EDTA and 5 g of alumina were added. The pH was adjusted to 8·5 by adding 2 M NH_4OH dropwise and the mixture stirred for 5 min. The alumina suspension was transferred to a column (3·5 cm in dia.), and washed with 100 ml of water to remove *p*-coumaric acid. Caffeic acid was then eluted with 30 ml of 0·6 M acetic acid, and the eluate extracted with ether. The extract was evaporated to dryness and taken up in a minimum volume of dilute ethanol. The caffeic acid obtained was recrystallized three times (7 mg); the m.p., 237·5°, was identical with that of the authentic sample.

Condition of the Reaction

The standard reaction mixture contained for each 1 ml 5 μmoles of *p*-coumaric acid, 10 μmoles of ascorbic acid, 40 μmoles of phosphate buffer and 0·224 units of enzyme. This solution was prepared by mixing 2 vols of $1{\cdot}25 \times 10^{-2}$ M *p*-coumaric acid dissolved in 0·1 M phosphate buffer, pH 6·8, 1 vol of 5×10^{-2} M ascorbic acid and 2 vols of enzyme solution (25 times dilution of the original solution). The reaction was run at 30°.

[23] T. NAGATSU, M. LEVITT and S. UDENFRIEND, *J. Biol. Chem.* **239**, 2910 (1964).

Manometric Method

Oxygen uptake was measured by an ordinary manometric method, using 1 ml of the standard solution unless otherwise stated. The enzyme solution in the side-arm and the other components in the flask were preincubated at 30° for 20 min. The flask oscillated 120 times per min. No oxygen consumption was observed with ascorbic acid alone.

Measurement of p-*Coumaric Acid Conversion*

The standard reaction mixture was used except that radioactive *p*-coumaric acid (-2-C^{14}, 0·27 μc per mg) was employed. To stop the reaction, one-fifth volume of 0·1 M HCl was added to the reaction mixture, and an aliquot (10 μl) was chromatographed on Whatman No. 1 paper as a band in 5% acetic acid. The substances were located under u.v. lamp, and pieces of the corresponding band were placed on planchets, the radioactivity being measured directly with a counter (Aloka low-background β-counter). After these procedures, 1 μmole of *p*-coumaric acid corresponded to 90 cpm, and the amount of *p*-coumaric and caffeic acids was calculated from the radioactivity.

Simultaneous Determination of Oxygen Consumption and p-*Coumaric Acid Conversion*

The experiment described in Table 2 was carried out using manometric flasks with two side-arms. In one of them were placed 0·2 ml of 0·1 M HCl and in the other the enzyme solution. After the reaction was started with 1 ml of the standard mixture (ascorbic acid was added in varied amount), the gas change was recorded every minute. Just as the reaction mixture developed a green colour, the time of this point was recorded by a stop-watch and the reaction stopped rapidly (within 5 sec) by transferring HCl in the side-arm to the flask. The final oxygen uptake was obtained by extending the oxygen uptake curve to the time of coloration. The determination of the converted *p*-coumaric acid was carried out as mentioned before.

TABLE 2. THE AMOUNTS OF *p*-COUMARIC ACID AND OXYGEN CONVERTED UNTIL THE ORIGINALLY SUPPLIED ASCORBIC ACID WAS JUST USED UP

Ascorbic acid supplied (a)	*p*-Coumaric acid converted (x)	Oxygen converted (y)	$2y$	$a+x$
4·00	1·78	2·86	5·72	5·78
7·00	2·40	4·66	9·32	9·40
10·00	3·01	6·47	12·94	13·01

The standard reaction mixture was employed except the amount of ascorbic acid was varied. Values are expressed in μmoles per 1 ml of the reaction mixture.

Chemicals

Radioactive *p*-coumaric acid was synthesized according to the literature.[24] Ascorbic acid was freshly recrystallized before use.

Acknowledgement—The author thanks Professor M. Hasegawa of this laboratory for his useful criticism throughout this study.

[24] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **34**, 769 (1956).