A KINETIC STUDY OF THE HYDROXYLATION OF MONOPHENOLS CATALYSED BY SPINACH LEAF PHENOLASE.

by

ROSEMARY J. McINTYRE (née ROBERTS).

Presented for the degree of Doctor of Philosophy

Department of Biochemistry,
University of Glasgow.

August, 1973.

ACKNOWLEDGEMENTS.

I would like to thank all those who have helped me in the course of my postgraduate work, especially

the late Professor J.N. Davidson and Professor R.M.S. Smellie for their encouragement and provision of facilities,

my immediate supervisor Dr. P.F.T. Vaughan for his helpful enthusiasm throughout the course of this work,

Dr. J.G. Lindsay for his help with carbon monoxide studies and Mr. A. Wilson for technical assistance,

the S.R.C. for granting me a studentship during the course of this work,

my husband and family for their continued support and encouragement,

Mrs. Jean Roberts for typing this manuscript.

I N D E X.

Section		Page No.
I	Introduction	1
1.2.	Phenolase	2
1.3.	Role of phenolase *in vivo*	6
1.4.	Enzyme kinetics	10
1.4.4.	Three substrate reactions	17
1.5.	Inhibitor studies	20
1.7.	Estimation of enzyme activity	23
II	Materials and methods	25
2.2.1.	Enzyme preparation	30
2.2.4.	Determination of caffeic acid	35
2.2.5.	Assay conditions	46
III	Results	51
3.1.	Preliminary studies	52
3.2.1.	Initial velocity studies	71
3.2.2.	Effect of product on initial velocity patterns	81
3.3.1.	Inhibition studies with substrate analogues and alternate substrates	87
3.3.2.	Inhibition studies with copper specific reagents	99
IV	Discussion	111
4.1.	Preliminary work	112
4.2.	Initial velocity studies	117
4.3.	Inhibition studies	119
4.4.	Copper specific inhibitor studies	121
4.5.	Proposed model for hydroxylation	126
	Appendices	134
	Bibliography	143
	Summary	150

FIGURE INDEX

Figure No.		Page No.
1.	Reaction catalysed by phenolase	4
2.	Production of cinnamic acid derivatives	7
3.	Evaluation of Dalziel constants	18
4.	Elution of phenolase from CM Cellulose	32
5.	Elution of phenolase from hydroxyapatite	34
6.	Standard curve for protein estimations	36
7.	Standard curve for colorimetric estimation of caffeic acid	37
8.	Absorption spectra	39
9.	Comparison of absorbances detected at 340 and 420 nm.	40
10.	Standard curve for DMDP estimation	45
11.	Variation of oxygen concentration	49
12.	Polyacrylamide gel electrophoresis	54
13.	Effect of pH	56
14.	Effect of catalase and EDTA on reaction rate	58
15.	Effect of enzyme concentration	60
16.	Substrate saturation - hydroxylase activity	61
17.	Substrate saturation - catechol oxidase activity	63
18.	Lineweaver - Burk plots	65
19.	Effect of caffeic acid on lag and reaction rate	69
20.	Pomerantz and Warner plot	70
21.	Initial velocity studies with ascorbic acid	72
22.	Initial velocity studies with DMTP	73
23.	Initial velocity studies in the absence of electron donor	74

24.	Secondary replots of initial velocity data	78
25.	Initial velocity studies with ascorbic acid - effect of caffeic acid	82
26.	Secondary replots - effect of caffeic acid	84
27.	Effect of caffeic acid on absorption spectrum of DMDP	86
28.	Inhibition curves obtained with substrate analogues and alternate substrates	89
29.	Effect of p-hydroxybenzoic acid on initial velocity patterns	91
30.	Secondary replots - effect of p-hydroxybenzoic acid	92
31.	Effect of benzoic acid on initial velocity patterns	93
32.	Secondary replots - effect of benzoic acid	94
33.	Effect of 3,4 - dihydroxybenzoic acid on initial velocity patterns	96
34.	Secondary replots - effect of 3,4 - dihydroxy-benzoic acid	98
35.	Difference spectra obtained on addition of copper specific reagents to phenolase	100
36.	Inhibition curves obtained with copper inhibitors	101
37.	Absorption spectrum of BCS	103
38.	Effect of carbon monoxide on initial velocity patterns	104
39.	Secondary replots - effect of carbon monoxide	105
40.	Effect of DIECA on initial velocity patterns	106
41.	Secondary replots - effect of DIECA	107
42.	Effect of BCS on initial velocity patterns	109
43.	Secondary replots - effect of BCS.	110
44.	Suggested model for phenolase action	127
45.	Role of copper in hydroxylation of monophenols	133

TABLE INDEX

Table No.		Page No.
1.	Types of inhibition - comparison of definitions	22
2.	Comparison of literature and observed melting points	28
3.	Molar extinction coefficients	44
4.	Enzyme purification	53
5.	Comparison between spectrophotometric and colorimetric methods for caffeic acid determination	55
6.	Effect of catalase and EDTA on NADH assay	59
7.	K_M and V values obtained for all substrates	67
8.	Computer analysis of initial velocity data	76
9.	Assignation of data to kinetic models	77
10.	Computer analysis of initial velocity data obtained in presence of caffeic acid	83
11.	Comparison of substrates and substrate analogues	88
12.	Comparison of predicted and observed inhibitor patterns	122

I. INTRODUCTION

If Haldane's suggestion that life is just one enzymic reaction after another is true, then a knowledge of the mechanism of action of enzymes is essential if life processes are to be understood. Some groups of enzyme, for example the hydrolases, have been extensively studied, and much is known about their mechanism of action. Hydrolases, such as lysozyme, are small readily crystallised proteins which are easily purified in bulk, and hence are suitable for physical techniques such as x-ray diffraction. Very little is known about the mechanism of action of many other groups of enzymes which are either difficult to purify, or not amenable to the techniques at present in use. An important class of enzymes which fall into this category are the oxidases, the best studied of which is laccase. The area of biochemistry covered by enzymes utilising molecular oxygen is vast, ranging from anabolic functions to energy production via electron transport. One oxidase widely distributed in plants is phenolase, and although considerable effort has been expended on this enzyme, little is known about its mechanism of action.

1.2.1. PHENOLASE

Although phenolase (EC 1.3.10.1 : o-diphenol oxygen oxidoreductase) has been known to exist for some time (Bertrand, 1895), it was not until 1937 that Kubowitz effected a large scale purification of potato phenolase. Purification has since been achieved from many other sources including mushroom (Keilin and Mann, 1938) spinach beet (Vaughan and Butt, 1969) and mouse melanoma (Lerner and Fitzpatrick, 1950). Phenolase has been thought of as a soluble enzyme (Bonner, 1957) but early work showed that it was associated with chloroplast fragments (Arnon, 1949). Recently Sato, (1966) and Bartlett et al (1971) have shown that the enzyme is associated with intact isolated chloroplasts, while Parish (1972)* has demonstrated mitochondrial and plastid phenolase in leaf and stem tissue

* and Mayer and Friend (1960).

of spinach beet.

1.2.2. Nature of Reaction Catalysed.

Spinach beet phenolase catalyses two reactions (Fig. I)

1) hydroxylation of monophenol to o-dihydricphenol.
2) oxidation of o-dihydricphenol to o-quinone.

1.2.2.1. Cresolase of hydroxylase activity.

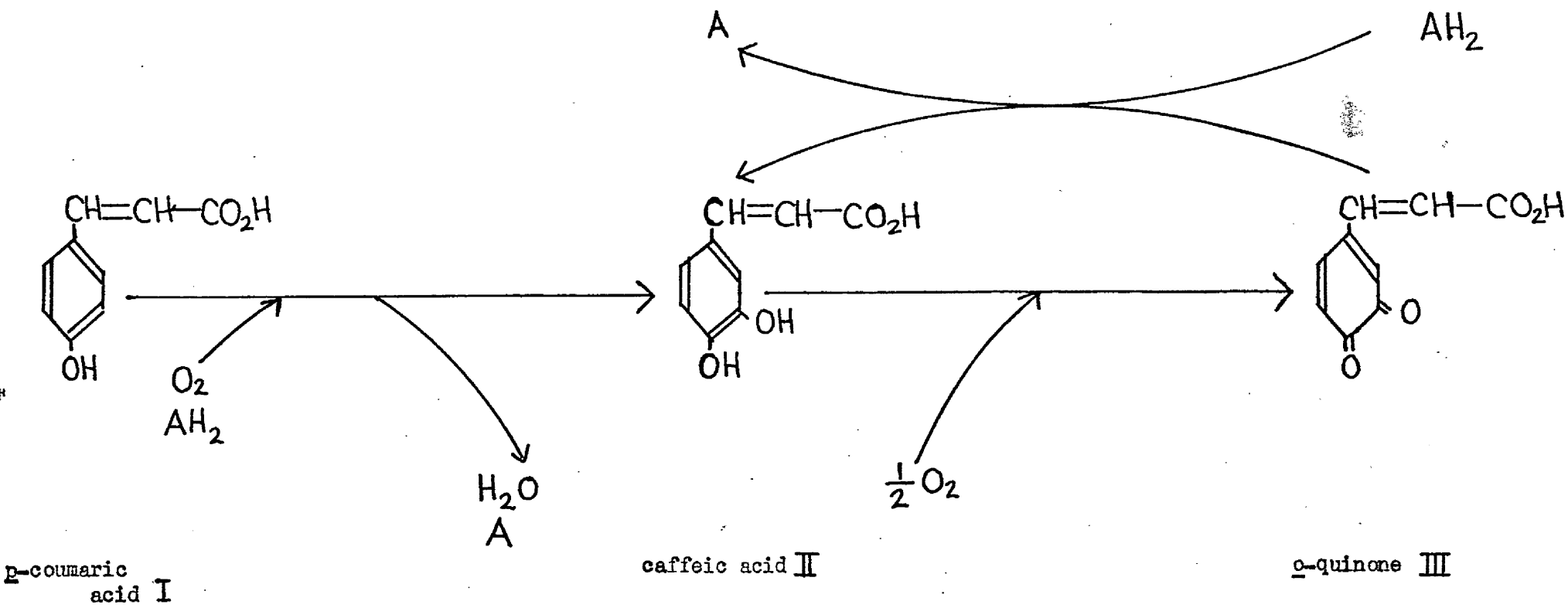
Phenolase catalyses the hydroxylation of a monophenol, such as p -coumaric acid I, to the o-dihydricphenol caffeic acid II, with concommit ant reduction of oxygen to water and oxidation of electron donor. Mason et al (1955) showed, by heavy isotope studies with O^{18}, that the oxygen incorporated into o-dihydricphenol came from molecular oxygen and not from water. It was deduced that one atom of oxygen was reduced to water while the other was incorporated into product, and thus phenolase could be classified as a mixed function oxidase (Mason, 1955).

1.2.2.2. Catechol oxidase activity.

The phenolic product of the hydroxylation reaction, caffeic acid, can be oxidised to o-quinone with simultaneous reduction of one atom of oxygen to produce one molecule of water (Nelson and Dawson, 1944). In this activity phenolase acts as an aerobic transelectronase (Hayaishi et al, 1956) or a four electron transferase (Mason, 1957). In the absence of electron donor the o-quinone undergoes further oxidation to form brown pigments, and reaction inactivation frequently occurs, (Nelson and Dawson, 1944). In the presence of electron donor, however, the o-quinone is non-enzymically reduced (Wagreich and Nelson, 1938) and browning does not occur until the reducing agent has been exhausted.

Fig. 1. Reactions catalysed by phenolase.

AH_2 = ascorbic acid, DMTP or NADH.



p-coumaric acid I

caffeic acid II

o-quinone III

These two activities, cresolase and catechol oxidase, would appear to be associated with one enzyme, as they cannot be separated by normal protein separation techniques.

1.2.2.3. Substrate Specificity.

Although animal phenolases (tyrosinases) are relatively specific for tyrosine and dihydroxyphenylalanine, the plant enzymes will catalyse the oxidation of a wide range of o-dihydricphenols and monophenols. From a consideration of the substrate structures (Mason, 1955) the criteria for specificity are clear; hydroxylation will only occur ortho to an existing phenolic group, while for catechol oxidase activity a substrate must have at least two hydroxyl groups ortho to one another.

1.2.2.4. Copper.

Kubowitz (1938) showed that phenolase is a metalloprotein, containing tightly bound copper. The copper has been implicated in the hydroxylation reaction on the basis of the reversible loss of activity on dialysis against cyanide (Kubowitz, 1938). The inhibition of enzyme activity by copper inhibitors such as diethyldithiocarbamate (DIECA; Vaughan and Butt, 1970) is further evidence to support this view.

1.2.2.5. Electron Donor.

p-coumaric acid can be hydroxylated to caffeic acid in the absence of any added reducing agent, the final product of the reaction being brown pigments. A characteristic aspect of this hydroxylation reaction of phenolase is the occurrence of an induction period of variable length before the true rate of hydroxylation is obtained. This lag is susceptible to many variables, among them the degree of enzyme purification (Keilin and Mann, 1938) and the addition of a catalytic amount of o-dihydricphenol,

(Nelson and Dawson, 1944; Pomerantz and Warner, 1967; and Vaughan and Butt, 1970). The rate of hydroxylation obtained after the lag is independent of the amount of o-dihydricphenol added and the lag can be completely eliminated. The hydroxylation observed in the absence of electron donor has led to the hypothesis that the reaction product, caffeic acid, can act as an internal electron donor for the reaction.

Ascorbic acid, nicotinamide adenine dinucleotide (NADH) and dimethyltetrahydropteridine (DMTP) have all been shown to increase the hydroxylation rate after the lag (Vaughan and Butt, 1969). These are the external electron donors for the reaction, and, unlike caffeic acid, these can shorten but not eliminate the lag. (Kreuger, 1953, 1958; Vaughan and Butt, 1970). Other electron donors such as potassium ferricyanide and hydroquinone (Bordner and Nelson, 1939) have also been shown to decrease the length of the lag, but not eliminate it. It has been suggested that the internal electron donor, o-dihydricphenol, is the reducing agent at the active site of phenolase and that this is kept in the reduced form by the external electron donor, ascorbic acid, NADH or DMTP.

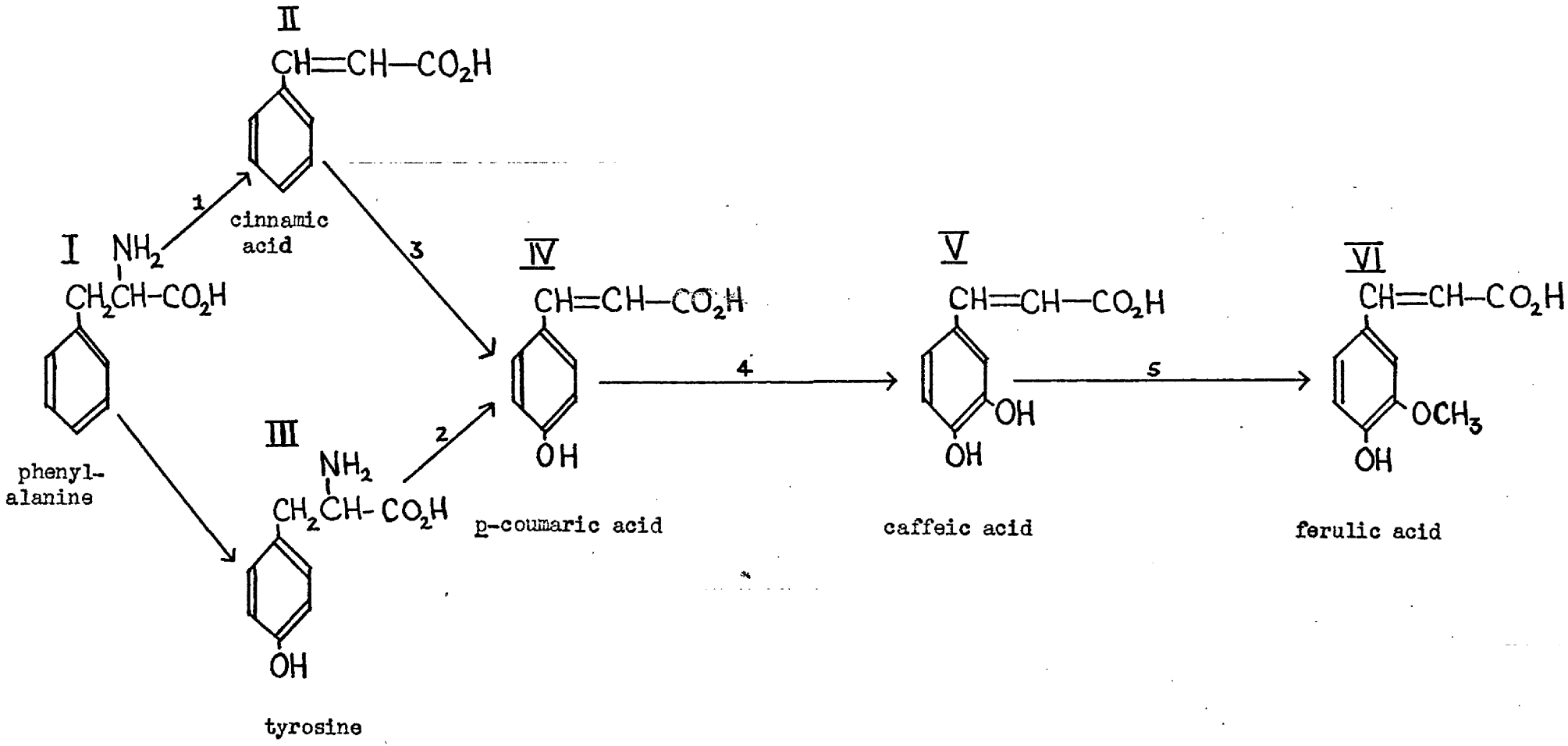
1.3.0. Role of phenolase in vivo.

Several suggestions have been put forward concerning the role of phenolase in the plant, but its function is still not understood.

1.3.1. Formation of cinnamic acid derivatives.

Lignins, flavonoids and coumarins form an important group of plant phenolic compounds which have a common hydroxylation and methoxylation pattern characteristic of p-coumaric acid, caffeic acid, and ferulic acid. On the basis of feeding experiments, McCalla and Neish, (1959) suggested the sequence of reactions shown (Fig. 2) for the synthesis of these intermediates. Considerable work has now been carried out on

Fig. 2. Production of cinnamic acid derivatives.



the enzymes involved in this pathway.

The ammonia lyase system (1) catalyses the conversion of phenylalanine I to cinnamic acid II in plants (Koukol and Conn, 1961), while in the Graminaceae a secondary pathway involves the production of p-coumaric acid IV from tyrosine III (Neish, 1961). Russell, (1971) has purified and partly characterised a cinnamic acid 4-hydroxylase from pea, while Nair and Vining (1965) have demonstrated the existence of a similar enzyme in spinach beet (3).

The in vivo tracer experiments of Brown and Neish (1956) and McCalla and Neish (1959) show that the 3'hydroxyl group is introduced after the formation of p-coumaric acid. This is substantiated by the work of Sato (1968) who showed that purified mushroom phenolase would carry out the hydroxylation of p-coumaric acid to caffeic acid, and the demonstration by Vaughan and Butt (1969) that this reaction occurred in the presence of purified spinach beet phenolase (4). The existence of caffeic acid and p-coumaric acid as esters in the plant (El Basyouni et al, 1964) is unimportant in this context, as Levy and Zucker (1960) have demonstrated the hydroxylation of p-coumaroylquinic ester to caffeoylquinic ester (chlorogenic acid) by partially purified potato phenolase.

The further oxidation of o-dihydricphenol to o-quinone by the catechol oxidase activity of phenolase would suggest an accumulation of o-quinone in the absence of electron donor, and would seem to rule out phenolase as the in vivo catalyst of the hydroxylation reaction. The close association between chloroplast and phenolase would, however, suggest an in vivo environment rich in electron donors, and thus regeneration of o-dihydricphenol would occur. The observation (Vaughan and Butt, 1969) that the catechol oxidase activity is suppressed in the presence of DMTP at high levels of p-coumaric acid provides a further mechanism for the in vivo control of this activity. It is therefore suggested that phenolase catalyses the hydroxylation of p-coumaric acid in the plant.

Finkle and Nelson (1963) and Brown (1966) have demonstrated the existence in wood of an enzyme which will methylate the 3' hydroxyl group of caffeic acid in the presence of S-adenosyl methionine. (5).

Thus the enzymes necessary for the cinnamic acid pathway have been demonstrated *in vivo* and the cinnamic acid derivatives produced act as intermediates in the formation of lignins, flavonoids and coumarins.

1.3.2. Plant Respiration.

Phenolase has also been implicated in plant respiration. Early work (Bonner and Wildman, 1946) suggested that phenolase mediated a large proportion of the oxygen uptake during respiration, catalysing the terminal step with oxygen as the electron receptor, but inhibitor studies with cyanide, azide, carbon monoxide and metal inhibitors such as DIECA (Beevers, 1961) show that most, but not all, of the oxygen fixed can be accounted for by cytochrome oxidase. It has been suggested (Pshenova, 1956) that cytochrome oxidase fulfils this function in the young plant, but that this role is taken over by phenolase on ageing. Consequently, the part played by phenolase in terminal oxidation is not clear, but may be dependent on the maturity of the plant.

1.3.3. Response to injury or attack.

The observation that damaged plant tissue turns brown suggests another function for phenolase; defence against injury or infection. Uritani and Hyodo (1967) observed the formation of two new phenolases on wounding of sweet potato. The observation that phenolase activity increased on infection of *Nicotania glutinosa* with tobacco mosaic virus (John and Weintraub, 1967) supports the suggestion that phenolase is involved in the plant response to infection by pathological agents (Williams, 1963)

Thus phenolase appears to be involved in several aspects of plant metabolism and hence a study of its mechanism of action would be of

interest. An initial stage in the elucidation of the reaction is a determination of the order of substrate binding and product release. Such information can be obtained from a steady state kinetic analysis, and it was therefore decided to use this approach in an attempt to clarify the nature of the reaction catalysed.

1.4. ENZYME KINETICS.

Since interest in enzymology stems from the ability of an enzyme to drastically alter the rate of a chemical reaction under *in vivo* conditions of temperature, pressure and pH, it is not surprising that the theories and techniques of chemical kinetics have been widely applied. The first rate equation was derived by Wilhelmy in 1850, but it was not until the beginning of the twentieth century (Brown, 1902; Henri, 1901, 1902) that enzyme kinetics, as we know it today, began to take shape.

1.4.1. Single substrate reactions.

Michaelis and Menten (1913) proposed the following model for enzyme catalysis.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P \qquad \text{eqn 1.1}$$

where E represents the enzyme, A the substrate, EA the enzyme-substrate complex and P the product. The assumptions made were:-

1) The enzyme exists only as E and EA, there being only one form of enzyme-substrate complex.

2) EA is in equilibrium with E and A.

Both these assumptions are approached if k_3 is very much smaller than the other rate constants.

On application of the Law of Mass Action to eqn 1.1, it was shown that

$$v = \frac{V.[A]}{K_A + [A]} \qquad \text{eqn 1.2}$$

where v is the rate of reaction, K_A is the dissociation constant for $EA = k_2/k_1$ and V is the maximum velocity $= k_2 E$.

Hence the scheme depicted in eqn 1.1 gives rise to the equation of a hyperbola (eqn 1.2.) which is characterised by two parameters K_A and V. V is the maximum velocity of the reaction which v approaches asymptotically as $[A]$ tends to infinity, and K_A is the Michaelis constant, a measure of the affinity of enzyme for substrate.

Lineweaver and Burk (1934) rearranged equation 1.2. into a linear form

$$\frac{1}{v} = \frac{K_A}{V} \cdot \frac{1}{[A]} + \frac{1}{V} \qquad \text{eqn 1.3}$$

K_A and V can be determined from a graph of $1/v$ against $1/[A]$.

A more realistic approach was adopted by Briggs and Haldane (1925) who assumed that EA reached a steady state level, thus avoiding the assumption that k_3 was much smaller than k_1 and k_2. This treatment gives rise to an equation of similar form to eqn 1.2.

$$v = \frac{V \cdot [A]}{K_M + [A]} \qquad \text{eqn 1.4}$$

but now $K_M = (k_2 + k_3) / k_1$.

Equations similar to eqn 1.4 can be derived for more complex cases but then both K_M and V are functions of the individual rate constants and K_M does not reflect the enzyme substrate affinity.

1.4.2. The steady state.

Steady state theory has since been widely applied in the

derivation of kinetic models and is especially useful in its applicability to seemingly complex situations. The assumptions made are

1) The rate of change of complex concentration is zero i.e. $d[EA] / dt = 0$.
2) complex formation occurs rapidly, thus $d[P] / dt$ quickly becomes constant i.e. the initial rate of reaction is attained before an appreciable amount of product is formed.
3) the concentration of complexes formed is low relative to those of reactant and product.

These assumptions are found to be applicable when, as Laidler (1955) has shown

a) $S_o \gg e_o$

b) $e_o \gg S_o$

c) $k_2 + k_3 \gg k_1 e_o$

d) $k_2 + k_3 \gg k_1 S_o$

Initial velocity studies (assumption 2) are extensively used as the effect of the back reaction can be generally disregarded, thus simplifying the analysis.

Heineken et al (1967) showed mathematically that the steady state assumptions were "somewhat scandalous" as in a strict sense $d[EA] / dt = 0$ at only one instant. Walter (1966), however, demonstrated by analytical and analogue computer analysis that the steady state assumption, while imperfect theoretically, is suitable for use in most experimental work, and Wong (1965) has laid down further guidelines on the applicability of steady state theory. If the errors due to the steady state assumption are within experimental error, then a graph of velocity against enzyme concentration will be linear, and steady state theory can be applied.

The most common condition under which steady state theory can be applied is $e_o \ll S_o$ i.e. the initial concentration of substrate is much greater than that of enzyme, and this condition was enforced throughout this study.

1.4.3. Multisubstrate systems

Kinetic analysis becomes more complicated when it is applied to enzyme catalysed reactions involving more than one substrate. The principles involved can, however, be illustrated by a consideration of the two substrate case, and can then be extended to cover more complex situations.

1.4.3.1. Two substrate case.

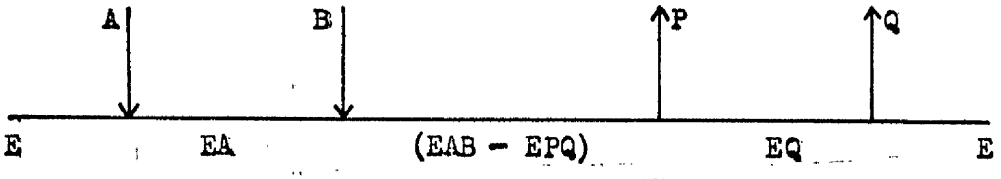
Two substrate reactions with linear mechanisms (i.e. no alternative or branched pathways) fall into two categories, being examples of either

1) ordered mechanisms, in which substrate addition follows a definite pattern, one substrate being bound before addition of the next is possible.

or 2) random mechanisms, for which no such pattern exists.

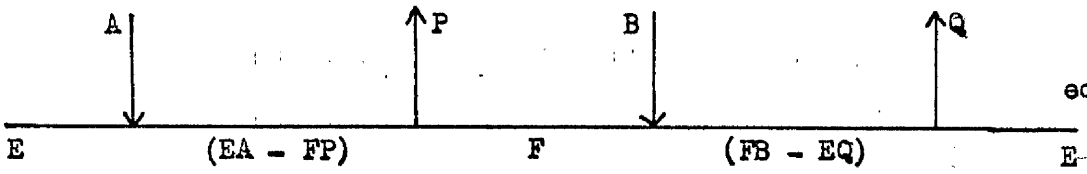
Ordered mechanisms, can be further classified into two types which can be described using Cleland's (1963 a,b,c) nomenclature. These are

a) a Sequential mechanism.



eqn 1.5

All substrates must bind to the enzyme before any reaction can occur, and b) a ping-pong or enzyme substitution mechanism



eqn 1.6

In this case addition and reaction of one substrate results in the formation of a new stable enzyme form F, which then reacts with the other substrate.

The linear two substrate case has therefore been well defined (Alberty, 1953, 1958, and Dalziel, 1957, 1958) and the theory of branched or alternative reaction pathways has also been studied in detail (Dalziel, 1958, and Wong and Hanes, 1962).

1.4.3.2. Reaction Analysis

A comparison between the rate equations for the various reaction schemes is a necessary step in the determination of the mechanism for any enzyme. The derivation of these rate equations is complex but the determinant technique of King and Altman (1956) considerably lessens this task. This procedure has been further simplified by the graphical method of Volkenstein and Goldstein (1966) and by the technique of Orsi (1971) which further reduces the algebraic manipulation required.

On treatment of eqns 1.5 and 1.6 in this way the following equations are derived if the product concentration is zero i.e. if initial rate measurements are used

1)
$$v = \frac{V[A][B]}{K_{iA} K_B + K_B[A] + K_A[B] + [A][B]}$$ eqn 1.7

2)
$$v = \frac{V[A][B]}{K_B[A] + K_A[B] + [A][B]}$$ eqn 1.8

These equations can also be expressed using Dalziel's Nomenclature

1) $$\frac{v}{[E_o]} = \frac{[A][B]}{\phi_{12} + \phi_2[B] + \phi_1[A] + \phi_o[A][B]}$$ eqn 1.9

2) $$\frac{v}{[E_o]} = \frac{[A][B]}{\phi_2[B] + \phi_1[A] + \phi_o[A][B]}$$ eqn 1.10

where E_o is the concentration of active sites and the values of the Dalziel constants can be seen by inspection of these equations

$$\phi_o = 1/k_2 \qquad \phi_2 = K_A/k_2$$
$$\phi_1 = K_B/k_2 \qquad \phi_{12} = K_{iA} K_B/k_2$$

Rearrangement of eqns 1.7 and 1.8 by the Lineweaver - Burk method demonstrates this point more clearly. The equations then become

$$\frac{1}{v} = \frac{1}{[A]}\left[\frac{1}{V}\left\{K_A + \frac{K_{iA} K_B}{[B]}\right\}\right] + \frac{1}{V}\left[1 + \frac{K_B}{[B]}\right]$$ eqn 1.11

and

$$\frac{1}{v} = \frac{1}{[A]}\left[\frac{K}{V}\right] + \frac{1}{V}\left[1 + \frac{K}{[B]}\right]$$ eqn 1.12

Inspection of these equations shows that, in the sequential mechanism, (eqn 1.11) the slope of the line obtained when $1/[A]$ is plotted against $1/v$ is dependent on $[B]$, but this is not so for a ping pong mechanism (eqn 1.12). Both types of mechanism, however, give rise to an intercept term which is dependent on $[B]$. Hence it is possible to distinguish between these two types of mechanism on the basis of initial rate studies.

The difference observed in the rate equations means that it is possible to predict the expected initial velocity patterns and hence select which kinetic model the initial velocity data fits best,

(Cleland, 1963, a,b,c). If A is varied at a number of fixed concentrations of B, the changing fixed variable (Cleland, 1970), and a Lineweaver-Burk plot constructed, then the resulting initial velocity pattern will be either parallel or can converge towards the abscissa, depending on the variation of slope and intercept with B.

This variation can be explained in terms of the rate equations as follows

1) Intercept effects. The vertical intercept of a reciprocal plot is the reciprocal velocity when the variable substrate A is at infinite concentration. Thus any substrate which affects the rate in the presence of saturating A will affect the intercept observed. This is generally true for a second substrate, as it combines with a different enzyme form than the variable substrate and will therefore affect the reaction rate at infinite concentrations of A.

2) Slope effects. At low concentration of substrate A the slope of a reciprocal plot is K_A/V where $K_A = [EA] / [E][A]$. In order for the rate of combination of E and A to be maximum when [A] is low, [E] must be as high as possible and [EA] as low as possible. Thus any factor which affects the ratio of [EA] to [E] will alter the slope obtained. If the changing fixed variable B is then added it will combine with EA thus altering the [EA] / [E] ratio. Increasing amounts of B will therefore lower the slope of the reciprocal plot, giving rise to the converging pattern observed with a sequential mechanism.

If the ping pong mechanism is considered (eqn 1.8) it can be seen that product release occurs after each substrate addition. In the absence of added product these partial reactions are irreversible under the conditions used for determination of initial velocities. Hence if A is the variable substrate raising the level of B will convert F to the second central complex (FB-EQ) but will not affect the ratio of E to (EA-FP). The slope of the reciprocal plot is therefore not altered and this gives rise to the characteristic

parallel velocity pattern. If, however, a fixed concentration of product is initially added to the system then combination with F will occur and the concentration of (EA - FP) will rise. B will then compete for F producing (FB - EQ) and preventing P from affecting (EA - FP), the level of which will drop specifically with respect to E, and a slope effect will be observed. The conversion of a parallel to an intersecting pattern by a fixed level of product is a diagnostic test for ping pong mechanisms involving product release.

Other situations that can give rise to parallel pattern are

1) when partial reactions involve a large free energy change thus making a step irreversible.
2) when the substrate dissociation constant is less than 10% of the concentration used in kinetic experiments (Kelley et al, 1967).

1.4.3.3. Evaluation of Kinetic Parameters.

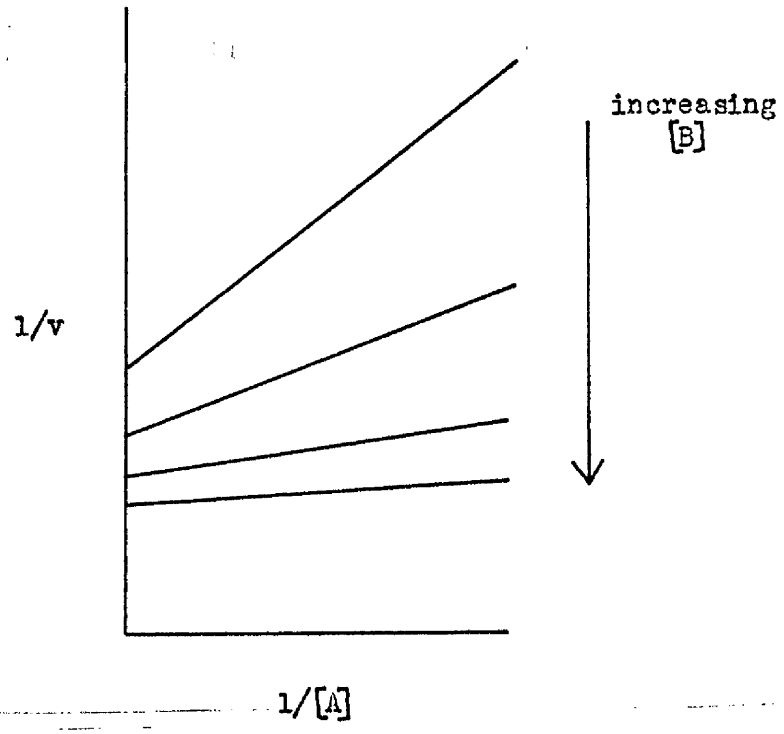
The kinetic constants involved in these types of mechanism can be evaluated by replotting the slopes and intercepts of the reciprocal plots (Fig 3a) against the reciprocal of the changing fixed variable, (Fig 3b). All of the constants for a two substrate reaction (eqns 1.5 and 1.6) can be calculated from such a graph, the slope of an intercept replot giving ϕ_2 and the intercept ϕ_0 while a slope replot yields ϕ_{12} and ϕ_1.

1.4.4.1. Three substrate reactions.

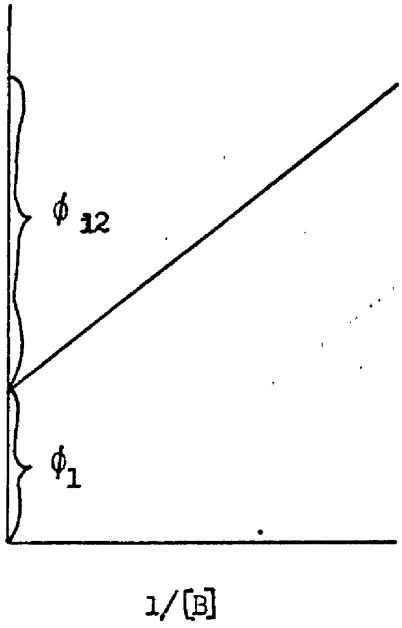
The extension of steady state kinetics to the multisubstrate, and particularly, the three substrate case, has been carried out by Cleland (1963 a, b, c); Wong and Hanes, (1969) and Dalziel, (1969). In this case three types of ordered reaction can be distinguished, the straight forward sequential and ping pong mechanisms of the two substrate case,

Fig. 3. Evaluation of Dalziel constants.

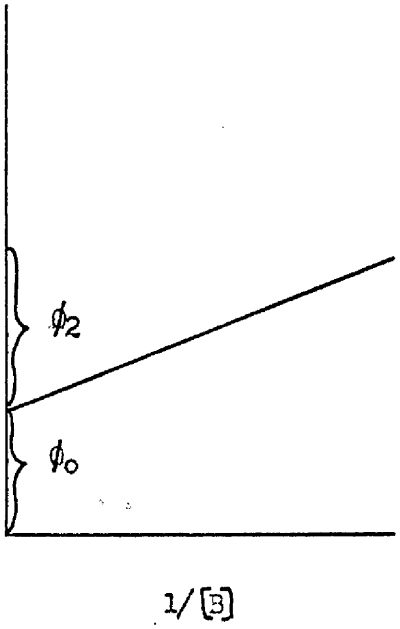
a) Lineweaver - Burk plot



b) Slope replot

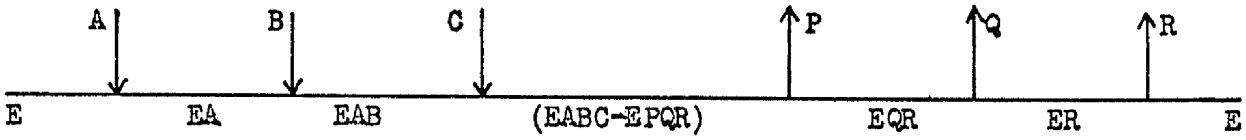


Intercept replot

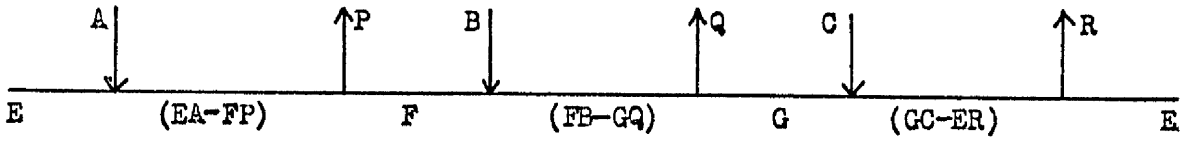


and a mechanism involving a mixture of the two. These can be described using Cleland's nomenclature.

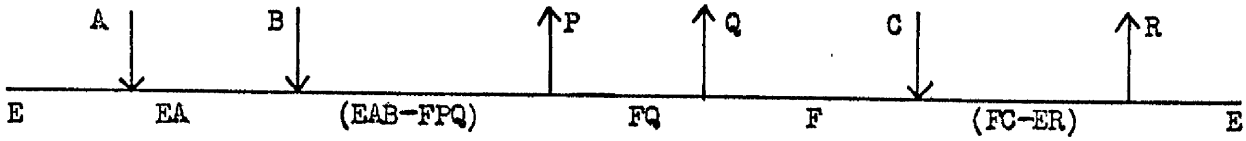
1) Sequential eqn 1.14



2) Ping Pong eqn 1.15



3) Mixed eqn 1.16



This is an example of a bi bi uni uni ping pong mechanism, but other permutations are possible e.g. bi uni uni bi ping pong.

The rate equations for these mechanisms have been worked out and if product concentration is zero then:-

1) $$v = \frac{V[A][B][C]}{K_{iA}K_{iB}K_C + K_{iA}K_C[A] + K_{iA}K_B[C] + K_C[A][B] + K_B[A][C] + K_A[B][C] + [A][B][C]}$$

2) $$v = \frac{V[A][B][C]}{K_C[A][B] + K_B[A][C] + K_A[B][C] + [A][B][C]}$$

3) $$v = \frac{V[A][B][C]}{K_{iA}K_B[C] + K_C[A][B] + K_B[A][C] + K_A[B][C] + [A][B][C]}$$

and hence it is again possible to distinguish between these varying types of mechanism on the basis of initial velocity studies.

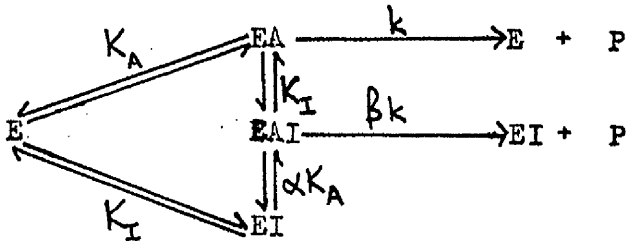
1.4.4. Experimental methodology.

There are two types of experimental approach to the problem of distinguishing between these mechanisms. In the first case A is varied at a number of fixed concentrations of B, in the presence of a saturating amount of C. Secondly A may be varied at a number of fixed concentrations of B and C which are in turn fixed in the ratio of their Michaelis constants. (Rudolph et al, 1968). The values obtained from either of these methods can be used to construct Lineweaver-Burk plots and the resulting initial velocity pattern examined for parallel or converging patterns. The technique of product conversion of a parallel to an intersecting pattern can be used, as for the two substrate case, and the kinetic parameters, although more numerous, can be evaluated from replots of slope and intercept. (Dalziel, 1969).

1.5. Inhibitor Studies.

Additional information on mechanisms of enzyme action can be obtained from initial velocity studies in the presence of reversible inhibitors. The effect of substrates, products and other inhibitors of enzyme activity can be differentiated and several classifications have been described. A general reaction scheme for the one substrate case based on equilibrium assumptions has been proposed by Webb (1963). This is useful in that it provides a basis for discussion on the common types of inhibition arising in more complex systems.

The following scheme for inhibition is suggested



where α represents the change in affinity and β the change of rate of complex decomposition produced by the inhibitor, these being the two methods by which activity can be affected.

An alternative classification is that of Cleland (1970) who uses the effect of inhibitor on the slope or intercept of a reciprocal plot to define its type. These definitions of inhibition are compared with those of Webb in Table I. Cleland's derivation is experimentally useful as results obtained from initial velocity studies can be used to predict the expected inhibitor patterns, and experimental data can be fitted to the best model.

Hence an inhibitor which was competitive with A would be expected to be uncompetitive with B and C in eqn. 1.15, noncompetitive with B and C in eqn. 1.14, and noncompetitive with B and uncompetitive with C in eqn 1.16. Thus predictions made from steady state kinetic analysis can be verified by inhibitor studies of this kind.

Various types of inhibition have been described and these are designated as

1) Product inhibition (Plowman, 1972)
2) Substrate inhibition (Cleland, 1970)
3) Alternate substrate inhibition - due to the presence of other less efficient substrates (Purich and Fromm, 1971)
4) Dead end inhibition:- due to a compound which is not acting as a substrate or product but which forms complexes with one or more enzyme forms (Plowman, 1972).

Table 1. Types of Inhibition : comparison of definitions.

Inhibition	Webb	Cleland
Competitive	substrate binding affected $\frac{d[EA]}{dt}$ not affected	effect on slope but not intercept inhibitor binds to same enzyme form as variable substrate
Non Competitive	substrate binding not affected $d[EAI]/dt$ lower than $d[EA]/dt$.	effect on intercept and slope inhibitor binds to different enzyme form reversibly connected to that which binds variable substrate.
Uncompetetive	substrate binding increased $d[EAI]/dt$ is decreased	effect on intercept but not slope inhibitor binds to different enzyme form not reversibly connected to that which binds variable substrate.

Product inhibition is not suitable for use with phenolase as the reaction products are water, o-dihydric phenol and the oxidised electron donor, probably o-quinone. Caffeic acid, if added in large amounts, would act as substrate for the catechol oxidase reaction, while addition of o-quinone would result in irreversible reaction inactivation rather than reversible inhibition. A further problem would be the difficulty in working with o-quinones due to their instability.

Alternate substrates or substrate analogues were thus chosen as the most suitable inhibitors for use with phenolase.

1.6. Statistical analysis of Kinetic Data.

Traditionally the kinetic parameters involved in rate equations have been evaluated by graphical analysis, which although simple and rapid does not give any estimate of the reliability of the constants determined. A knowledge of the precision of these measurements is necessary for a proper evaluation of the results in relation to the various kinetic models under consideration, and this can be obtained from a statistical analysis of the data. The use of least-squares analysis in fitting data to eqn 1.4 has been described by Johansen and Lumry (1961) and Wilkinson (1961), and the extension of these procedures to more complex systems has been carried out by Cleland (1967). Data obtained throughout this work were therefore subjected to least squares analysis, using the programs shown in Appendix I, the data being fitted directly to the equation of the hyperbola eqn 1.4 or more complex cases.

1.7. Estimation of enzyme activity.

The determination of accurate initial rates is very important as the steady state approach is based on these measurements. A major problem in the study of phenolase is the determination of hydroxylation

in the presence of the catechol oxidase activity. Because of this dual function, assays involving oxygen uptake (Keilin and Mann, 1938) or disappearance of electron donor (Dawson and Magee, 1955) are not applicable. Hydroxylation must therefore be measured by monophenol utilisation or product formation. The colorimetric method of Vaughan and Butt (1969) is specific for the formation of o-dihydrophenol but difficulties and errors arising from sampling make this unsuitable for the determination of initial rates. It was therefore decided to devise a continuous spectrophotometric assay as this would reduce the error involved and would be more convenient for extensive use.

I.I. MATERIALS AND METHODS

2.1. Materials.

Fine chemicals were obtained as follows

Proteins and Enzymes.

Bovine Plasma Albumin (3x recrystallised)	Armour Pharmaceutical Co. Eastbourne, Essex, U.K.
Catalase	Calbiochem Ltd., 10 Wyndham Place, London, WHIAS

Reducing Agents.

Ascorbic Acid (Biochemical Grade)	BDH Chemicals, Ltd., Poole, England.
6,7 - dimethyl - 5,6,7,8 - tetra-hydropteridine (DMTP)	Calbiochem Ltd.
Nicotinamide Adenine dinucleotide	

Substrates:

Catechol	BDH Chemicals, Ltd.
Caffeic Acid	Koch Light Laboratories, Colnbrook, Bucks., England.
p-coumaric acid	

Inhibitors.

Benzoic Acid	BDH Chemicals, Ltd.
3,4-dihydroxybenzoic acid	
p-hydroxybenzoic acid	
Bathocuproine Sulphonate	
DIECA	

Other Chemicals.

Coomassie Brilliant Blue G250	Serva Micro Labs, Ltd., 46 Pembridge Road, London, W.11.
Acrylamide	BDH Chemicals, Ltd.
L - proline	

The following gases were obtained from the British Oxygen Company, Polmadie Estate, Glasgow.

Oxygen

Oxygen free nitrogen

air

carbon monoxide

5% oxygen / 95% oxygen free nitrogen

2.1.2. Further Purification of Materials.

p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and benzoic acid were recrystallised from 40% ethanol to constant melting point (Gallenkamp melting point apparatus, Gallenkamp, London, England). A comparison between the corrected observed values and those in the literature (Handbook of Chemistry and Physics, 1948) is shown in Table 2. Acrylamide was recrystallised from benzene before use.

All other chemicals were analytical grade, and all solutions were made up in distilled deionised water.

2.1.3. Unstable Materials.

DMTP has been shown to auto-oxidise in air (Nielsen, 1968). In order to minimise this effect, samples of DMTP were kept under constantly bubbling oxygen free nitrogen. Standard amounts of DIECA were diluted

Table 2. Comparison of literature and observed melting points.

Compound	Observed melting point (°C)	Literature melting point (°C)
caffeic acid	209	211
p-coumaric acid	215	217
Benzoic acid	121.5	122.4
p-hydroxybenzoic acid	212	215
3,4-dihydroxybenzoic acid	203	205

to make up fresh solutions every 45 min., thus surmounting the instability problem (Hallaway, 1959).

2.2.1. Enzyme preparation.

Phenolase was prepared by the method of Vaughan and Butt (1969), modified to handle much larger amounts of material. All operations were carried out as near $0^{o}C$ as possible.

2.2.1.2. Release of phenolase from chloroplasts.

Freshly picked, washed leaves of Beta Vulgaris L. were macerated in 0.01M Na_2HPO_4 - 0.005M citric acid buffer, pH 5.3 in a one gallon Waring blender (Waring Products Division, New Hartford, Connecticut, U.S.A.), and fibrous stalk tissue removed by centrifugation through a continuous action basket head using an MSE super Medium centrifuge (Measuring and Scientific Equipment, Ltd., London, England.) The crude macerate was centrifuged for 30s at 1,500g in an MSE Mistral 6L, the supernatant (17 l) collected, and 4g/l cetyl trimethyl ammonium bromide (Cetavlon) added to liberate particle bound enzyme (Ritchie, 1972).

2.2.1.3. $(NH_4)_2$ SO_4 Fractionation.

210 g $(NH_4)_2$ SO_4 per litre of solution were added slowly, with stirring, to give 35% saturation, the pH of the solution being maintained at 5.3 by addition of 3M H_2SO_4. The mixture was left to equilibrate for 30 min, centrifuged for 30 min at 10,000 g in an MSE Mistral 6L and the supernatant retained (16 l). A further 230 g of $(NH_4)_2SO_4$ per litre of solution - equivalent to 70% saturation - was added as before, the pH checked and the solution left to re-equilibrate. Centrifugation was carried out in an MSE Mistral 6L at 10,000 g for 30 min, and the precipitate resuspended in 0.01M Na_2HPO_4 - 0.005 M citric acid buffer, pH 5.3.

2.2.1.4. Heat treatment and Dialysis.

The sample (1.5l) was heated at 60°C for 10 min, the conductivity adjusted to 150 millimhO, the conductivity of 35% saturated $(NH_4)_2SO_4$, and 4g/l Cetavlon were added. Under these conditions phenolase was found to be soluble, and did not reassociate with denatured protein. Centrifugation was carried at 10,000g for 30 min in an MSE Mistral 6L, and the supernatant was dialysed against 50 l of distilled water until the conductivity was less than 4 millimho, the conductivity of 0.1M Na_2HPO_4- 0.05 M citric acid buffer, as at higher ionic strengths the enzyme did not bind to CMCellulose, and column chromatography could not be carried out.

2.2.1.5. Column Chromatography.

2.2.1.5.1. CM Cellulose.

CM Cellulose (Whatman CM22 from W.R. Balston (Modified Celluloses) Ltd., Maidstone, Kent) was pretreated with acid and alkali in accordance with the makers instructions. A column (100 x 2.5 cm) was packed and equilibrated with 1mM Na_2HPO_4- 0.5 mM citric acid buffer pH 5.3, the sample (2.1) applied, and the column washed with 0.1M Na_2HPO_4- 0.05M citric acid buffer, pH 5.3. The effluent was monitored for absorption at 280 nm and washing was continued, removing tanned material, until a basal level was established. The column was eluted with a linear gradient from 0 to 2M NaCl in the washing buffer. 6 ml fractions were collected and the effluent assayed for activity. (Fig. 4) The shape of the elution profile obtained (Fig. 4), and the low specific activity compared with that of small preparations (P.F.T. Vaughan, personal communication), suggested that further purification was required and the method was therefore extended to include Hydroxyapatite column chromatography.

Fig. 4. Elution of phenolase from CM Cellulose.

and Fig. 5. Elution of phenolase from hydroxyapatite.

The procedure followed is described in Materials and Methods (2.2.1.5).

———— E_{280}

- - - - - - - Enzyme activity - expressed as caffeic acid produced (E_{525} / 15 min).

— — — Salt gradient applied.

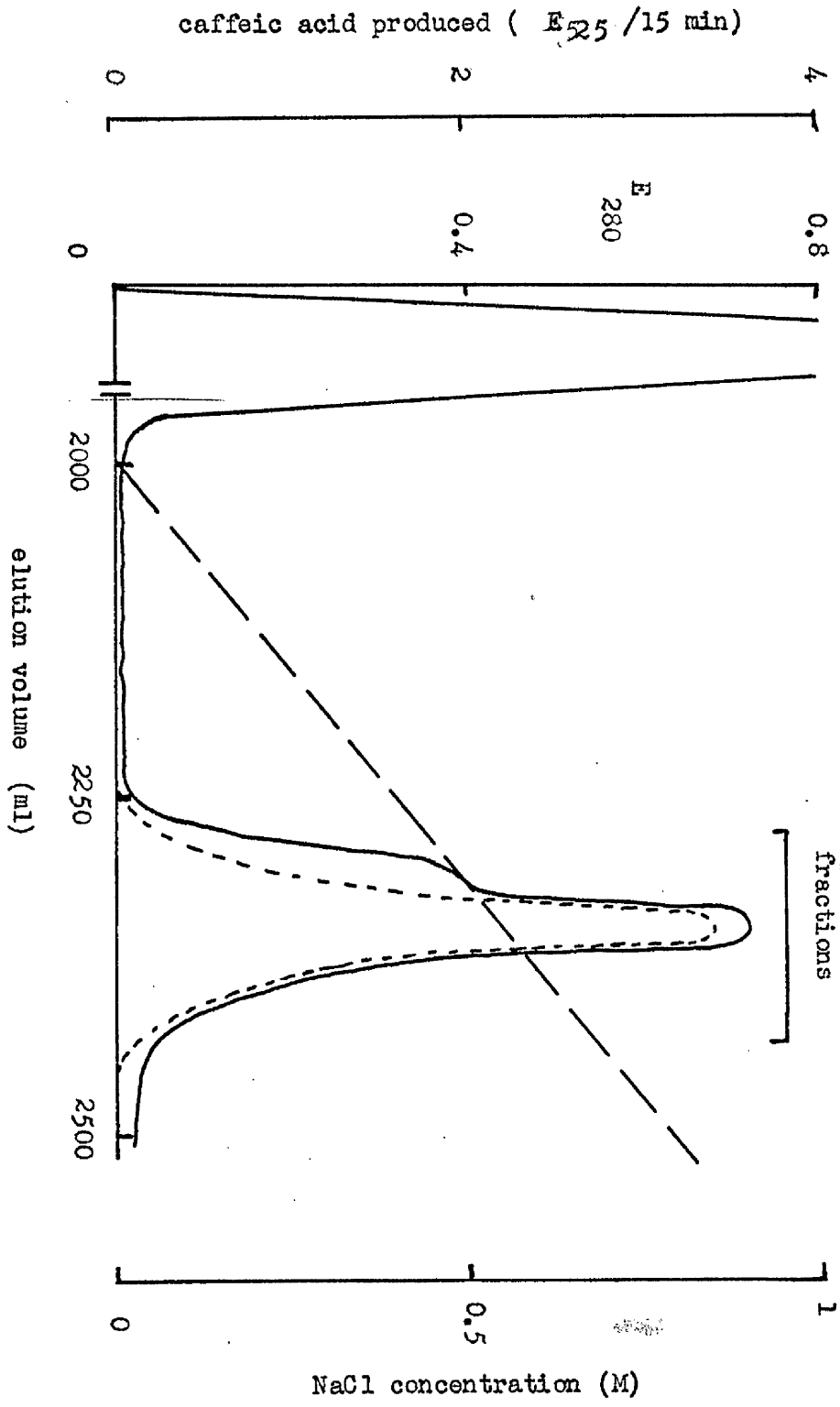


Fig. 4. Elution of phenolase from CM Cellulose.

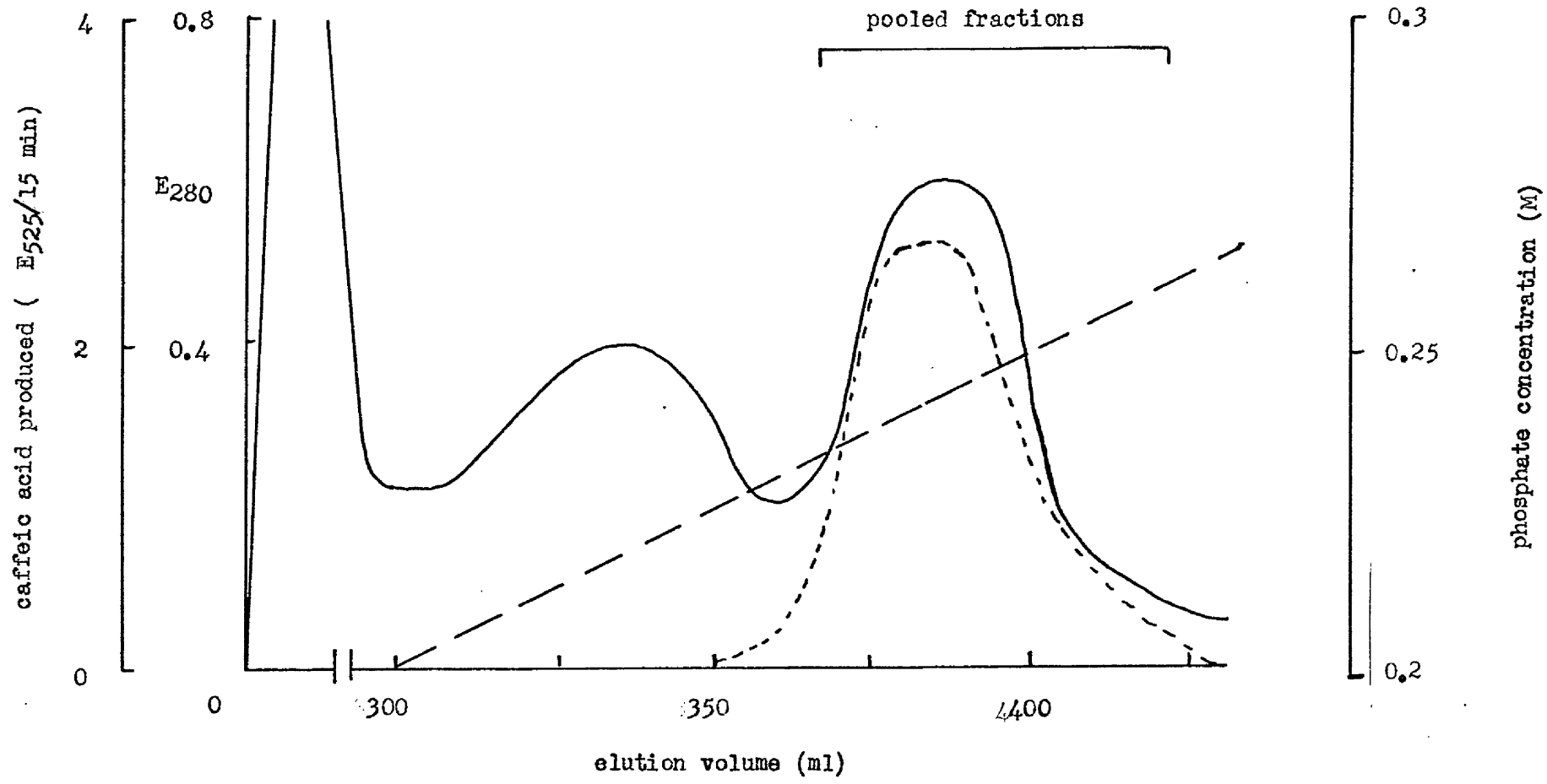
2.2.1.5.2. Hydroxyapatite.

The active fractions from the CM Cellulose column were pooled and dialysed against two changes of 1 mM KH_2PO_4 - K_2HPO_4 buffer, pH 6.8. Hydroxyapatite (Biorad Laboratories, Richmond, California, U.S.A.) was suspended in this buffer and a column (2.5 x 2.5 cm) poured and equilibrated. After application of the sample (175 ml) the column was washed with 0.2M phosphate buffer pH 6.8, removing further amounts of tanned material, until a basal effluent absorption at 280 nm was obtained. The enzyme was eluted with a linear gradient from 0.2M to 0.4M phosphate buffer pH 6.8, and 3ml samples collected. The effluent was assayed for activity and the enzyme containing fractions pooled. The elution profile shows an apparently homogeneous enzyme sample (Fig 5). After dialysis against saturated $(NH_4)_2SO_4$ pH 7.3, the concentrated protein suspension could be stored for at least 2 years at $0^{\circ}C$ without loss of activity. Before use the enzyme was diluted with 2mg/ml bovine plasma albumin, a precaution necessary to stabilise the purified enzyme at a concentration convenient for assay (Mallette and Dawson, 1947).

2.2.2. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was carried out as described by Maurer (1971) using 7.5% acrylamide gel with 4% acrylamide spacer gel. Protein was stained using Coomassie Brilliant Blue (Weber and Osborne, 1969). Enzyme activity was detected by spraying gels with 0.4g catechol and 0.4g L - proline dissolved in 40 ml 1M $(NH_4)_2SO_4$ and 10 ml 0.1M Na_2HPO_4 - 0.05M citric acid buffer, pH 5.3, a brown colour being developed on standing.

Fig. 5. Elution of phenolase from hydroxyapatite.



2.2.3. Protein Estimations.

Protein estimations were carried out using Bailey's (1962) modification of the method of Lowry et al, (1951). Three times recrystallised bovine plasma albumin was used as standard (Fig. 6). Samples for assay contained up to 100 µg protein/ml.

2.2.4. Determination of Caffeic Acid.

2.2.4.1. Colorimetric assay.

Caffeic acid was estimated by a modification of the method of Hoepfner, (1932). 0.5 ml of solution, containing up to 0.66 µmole caffeic acid, were added to 2 ml 5% (v/v) acetic acid. After the addition of 2 ml 0.5% (v/v) $NaNO_2$, the solutions were mixed and allowed to stand for 5 min and the absorbance at 525 nm was then read using a Beckman DB spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, U.K.). A linear relationship between concentration of caffeic acid and absorbance has been shown over the range considered (Fig 7). Included in each batch of estimations was a standard containing 0.267 µmoles of caffeic acid.

2.2.4.2. Spectrophotometric assay

2.2.4.2.1. Determination of caffeic acid

The spectrophotometric estimation of caffeic acid was based on differences between the absorption spectra of

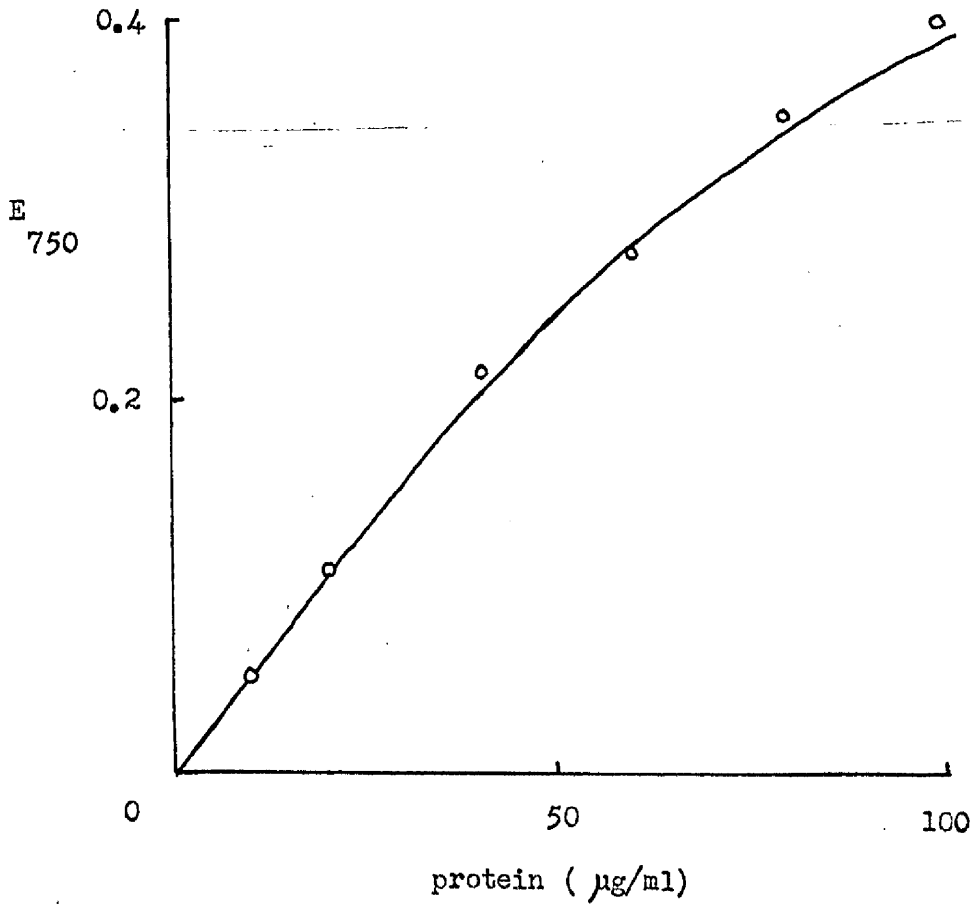
a) p-coumaric acid, caffeic acid and oxidised caffeic acid

b) DMTP and dimethyldihydropteridine (DMDP),

c) NADH and NAD^+ and

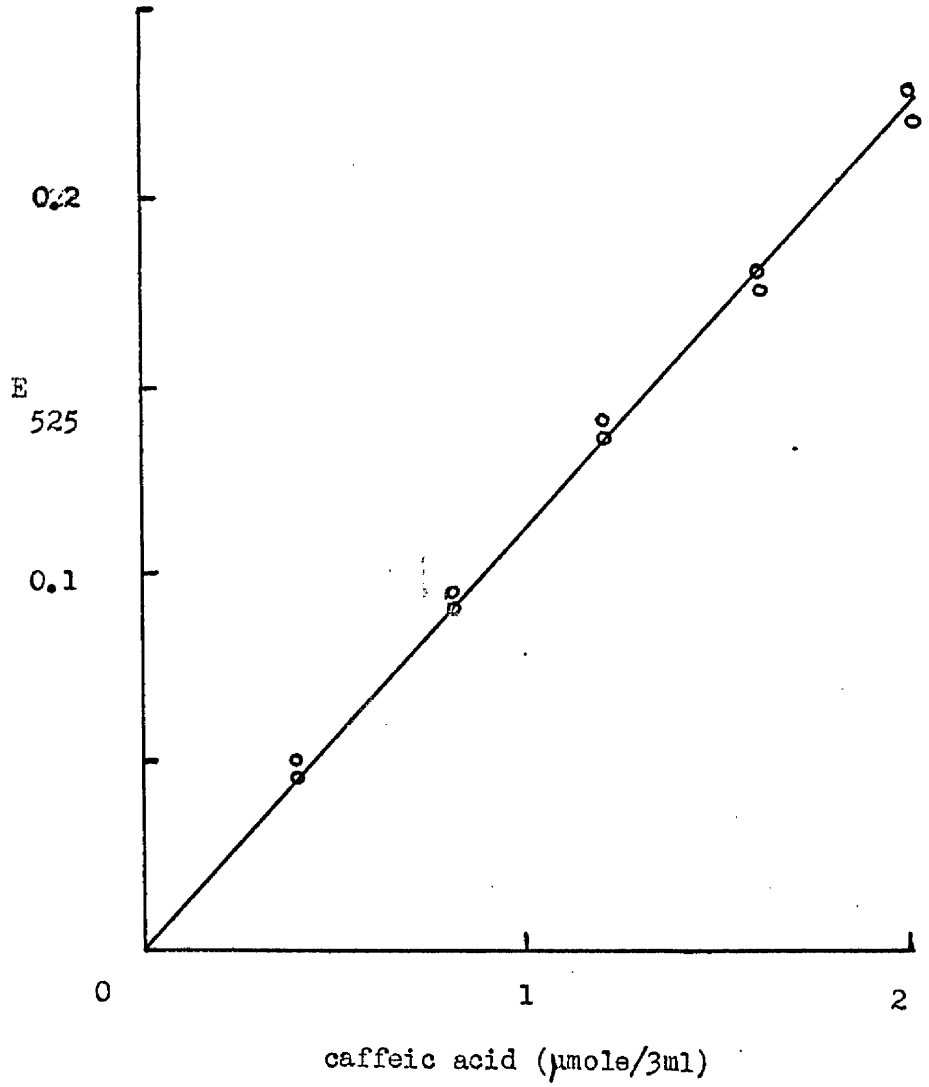
d) ascorbic acid and dehydroascorbic acid

Fig. 6. Standard curve for protein estimations.



Bovine plasma albumin standards were treated as described (2.2.3) and the E_{750} values were corrected for zero blank.

Fig. 7. Standard curve for colorimetric estimation of caffeic acid.



Caffeic acid standards were treated as described (2.2.4.1.) and the E_{525} values obtained were corrected for zero blank.

in 0.1M Na_2HPO_4 - 0.05 M citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$ (Fig 8). Spectra were obtained using a Cary 15 spectrophotometer (Cary Inst., California, U.S.A.). From these it was apparent that caffeic acid could be detected in the presence of p-coumaric acid at 340 nm (Fig 8a) and hence measurements at this wavelength were used to determine caffeic acid production with ascorbic acid as reducing agent. Comparison of the absorbance changes observed in the absence of electron donor, at 340 and 420 nm (Fig 9) shows that a linear rate of reaction was observed at 340 nm after 4.75 min. The initial velocity did not become linear at 420 nm until after 6 min. Thus the production of caffeic acid, in the absence of electron donor, could be detected earlier and with greater sensitivity at 340 nm. Both DMDP and NADH (Fig 8b,c) absorb at this wavelength and to correct for this, absorption was monitored at 340 and 370 nm in the case of DMTP, and 240 and 370 nm when the reducing agent present was NADH .

Changes in extinction at these wavelengths were related to caffeic acid concentration using the following expressions.

The relationship between caffeic acid concentration and change in absorbance is derived from equation (i)

$$\Delta E_{\lambda_{nm}} = \sum \epsilon_i . c_i^t - \epsilon_i . c_i^o \qquad \text{(i)}$$

where λ_{nm} is wavelength (nm) at which absorbance change is measured ϵ_i^{λ} is molar extinction coefficient for each species absorbing at nm and C_i^o and C_i^t are the concentrations (mol/litre) of species at times 0 and t seconds respectively.

Ascorbate

λ = 340 nm. p-coumaric acid and caffeic acid are the only components in the assay absorbing at 340 nm.

Fig. 8. Absorption spectra.

All spectra were determined under the conditions described (2.2.4.2).

a) ———— 0.067 mM caffeic acid
--------- 0.067 mM p-coumaric acid
— — — 0.188 mM oxidised caffeic acid - produced by reaction of phenolase on caffeic acid in the absence of electron donor.

b) ———— 0.54 mM DMTP
------- 0.54 mM DMDP

c) ———— 0.017 mM NADH
------ 0.017 mM NAD^+

d) ———— 0.163 mM ascorbic acid
------- 0.163 mM dehydroascorbic acid

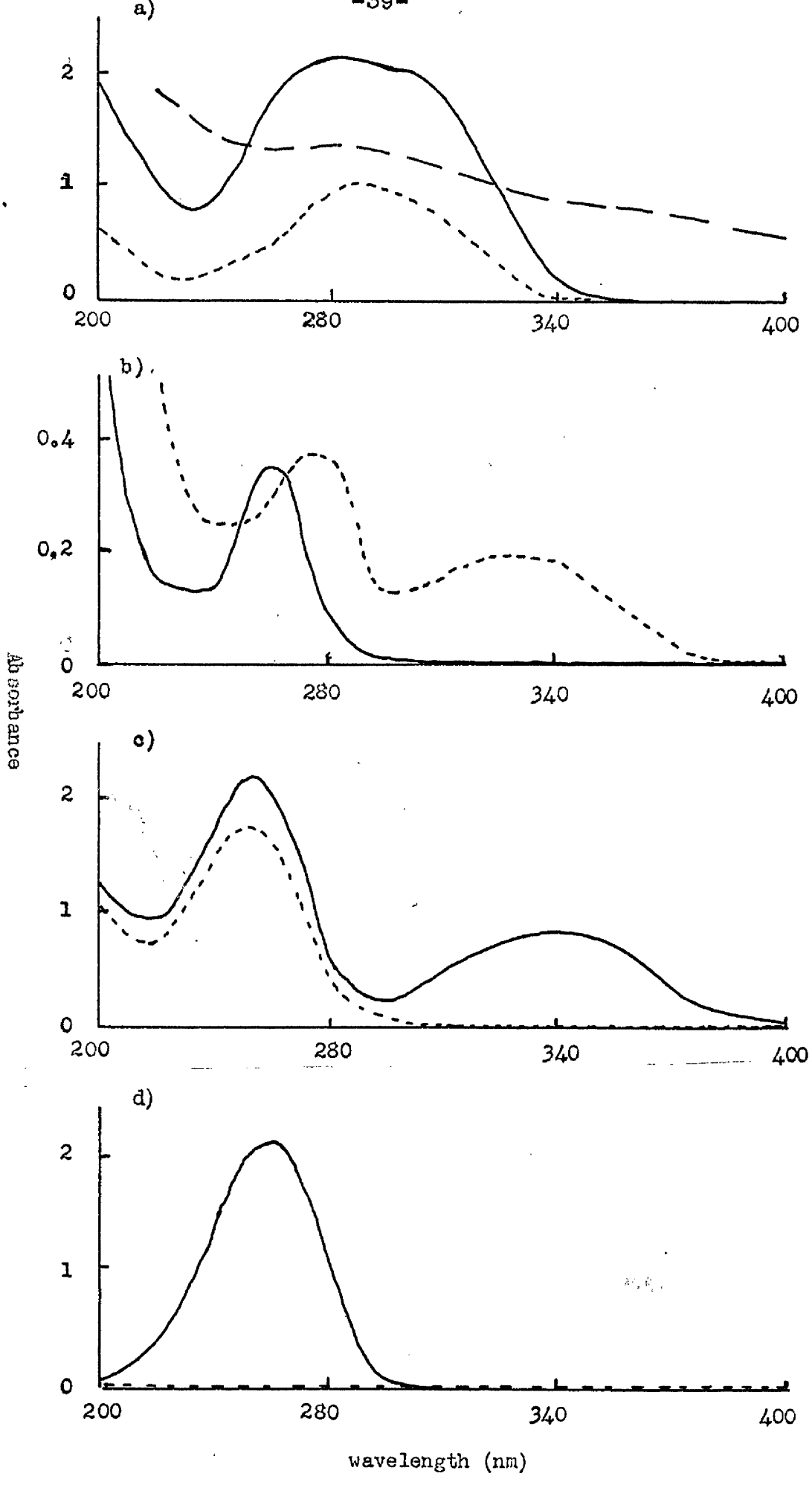
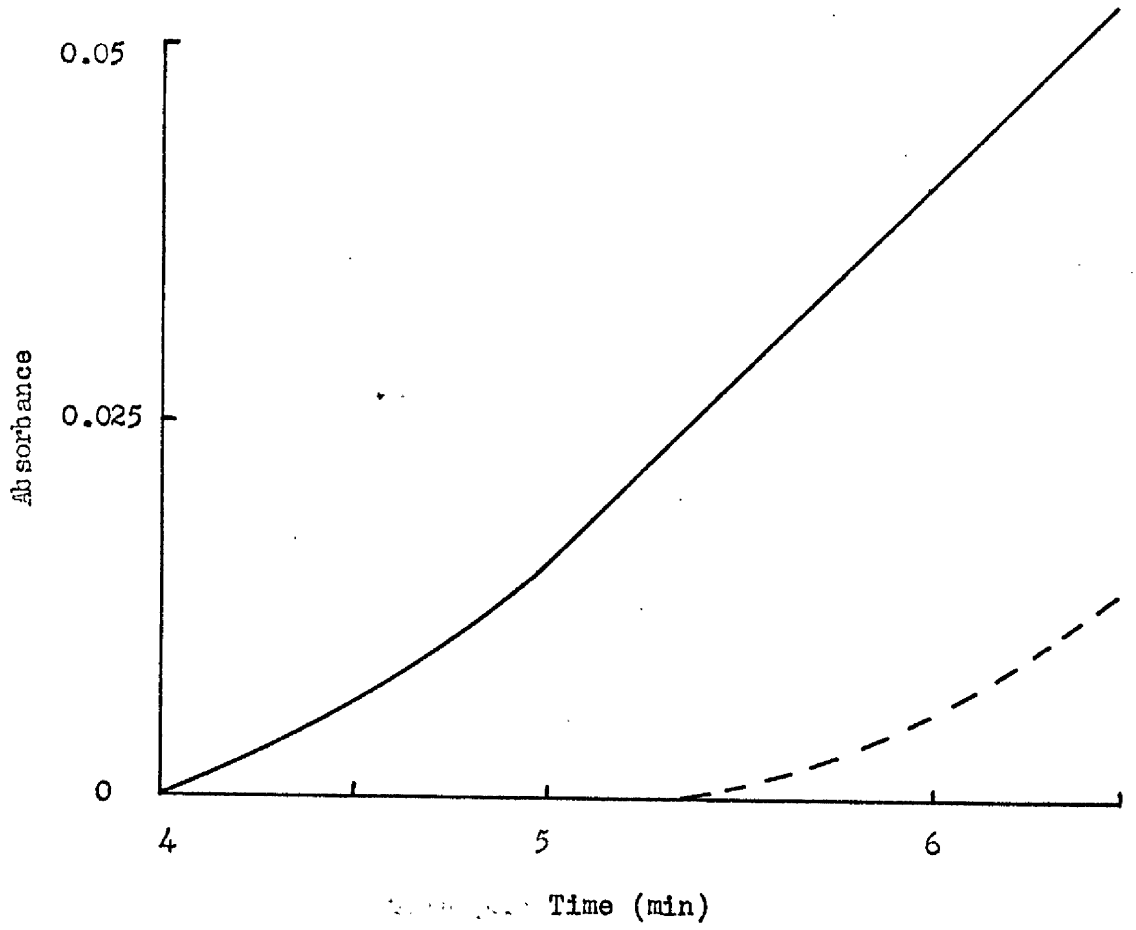

a)
2
1
0
200
280
340
400
b)
0.4
0,2
0
200
280
340
400
Absorbance
c)
2
1
0
200
280
340
400
d)
2
1
0
200
280
340
400
wavelength (nm)

Fig. 9. Comparison of absorbances detected at 340 and 420 nm.

1.67 mM p-coumaric acid was incubated with 76 m units of phenolase under standard conditions for continuous assay. The reaction was carried out in the absence of electron donor, the change in absorbance being followed at (———) 340 and (- - - -) 420 nm simultaneously.


0.05
0.025
0
Absorbance
4
5
6
Time (min)

From (i)

$$\Delta E_{340nm} = \epsilon^{340}_{pCA}.C^{t}_{pCA} + \epsilon^{340}_{caf}.C^{t}_{caf} - \epsilon^{340}_{pCA}.C^{o}_{pCA} - \epsilon^{340}_{caf}.C^{o}_{caf}$$

From the stoichiometry of the hydroxylation reaction (Vaughan and Butt, 1969), and since $C^{o}_{caf} = 0$.

$$C^{t}_{pCA} = C^{o}_{pCA} - C^{t}_{caf} \qquad \text{(ii)}$$

$$\Delta E_{340nm} = \epsilon^{340}_{caf}.C^{t}_{caf} - \epsilon^{340}_{pCA}.C^{t}_{caf}$$

$$\text{and} \quad C^{t}_{caf} = \frac{E_{340nm}}{(\epsilon^{340}_{caf} - \epsilon^{340}_{pCA})}$$

By substitution of molar extinction coefficients from Table 3,

$$C^{t}_{caf} = \frac{E_{340nm}}{2.61 \times 10^{3}} \quad M.$$

DMTP.

In addition to caffeic and p-coumaric acids, DMDP absorbs at 340nm. Absorbance changes at 370 nm due to DMDP were therefore determined and used to correct the 340 nm absorbance change for DMDP. From (i) and (ii)

$$\Delta E_{340} = \epsilon^{340}_{caf}.C^{t}_{caf} - \epsilon^{340}_{pCA}.C^{t}_{caf} + \epsilon^{340}_{DMDP}(C^{t}_{DMDP} - C^{o}_{DMDP}) \qquad \text{(iii)}$$

$$\Delta E_{370} \simeq \epsilon^{370}_{caf}.C^{t}_{caf} - \epsilon^{370}_{pCA}.C^{t}_{caf} + \epsilon^{370}_{DMDP}(C^{t}_{DMDP} - C^{o}_{DMDP}) \qquad \text{(iv)}$$

Since $C^{o}_{DMDP} = 0$, from (iii)

$$C^{t}_{DMDP} = \frac{\Delta E_{340} - C^{t}_{caf}\,(\epsilon^{340}_{caf} - \epsilon^{340}_{pCA})}{\epsilon^{340}_{DMDP}} \qquad (v)$$

Substitution of (v) into (iv) and rearrangement gives

$$C^{t}_{caf} = \frac{\epsilon^{340}_{DMDP}\Delta E_{370} - \epsilon^{370}_{DMDP}\Delta E_{340}}{\epsilon^{340}_{DMDP}\left[\epsilon^{370}_{caf} - \epsilon^{370}_{pCA}\right] - \epsilon^{370}_{DMDP}\left[\epsilon^{340}_{caf} - \epsilon^{340}_{pCA}\right]}$$

By substitution of values for molar extinction coefficients from Table 3,

$$C^{t}_{caf} = (0.403\Delta E_{340} - 0.85\Delta E_{370}) \times 10^{-3} M$$

NADH

Convenient wavelengths for following hydroxylation in the presence of NADH were 240 nm and 370 nm. From equations (i) and (ii) and as

$$C^{t}_{NAD^+} = C^{o}_{NADH} - C^{t}_{NADH}$$

then

$$E_{370} = C^{t}_{caf}\left[\epsilon^{370}_{caf} - \epsilon^{370}_{pCA}\right] - \epsilon^{370}_{NADH}.C^{t}_{NAD^+} \qquad (vi)$$

and

$$E_{240} = C^{t}_{caf}\left[\epsilon^{240}_{pCA} - \epsilon^{240}_{pCA}\right] - C^{t}_{NAD}\left[\epsilon^{240}_{NADH} - \epsilon^{240}_{NAD}\right] \qquad (vii)$$

from (vi)

$$C^{t}_{caf} = \frac{C^{t}_{caf}\left[\epsilon^{370}_{caf} - \epsilon^{370}_{pCA}\right] - \Delta E_{370}}{\epsilon^{370}_{NADH}}$$

Substitution in (vii) and rearrangement gives

$$C^{t}_{caf} = \frac{\epsilon^{370}_{NADH}\Delta E_{240} + \left[\epsilon^{240}_{NAD} - \epsilon^{240}_{NADH}\right]\Delta E_{370}}{\epsilon^{370}_{NADH}\left[\epsilon^{240}_{caf} - \epsilon^{240}_{pCA}\right] + \left[\epsilon^{240}_{NAD} - \epsilon^{240}_{NADH}\right]\left[\epsilon^{370}_{caf} - \epsilon^{370}_{pCA}\right]}$$

Substitution of values for molar extinction coefficients from Table 3 gives,

$$C^{t}_{caf} = (1.16\Delta E_{240} + 0.85\Delta E_{370}) \times 10^{-4}M$$

Throughout this analysis pCA represents p-coumaric acid and caf represents caffeic acid.

2.2.4.2.2. Determination of molar extinction coefficients.

The values of the molar extinction coefficients at 240, 340 and 370 were calculated from the absorbance at these wavelengths of 0.79 mM p-coumaric acid, and 0.44 mM caffeic acid, in 0.1 M Na_2HPO_4 - 0.05 citric acid buffer pH 6.5 and 0.5M. $(NH_4)_2SO_4$.

DMTP was oxidised to DMDP by Hydrogen peroxide (Nielsen, 1969). Hydrogen peroxide (800 µmoles) and catalase (600 units) were added to DMTP in 0.1M Na_2HPO_4 - 0.05 M citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$ in a total volume of 3 ml. When oxygen evolution ceased, the absorbance was measured and a linear relationship was observed between absorbance and concentration up to 1.4 mMDMTP (Fig. 10.). The molar extinction coefficient was calculated from this relationship.

Literature values (Data for Biochemical Research) of the molar extinction coefficients for NAD and NADH were used to determine the concentrations of samples of stock solutions under the conditions quoted. Similar samples were then scanned in 0.1M Na_2HPO_4 - 0.05 citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$ and the corrected molar

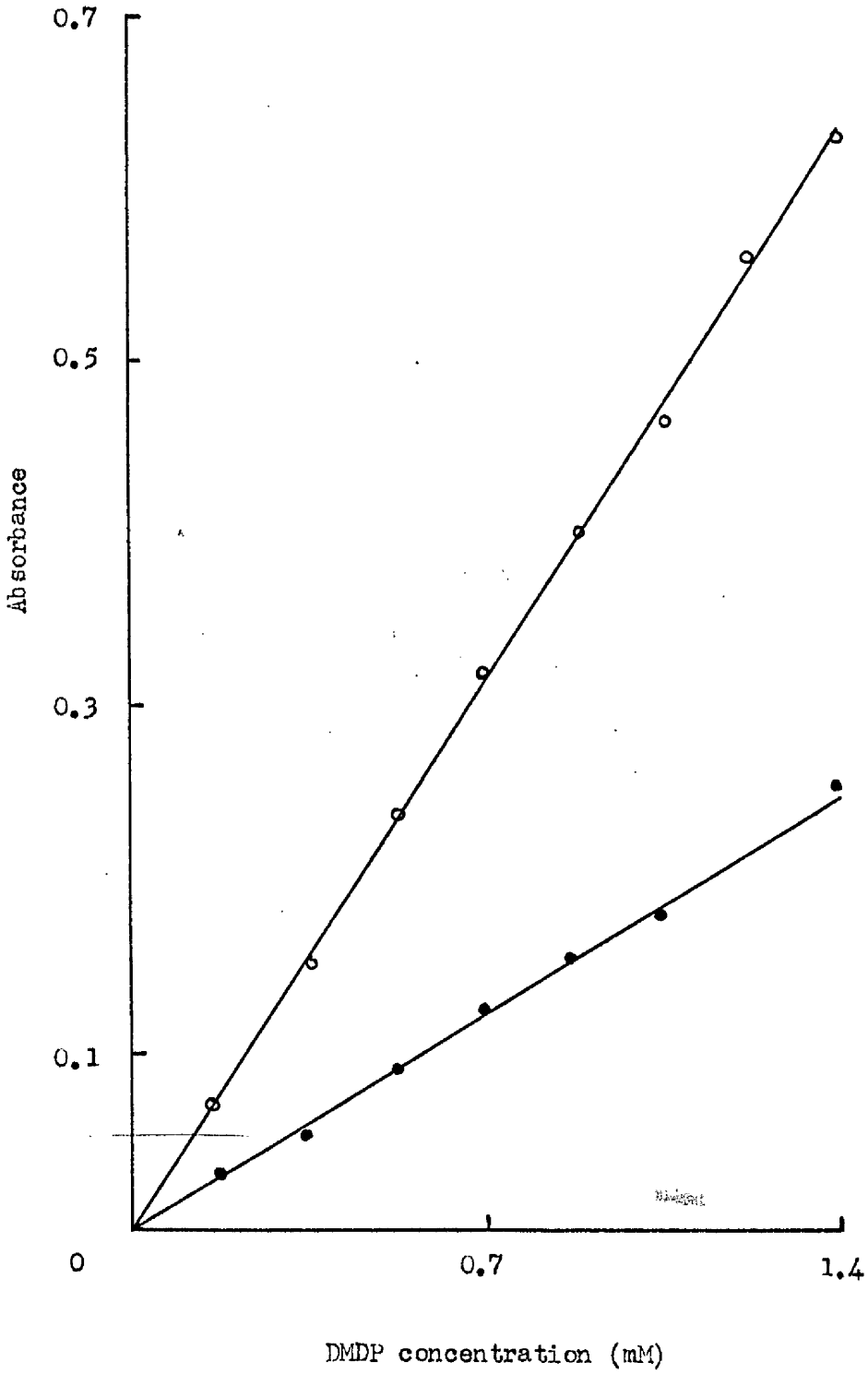
Table 3. Molar extinction coefficients for p-coumaric acid, caffeic acid, DMDP, NADH and NAD at wavelengths used in the spectrophotometric assay.

Molar extinction coefficients were determined by procedures described in Materials and Methods section (2.2.4.2.2.)

Component	λ nm	ϵ_λ (litre $mol^{-1}.cm^{-1}$)
p-coumaric acid	240	2.37×10^3
	340	3.84×10^2
	370	7.58
caffeic acid	240	1.09×10^4
	340	2.99×10^3
	370	6.63×10
DMDP	340	3.68×10^2
	370	1.75×10^2
NADH	240	6.92×10^3
	370	2.4×10^3
NAD^+	240	8.67×10^3

Fig. 10. Standard curve for DMDP estimation.

The standard curve was obtained as described (2.2.4.2.2.), sample absorbance being read at (○) 340 and (●) 370 nm, and corrected for zero blank.



extinction coefficients evaluated. The values obtained in all cases are shown in Table 3.

2.2.5. Assay Conditions.

The formation of caffeic acid was used as a measure of the hydroxylation activity of phenolase at 30°C, under aerobic conditions. The rate of caffeic acid production was determined in two ways.

1) Intermittant Assay. The reaction was carried out in 25ml conical flasks in a shaking water bath, samples being removed for colorimetric assay of caffeic acid. This method was used during enzyme purification in the determination of specific activities.

2) Continuous Assay. The reaction was carried out in quartz cuvettes in the thermostated cell compartment of a spectrophotometer. Caffeic acid production was determined continuously, using the appropriate spectrophotometric assay. Initial rate measurements for kinetic treatment were all obtained using this method.

2.2.5.1. Intermittent Assay.

Phenolase was incubated in 0.1M Na_2HPO_4 - 0.05M citric acid buffer pH 5.3 containing 3.33 mM p-coumaric acid, 3.33mM ascorbic acid and 0.5M $(NH_4)_2SO_4$, in a total volume of 3ml. 0.5ml samples were removed into 2ml 5% v/v acetic acid, at 0, 5 and 10 min., thus stopping the reaction. Any precipitated material obtained with crude enzyme extracts was removed by centrifugation (15 min. at 2,000 g in an MSE Super Medium) and the caffeic acid content determined. (2.2.4.1.) The enzyme unit has previously been defined (Vaughan and Butt, 1969) as the amount required to produce 1 μmole of caffeic acid per minute under these conditions.

2.2.5.2.1. Continuous Assay.

Enzyme was incubated in 0.1M Na_2HPO_4- 0.05M citric acid buffer pH 6.5 containing 0.5M $(NH_4)_2SO_4$, oxygen (0.056 - 1.12 mM), p-coumaric acid (0.166 - 3.33 mM) and either ascorbic acid (0.017 - 3.33mM), DMTP (0.13-1.0 mM), NADH (0.01 - 0.05 nm) or no electron donor. 3.33mM EDTA was included in ascorbic acid assays, while 200 units catalase /ml were added to reaction mixtures containing DMTP. Assays were carried out in 3ml, in 10mm cuvettes in a Beckman DB spectrophotometer with medium slit width, and with the cell compartment thermostatted to ±0.05°C by a Haake Cryostat F 4291 (Haake, Berlin, Germany.) High concentrations of p-coumaric acid and NADH resulted in strongly absorbing solutions, causing loss of resolution in the spectrophotometer, but short path length cells (e.g. 5 mm, 2 mm) and assay conditions equivalent to a total volume of 3 ml were used to overcome this problem.

The reference cuvette contained all assay components except enzyme. The reaction was started by addition of phenolase, and the cell contents mixed by aeration for 5s. Changes in extinction were measured as digital output by means of an Optilab Multilog and Printer Driver linked to an Addo X printer (Techmation Ltd., 58 Edgeware way, Edgware, Middlesex, HA8 8JP, U.K.)

Dual wavelength assays (those involving DMTP and NADH) could be carried out either by using duplicate cuvettes, or by altering the wavelength setting of the spectrophotometer between readings, (every 5s), obtaining absorbance values for both wavelengths from one cuvette. It was thought that the latter was more accurate as pipetting errors were minimised and, as the spectrophotometer wavelength adjustment was modified to set at the appropriate wavelengths (370 and 340, 370 and 240), errors in these settings were eliminated.

2.2.5.2.2. Variation of oxygen concentration.

As has already been stated (2.2.5.2.1.) the initial concentration of oxygen present in the reaction cuvette ranged from 0.056 to 1.12 mM. This was achieved as shown in Fig. 11. The ratio of oxygen free nitrogen to air or pure oxygen was varied by adjusting the gap meters and, after mixing, the gas was bubbled through a thermostated cell containing all the reaction components except enzyme and electron donor. The percentage of oxygen present was read using a Beckman oxygen electrode, and the oxygen content was expressed in moles/litre using the known oxygen content of oxygen saturated water at 30°C (1.12 mM: Handbook of Chemistry and Physics, 1948) and assuming that the relationship between concentration of oxygen in solution and percentage of oxygen in the gas phase was linear over the range of concentrations used. It was assumed that this gas mixture was equivalent to that passed through the assay cuvette.

Reaction components were equilibrated with gas mixtures for 5 min, as equilibration with oxygen free nitrogen for this time was shown to prevent any reaction occurring.

2.2.6. Measurement of Oxygen uptake.

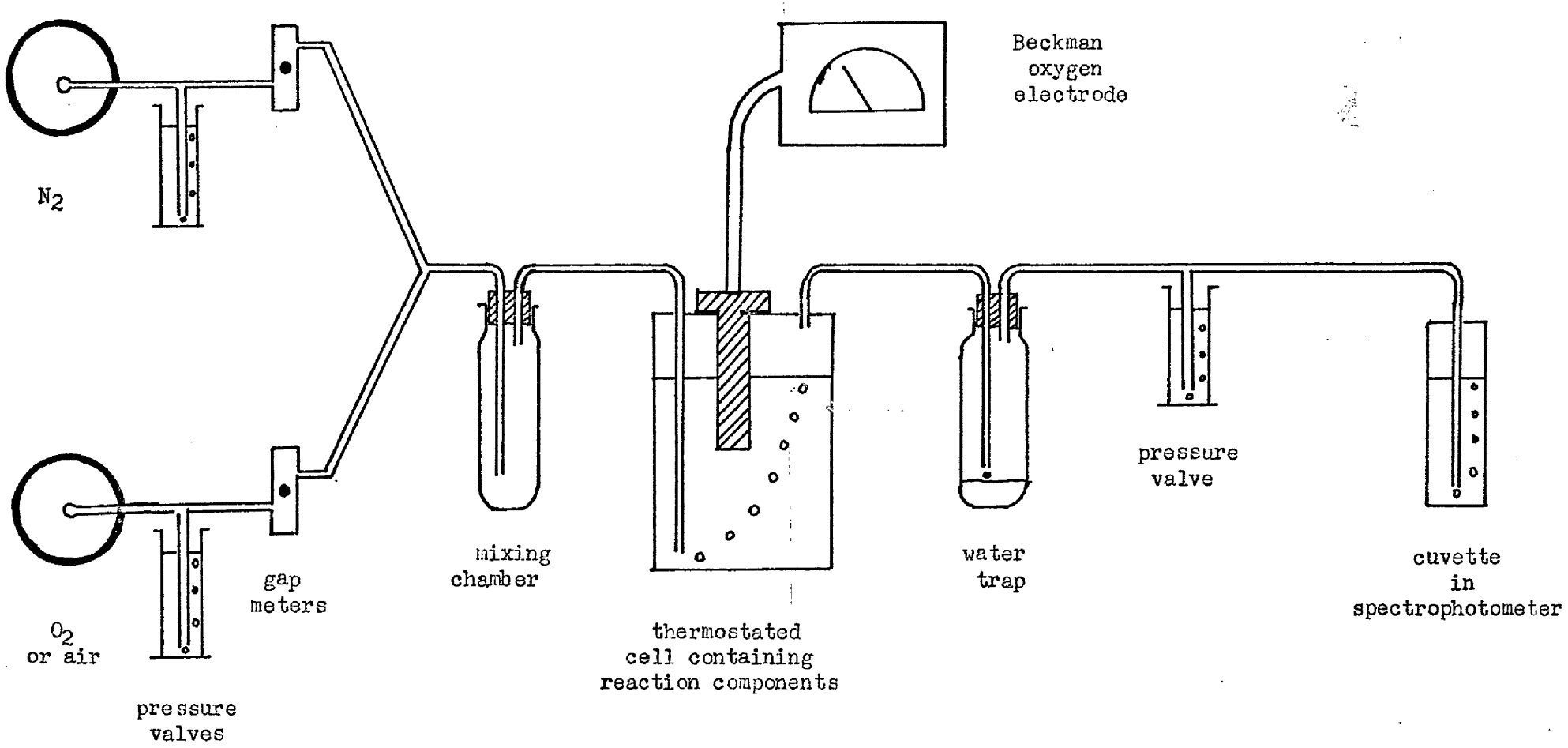
The rate of oxygen utilisation during the reaction at 30°C can be monitored using a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) coupled to a Servoscribe chart recorder (Smiths Industries Ltd., Wembley, Middlesex, U.K.) The recorder was calibrated by the method of Robinson and Cooper (1970.) This method was used in two ways.

1) as an assay for the catechol oxidase activity of phenolase.

2) to determine whether substrate and product analogues were in fact alternate substrates, and to determine their reactivity compared with that of p-coumaric acid and caffeic acid.

Fig. 11. Variation of oxygen concentration.

(not drawn to scale)



2.2.6.1. Catechol oxidase assay.

Caffeic acid (0.033 - 1.33 mM) was added to 0.1M Na_2HPO_4 - 0.05 M citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$ in a total volume of 3 ml, assay components equilibrated for 15 min, and the reaction started by addition of enzyme.

2.2.6.2. Check on reactivity of substrate analogues.

In this case, either caffeic acid (0.267 - 1.33 mM), p-coumaric acid (0.83 - 5 mM), p-hydroxybenzoic acid (2.5 - 10 mM) or 3,4-dihydroxybenzoic acid (1.08 - 4.33 mM) was added to 0.1M Na_2HPO_4 - 0.05 M citric acid buffer pH 6.5, containing 0.5M $(NH_4)_2SO_4$ and 3.33 mM ascorbic acid. The reaction was carried out as described (2.2.6.1.)

2.2.7. Inhibitor studies.

The assay conditions used were those already defined (2.2.5.2.1.), but varying amounts of the inhibitors p-hydroxybenzoic acid (0.66 - 2.60 mM), 3,4 - dihydroxybenzoic acid (0.33 - 3.33mM), benzoic acid (0.33 - 3.33 mM), carbon monoxide (0.05 - 0.33mM), DIECA (1.0 - 10.0 µM) and bathocuproine sulphonate (BCS; 0.033 - 0.33mM) were added. The required carbon monoxide concentration was obtained by the addition of varying amounts of a saturated solution of the gas, which was assumed to be 2 mM. Ascorbic acid was used as electron donor throughout these experiments.

III. RESULTS.

3.1. Preliminary Studies

3.1.1. Enzyme purification

Table 4 summarises the results of a typical enzyme purification (2.2). The enzyme was purified 1,600 fold compared with the crude extract with recovery of 33% of the hydroxylase activity. Polyacrylamide gel electrophoresis of the enzyme after hydroxyapatite chromatography shows one band, staining for both protein and enzyme (Fig. 12). This enzyme fraction, homogeneous by these criteria, was used in all experiments, the specific activity of the sample being 30,200 m units /mg protein.

3.12. Comparison of colorimetric and spectrophotometric assays.

The agreement between the colorimetric and spectrophotometric methods for the estimation of caffeic acid is within 10% with ascorbic acid, DMTP or NADH as electron donor (Table 5a) and in the absence of electron donor (Table 5b). This has led to routine use of the spectrophotometric assay for determining initial rates of caffeic acid production.

3.1.3. Determination of assay conditions

3.1.3.1. Effect of pH.

In the presence of 0.5M $(NH_4)_2SO_4$ two approximately equal pH maxima, at pH 6.5 and pH 7.5, were detected using the colorimetric method of caffeic acid estimation (Fig. 13a), as pH was found to affect the absorption due to caffeic acid (Fig. 13b). Hence the spectrophotometrid assay could not be used unless the molar extinction coefficients involved were evaluated at each pH. All subsequent assays were carried out at pH 6.5 for maximum activity with minimum

Table 4. Enzyme Purification

Details of the purification are given in Methods Section (2.2.1.)

Sample	Volume (ml)	Hydroxylase activity (m units/ml)	Total units	Protein (mg/ml)	Total Protein (g)	Specific activity (m units/mg)	% Purification	% Recovery
Crude macerate	17,000	52.37	899.4	2.76	46.9	18.9	1	100
Supernatant from 0-35% saturated $(NH_4)_2SO_4$	16,000	55.43	886.9	0.72	11.52	77.3	4.1	99.6
Precipitate from 35-70% saturated $(NH_4)_2SO_4$	1,500	384.5	576.8	4.0	6.0	96.1	5.1	70
Heat treatment and dialysis	2,000	227.1	454.3	0.26	0.561	878.9	46.5	52
Eluate from CM Cellulose	150	1,893	284.0	0.157	0.024	12,058	638	34
Eluate from Hydroxyapatite	81	3,378	273.6	0.112	0.009	30,200	1,598	33

Fig. 12. Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis was carried out, and gels stained as described in Methods section (2.2.2.)

a) protein stain

b) enzyme stain

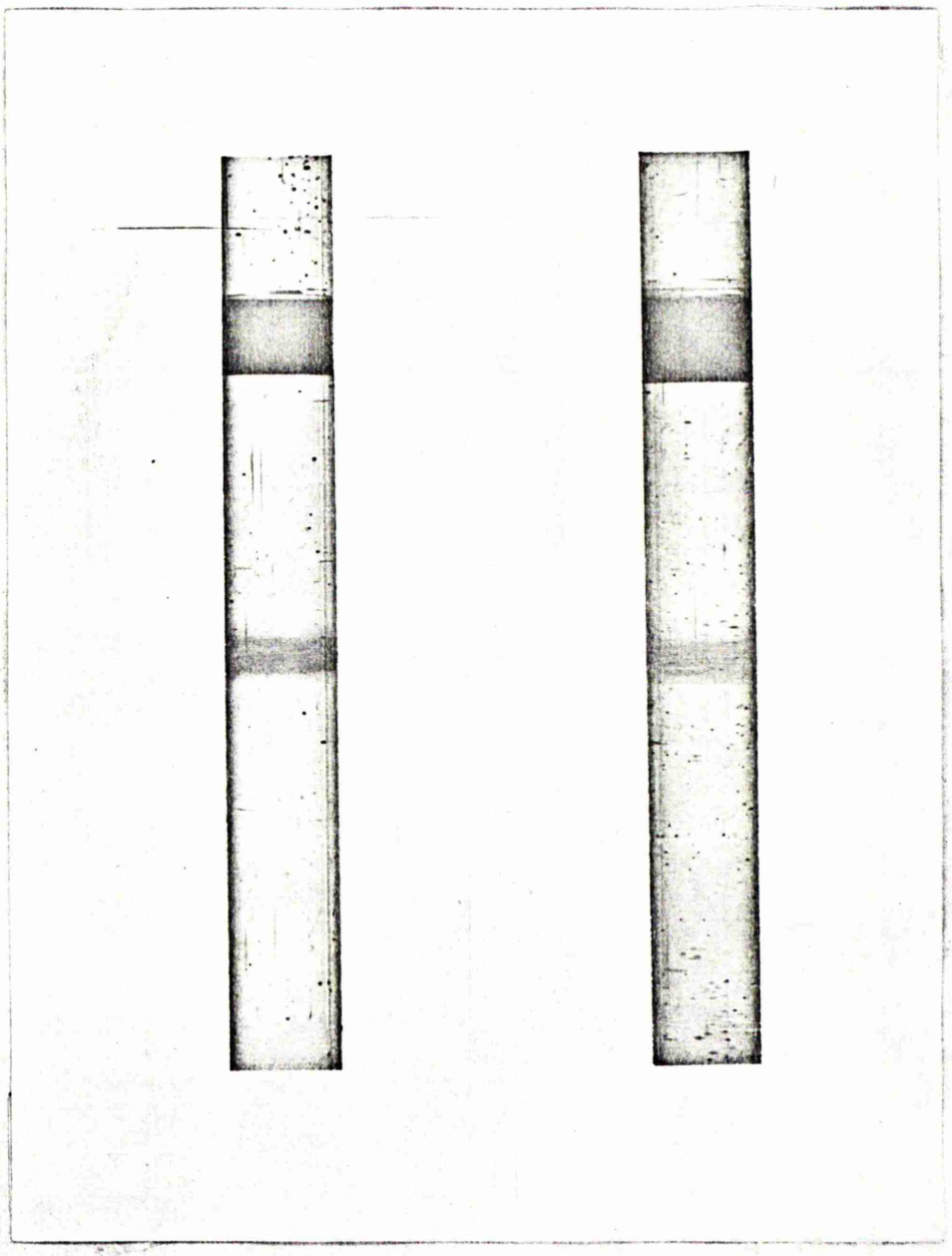


Table 5. Comparison between spectrophotometric and colorimetric methods for caffeic acid determination.

a) 3.33 mM p-coumaric acid was incubated with either 3.33 mM ascorbic acid, 3.33 mM NADH or 1 mM DMTP, and 76 m units of enzyme, in 0.1 M Na_2HPO_4- 0.05M citric acid buffer, pH 6.5, and 0.5 M $(NH_4)_2SO_4$ in a total volume of 6 ml under the conditions for the intermittent assay (2.2.5.1.). 0.5ml samples were removed, at the times stated, into 2ml 5% (v/v) acetic acid (C), or 5ml ice cold 0.1M Na_2HPO_4- 0.05M citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$ (S). Caffeic acid content was estimated by either the colorimetric method C (2.2.4.1.) or the spectrophotometric method S (2.2.4.2.). Results are expressed as µmole/3ml and are corrected for zero blank.

b) 3.33 mM p-coumaric acid was incubated with 76 m units enzyme under standard conditions for the continuous assay (2.2.5.2.). Once a linear rate of reaction was obtained - after 5.5 min - the absorbance was read, and the sample was then tipped into 0.1 ml of glacial acetic acid. The caffeic acid content was estimated by the colorimetric method C (2.2.4.1.), and by applying the derived formula for the spectrophotometric assay (2.2.4.2.) to the absorbance value obtained (S). The experiment was carried out in triplicate and the results expressed as µmole/3ml.

a)

Incubation time (min)	Electron Donor					
	Ascorbic Acid		NAIH		DMTP	
	C	S	C	S	C	S
2	0.10	0.11	0.07	0.06	0.15	0.15
4	0.18	0.19	0.14	0.13	0.19	0.17
6	0.26	0.25	0.23	0.21	0.22	0.24
8	0.33	0.33	0.33	0.32	0.29	0.28
10	0.36	0.36	0.40	0.40	0.35	0.35

b)

C	S
0.0477	0.0508
0.0477	0.0488
0.05	0.0546

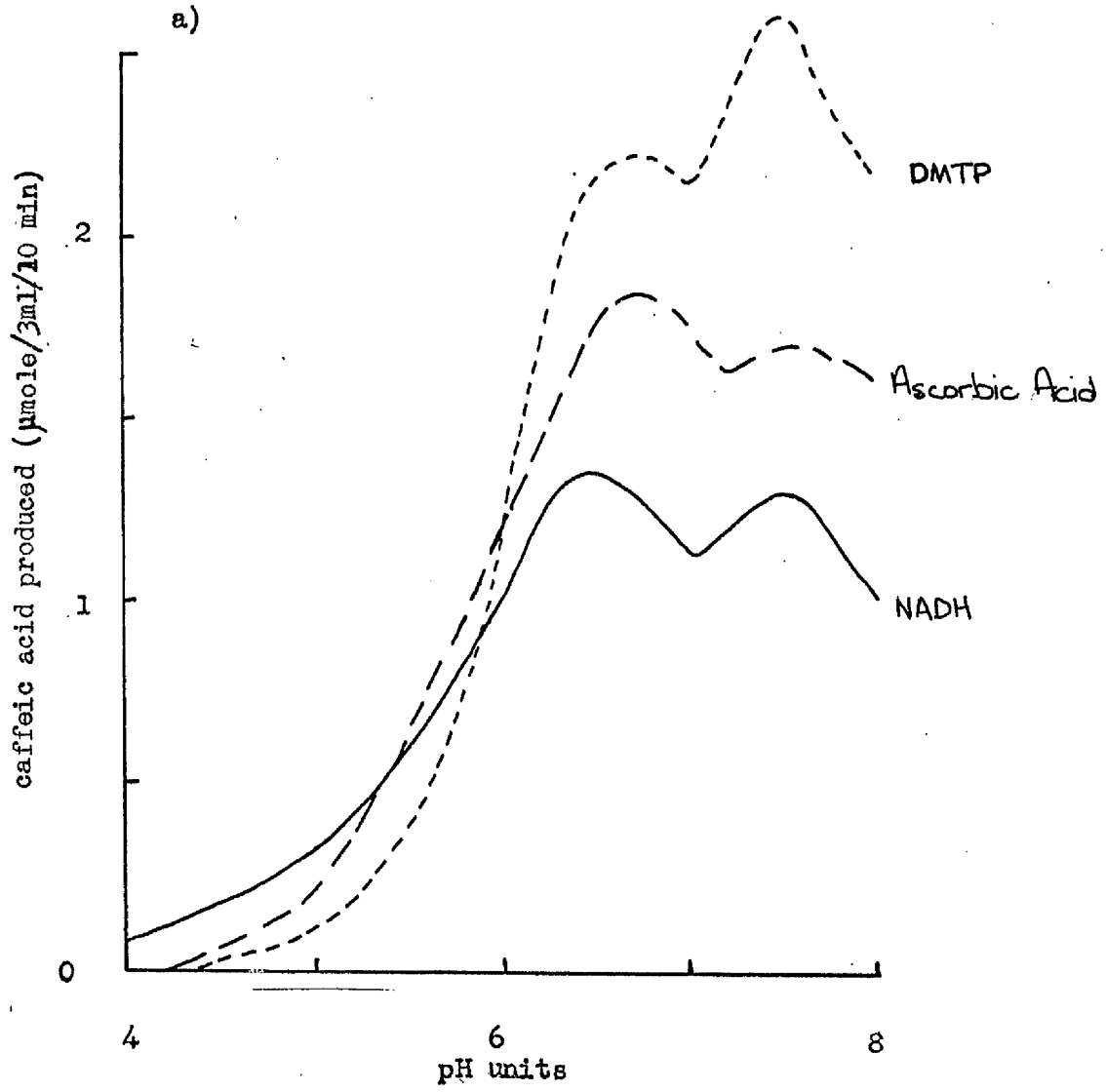
Fig. 13

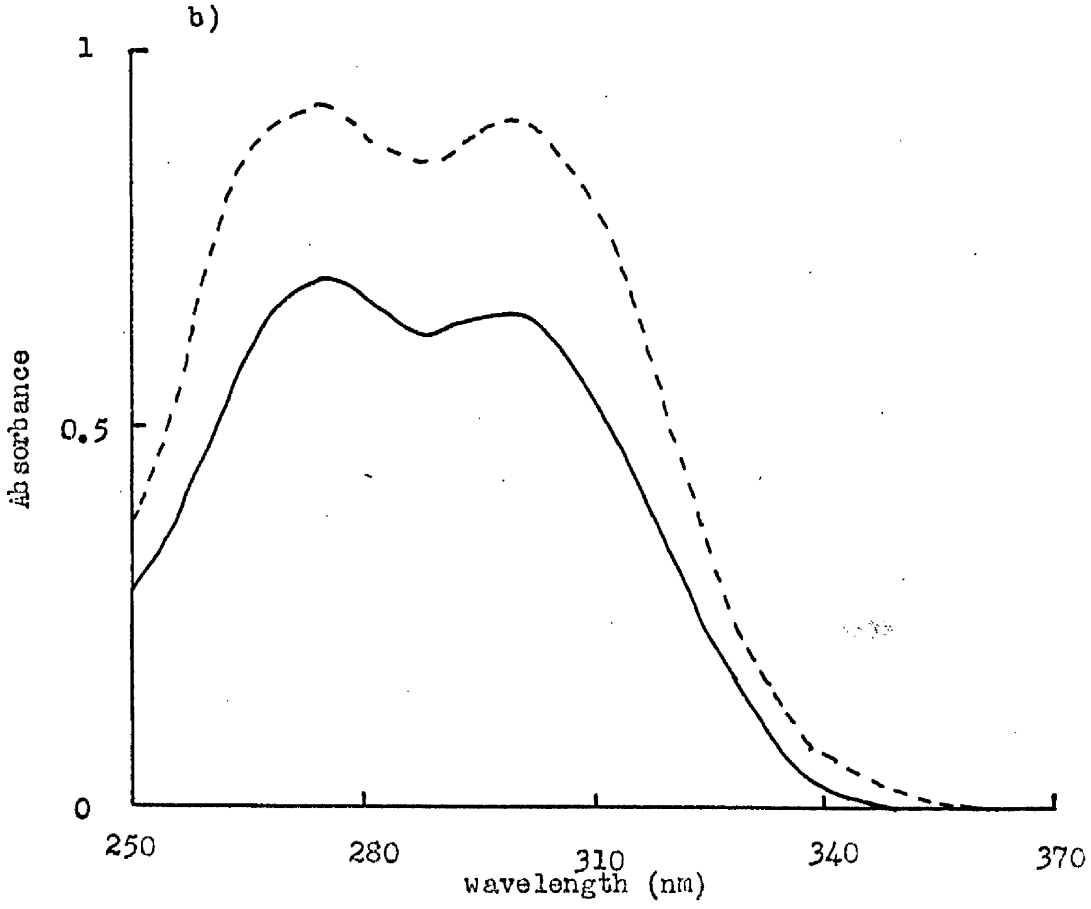
a) Effect of pH on reaction rate.

p-coumaric acid (1.67mM) was incubated with 3.33 mM ascorbic acid (76 m units enzyme), 1 mM DMTP (38 m units enzyme) and 3.33 mM NADH (95 m units enzyme), in 0.5M $(NH_4)_2SO_4$ at various pHs in the range 4-8. Experiments were carried out under the conditions for intermittent assay (2.2.5.1.) and caffeic acid was estimated colorimetrically (2.2.4.1.).

b) Effect of pH on spectrum of caffeic acid.

The absorption spectrum of 0.01 mM caffeic acid was determined in the presence of 0.5M $(NH_4)_2SO_4$ and 0.1M Na_2HPO_4 - 0.05M citric acid buffer, pH 5.3 (- - - -), or 0.1M Na_2HPO_4 - 0.05M citric acid buffer, pH 6.5 (——). Spectral measurements were made using a Cary 15 spectrophotometer.


a)
caffeic acid produced (µmole/3ml/10 min)
2
1
0
DMTP
Ascorbic Acid
NADH
4
6
8
pH units


b)
Absorbance
1
0.5
0
250
280
310
340
370
wavelength (nm)

alkali catalysed auto-oxidation of DMTP (Nielsen, 1969), and ascorbate (Butt and Hallaway, 1961).

3.1.3.2. Effect of catalase and EDTA.

In the presence of ascorbic acid or DMTP, catalase was shown to inhibit the rate of caffeic acid formation at pH 6.5, by 32% and 20% respectively. Addition of EDTA resulted in an inhibition of 29% (Fig. 14) with ascorbic acid as electron donor. Neither catalase nor EDTA affected the reaction velocity with NADH as electron donor (Table 6), when the reaction was followed by determining the rate of NADH utilisation at 370 nm. Although this could encompass changes due to both the cresolase and catechol oxidase activities of phenolase, it was taken as a measure of the rate of hydroxylation as, over the time course of the experiment, catechol oxidase activity is thought to be slight.

3.1.3.3. Effect of enzyme concentration.

A linear relationship (Fig. 15) was observed between rate of reaction and enzyme concentration up to 250 m units per assay in the presence of the three electron donors, or in their absence. All experiments were carried out within this range of enzyme concentration.

3.1.3.4. Substrate saturation

3.1.3.4.1. Hydroxylase assay

The variation of initial velocity with p-coumaric acid concentration in the presence of air and ascorbic acid (1.67 mM), DMTP (1mM), NADH (0.33mM) or no electron donor, is shown in Fig. 16a). 1.66 mM p-coumaric acid was sufficient to saturate the enzyme in the presence

Fig. 14 Effect of catalase and EDTA on reaction rate.

1.67 mM p-coumaric acid was incubated with 1mM DMTP and 76 m units enzyme in the presence of various amounts of catalase (●) under the conditions for spectrophotometric assay (2.2.5.2.). Similar experiments in the presence of 1.67 mM ascorbic acid were carried out in the presence of varying amounts of catalase (○) or EDTA (▲).

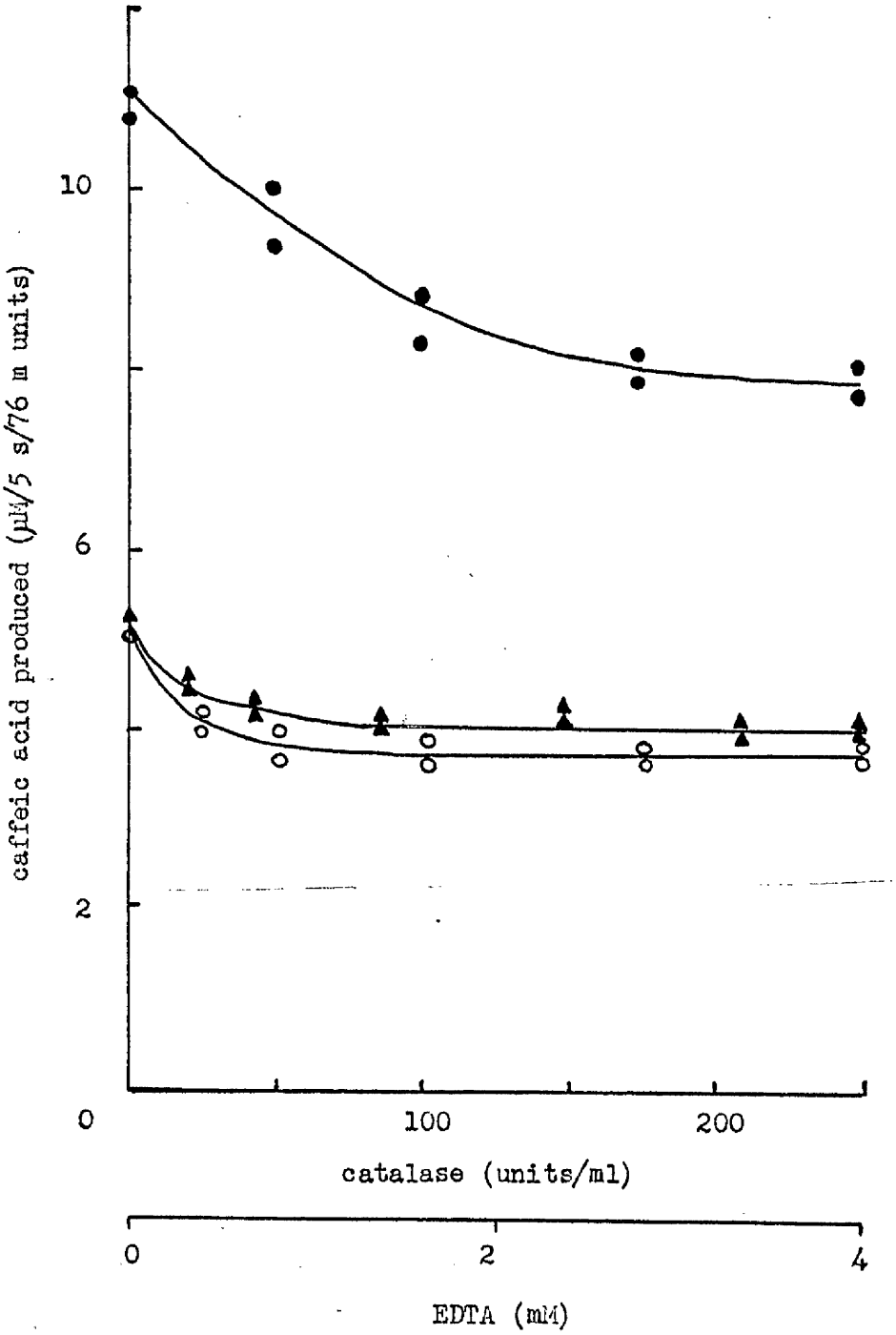

caffeic acid produced (µM/5 s/76 m units)
10
6
2
0
100
200
catalase (units/ml)
0
2
4
EDTA (mM)

Table 6. Effect of catalase and EDTA on NADH assay.

1.66mM p-coumaric acid was incubated with 0.66mM NADH and 76 munits of enzyme in 0.1M Na_2HPO_4 - 0.05M citric acid buffer pH 6.5 and 0.5M $(NH_4)_2SO_4$, in a total volume of 3 ml, under the conditions for continuous assay (2.2.5.2.), and the reaction followed at 370 nm. The assay was repeated in the presence of 3.33 mM EDTA, and 200 units/ml catalase.

Addition	Reaction Rate ΔE_{370}/ 10s	
-	0.072	0.073
3.33 mM EDTA	0.072	0.072
200 units/ml catalase	0.072	0.071

Fig. 15 Effect of enzyme concentration.

1.67 mM p-coumaric acid was incubated with varying amounts of phenolase in the presence of 1 mM DMTP (▲), 1.67 mM ascorbic acid (Δ), 0.33 mM NADH (●) and no electron donor (○) under the conditions for continuous assay (2.2.5.2.).

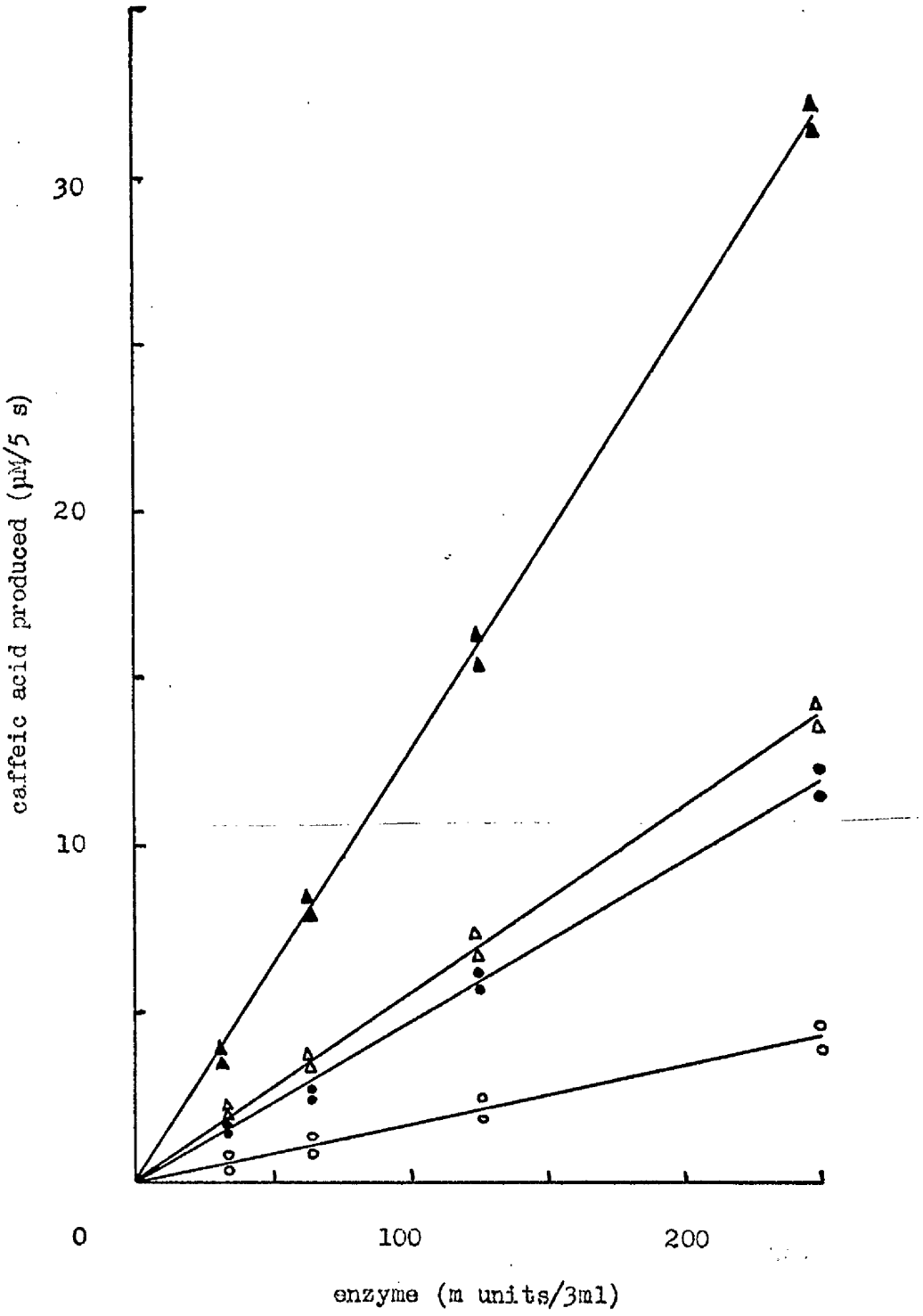

caffeic acid produced (μM/5 s)
30
20
10
0
100
200
enzyme (m units/3ml)

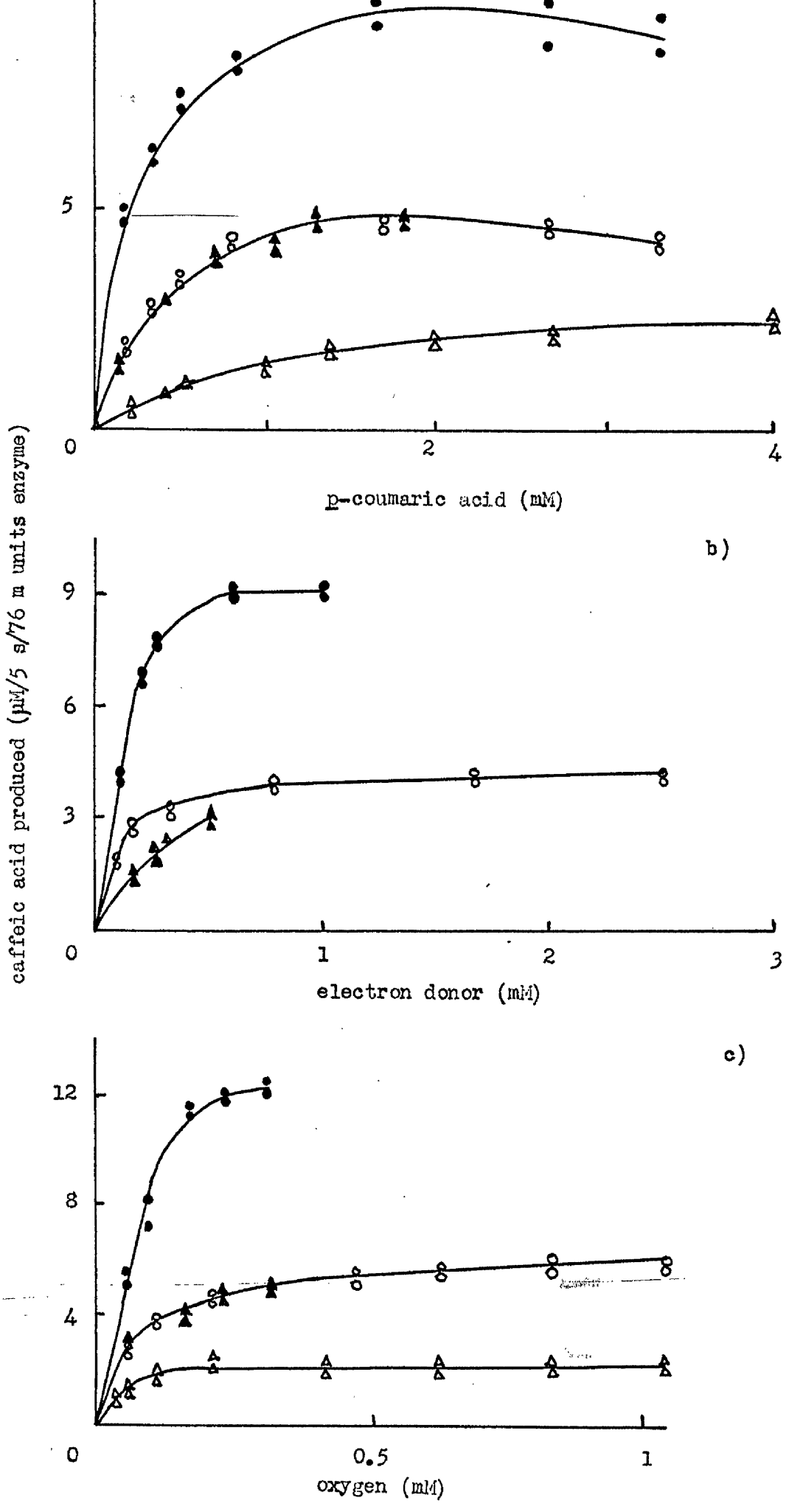
Fig. 16 Substrate saturation - hydroxylase activity.

76 m units phenolase were incubated under the conditions for continuous assay with

a) varying amounts of p-coumaric acid in the presence of air (0.230 mM oxygen) and 1 mM DMTP (●), 1.67 mM ascorbic acid (○), 0.33 mM NADH (▲) or no electron donor (Δ).

b) varying amounts of DMTP (●) and ascorbic acid (○) in the presence of air and 1.67 mM p-coumaric acid, and varying amounts of NADH (▲) in the presence of 0.92 mM p-coumaric acid and air.

or c) varying amounts of oxygen in the presence of 1.67 mM p-coumaric acid and 1 mM DMTP (●), 1.67 mM ascorbic acid (○), or no electron donor (Δ), and in the presence of 0.92 mM p-coumaric acid and 0.33 mM NADH (▲).


caffeic acid produced (µM/5 s/76 n units enzyme)
5
0
2
4
p-coumaric acid (mM)
b)
9
6
3
0
1
2
3
electron donor (mM)
c)
12
8
4
0
0.5
1
oxygen (mM)

of electron donor, but greater amounts (3.33 mM) were required in its absence. Slight inhibition was observed at concentrations of p-coumaric acid greater than 2 mM in the presence of DMTP and ascorbic acid. Saturation with electron donor, in air and 1.67 mM p-coumaric acid, (Fig 16b) was achieved with 1 mM DMTP and 1.66 mM ascorbic acid. The high absorbance of NADH at 240 nm prevented concentrations greater than 0.4 mM being used at the p-coumaric acid concentration used, 0.92 mM.

The graph of initial velocity against oxygen concentration (Fig. 16c) in the presence of 1.67 mM p-coumaric acid and ascorbic acid (1.67 mM), DMTP (1 mM) or no electron donor, or 0.92 mM p-coumaric acid and 0.33 mM NADH, shows that 0.224 mM oxygen was sufficient to saturate the enzyme in the absence of reducing agent, but almost 1.12 mM (100%) oxygen was required in ascorbic acid assays. Concentrations of oxygen above 0.336 mM could not be used in the presence of DMTP as the rate of auto-oxidation was too high to permit accurate determination of initial rates of caffeic acid production. The results with NADH would seem similar to those obtained with ascorbic acid over the range of oxygen concentrations studied.

3.1.3.4.2. Catechol oxidase activity.

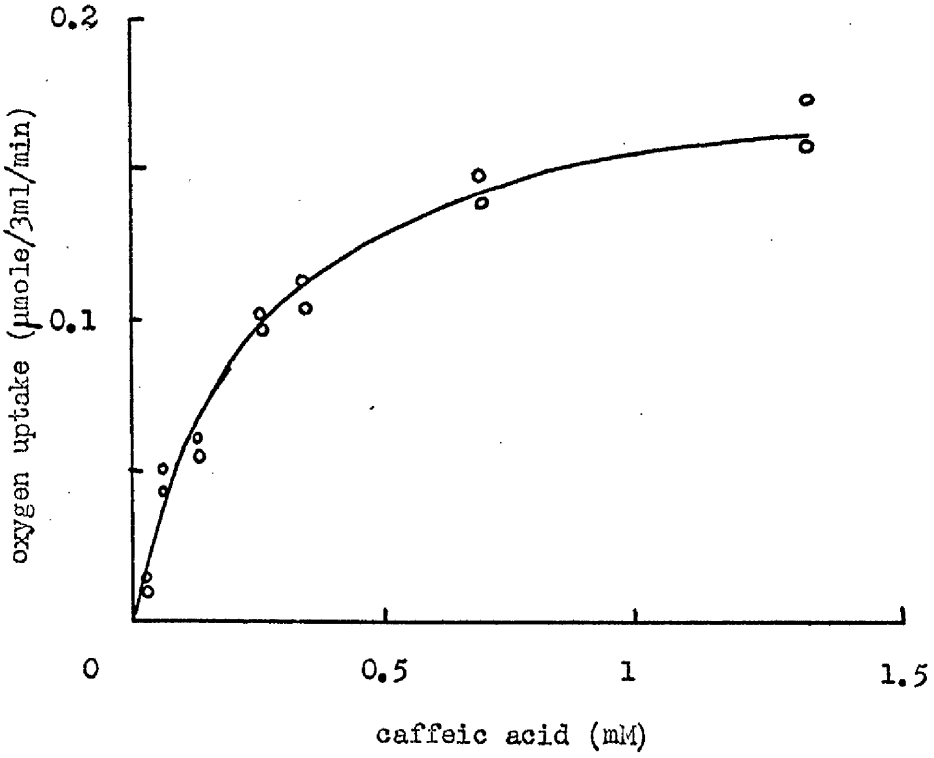
Caffeic acid can also function as a substrate for the catechol oxidase activity of phenolase. The oxygen uptake observed when caffeic acid was varied in air, in the absence of electron donor, is shown in Fig. 17, 1.33 mM caffeic acid being almost sufficient to saturate the enzyme.

3.1.3.5. Determination of Km and Vmax.

The results shown in Fig. 16 were analysed in two ways.

Fig. 17 Substrate saturation - catechol oxidase activity.

Phenolase (76 m units) was incubated with varying amounts of caffeic acid under the conditions for estimation of catechol oxidase activity (2.2.6.1.)


0.2
0.1
0
0.5
1
1.5
oxygen uptake (µmole/3ml/min)
caffeic acid (mM)

1) Graphical analysis. Linear Lineweaver-Burk plots were obtained in all cases (Fig. 18) and

2) Statistical analysis using Program 1 (Appendix I). The date were fitted to the equation of the hyperbola (eqn. 1.4).

The Km and V values obtained by graphical and computer analysis yield similar values (Table 7). The Km for oxygen is not much affected by the nature of the electron donor. This does, however, alter the Michaelis constant of the enzyme for p-coumaric acid, the Km being highest in the absence of reducing agent (1.103 mM) and becoming progressively lower in the presence of 0.33 mM NADH (0.366 mM), 1.67 mM ascorbic acid (0.268 mM) and 1 mM DMTP (0.192 mM). The Km for electron donor is approximately the same for ascorbic acid (0.133 mM) and DMTP (0.131 mM) but is higher for NADH (0.208 mM).

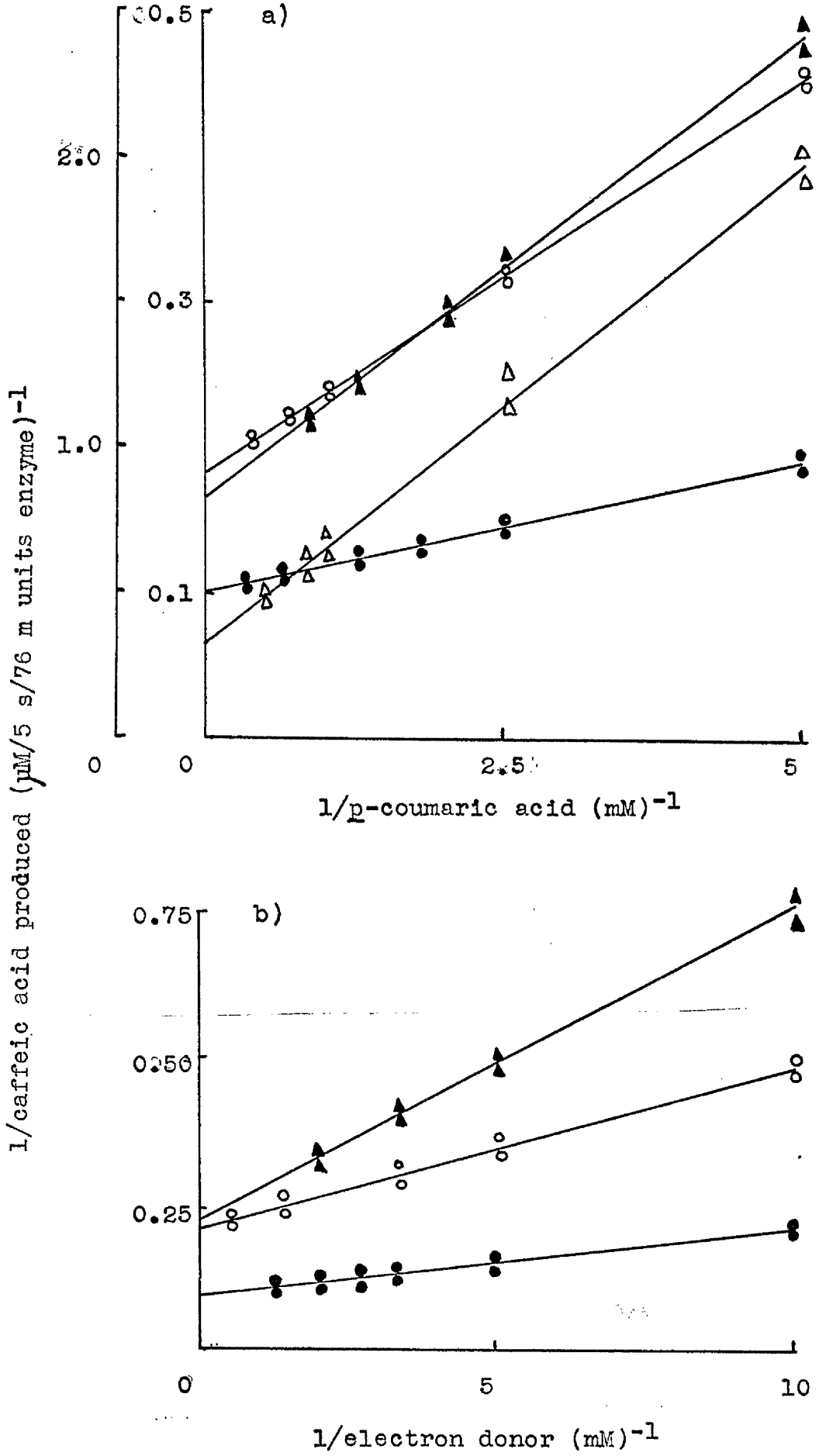
Under saturating conditions the maximum rate of reaction with DMTP (10.7 µM/5s/76 m units) is twice that observed with ascorbic acid (5.6 µM/5s/76 m units) and four times that observed with no electron donor (2.65 µM/5s/76 m units) It would also seem to be more than twice that observed with NADH (5.15 µM/5s/76 m units) but direct comparison of all values obtained with NADH is impossible, as saturation with this compound could not be achieved.

3.1.3.6. Effect of caffeic acid.

Fig. 19a shows the effect of addition of varying amounts of caffeic acid on the lag observed during hydroxylation in air, in the absence of electron donor. The induction period was totally eliminated by 0.033 mM caffeic acid, while the rate of reaction was increased by 23%. At concentrations of caffeic acid above 0.1 mM, inhibition was shown to occur. In the presence of electron donor, however, the reaction rate was not affected by the addition of 0.09 mM caffeic

Fig. 18. Lineweaver-Burk plots.

Experimental details are given in the legend to Fig. 16, experiments being carried out in the presence of DMTP (●), ascorbic acid (○), NADH (▲) and no electron donor (△). In Fig. 18a) the scale 0-0.5 applies to results obtained in the presence of reducing agent.


a)
0.5
2.0
0.3
1.0
0.1
0
0
2.5
5
1/p-coumaric acid (mM)-1
1/caffeic acid produced (μM/5 s/76 m units enzyme)-1
b)
0.75
0.50
0.25
0
5
10
1/electron donor (mM)-1

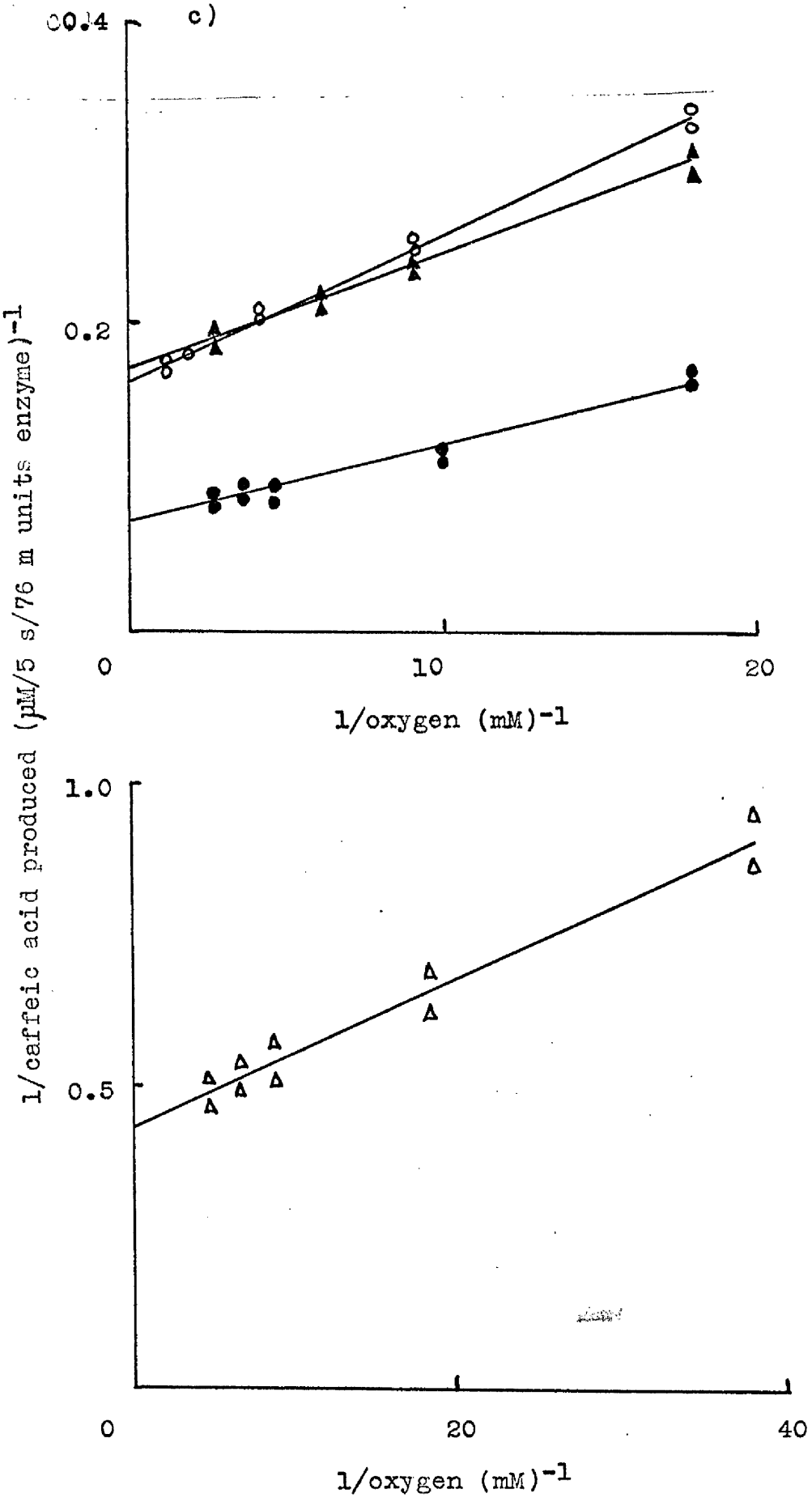

c)
0.4
0.2
0
10
20
1/oxygen (mM)-1
1/caffeic acid produced (µM/5 s/76 m units enzyme)-1
1.0
0.5
0
20
40
1/oxygen (mM)-1

Table 7. Km and V values obtained for all substrates.

electron donor	Variable substrate	Km (mM)		V (μM caffeic acid/5s/76 m unit)	
		Computer	Graph	Computer	Graph
ascorbic acid	oxygen	0.0666±0.0043	0.0544	6.13±0.0765	6.061
	p-coumaric acid	0.268 ± 0.027	0.323	5.245±0.145	5.814
	ascorbic acid	0.133 ± 0.0143	0.133	5.702±0.083	4.76
DMTP	oxygen	0.0513±0.0069	0.0645	10.796±0.253	15.38
	p-coumaric acid	0.192 ± 0.018	0.182	10.186±0.209	10.0
	DMTP	0.131 ± 0.0143	0.130	11.077±0.344	11.11
NADH	oxygen	0.0411±0.0055	0.0455	5.423±0.167	5.882
	p-coumaric acid	0.366 ± 0.041	0.364	5.835±0.228	5.56
	NADH	0.208 ± 0.035	0.233	4.17 ± 0.279	4.35
-	oxygen	0.0454±0.0107	0.0625	2.57 ± 0.165	2.43
	p-coumaric acid	1.103 ± 0.151	1.053	3.166±0.163	3.03

acid, although the shorter lag observed was eliminated by 0.05 mM caffeic acid (Fig. 19c). Inhibition occurred at concentrations of caffeic acid greater than 0.75 mM.

The Km for caffeic acid from the results obtained in the absence of electron donor was determined by the method of Pomerantz and Warner (1967). A graph of 1/ lag time against caffeic acid concentration (Fig. 20) is a straight line, the intercept with the ordinate giving a value for Km. This was found to be 1.8×10^{-3} mM, in contrast to that for caffeic acid as a substrate for catechol oxidase activity (Fig. 17) which was found by computer analysis to be 0.333 ± 0.03 mM. In the presence of electron donor the Km for caffeic acid reduction of the lag was 1.6×10^{-3} mM (Fig. 20).

Fig. 19. Effect of caffeic acid on lag and reaction rate.

1.67 mM p-coumaric acid was incubated with phenolase (76 m units) in the presence of varying amounts of caffeic acid, under the conditions for continuous assay (2.2.5.2.). The experiment was carried out in the presence (Fig. 19b) and absence (Fig. 19a) of electron donor, the rate of reaction (●) and length of lag (○) being measured at each concentration of caffeic acid. The results were plotted semi-logarithmically in order to cover the concentration range used. Ascorbic acid (1.67 mM) was used as electron donor.

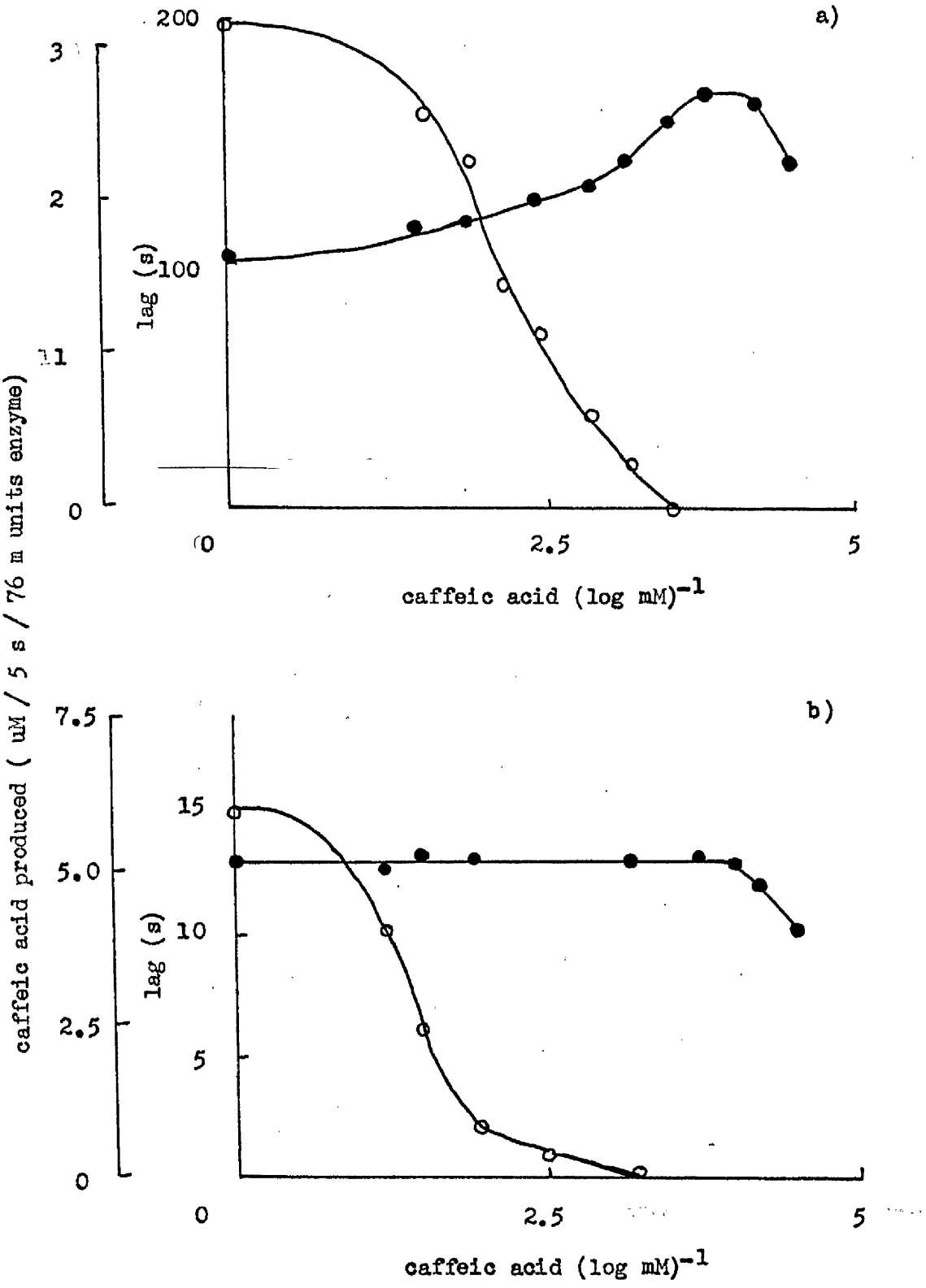
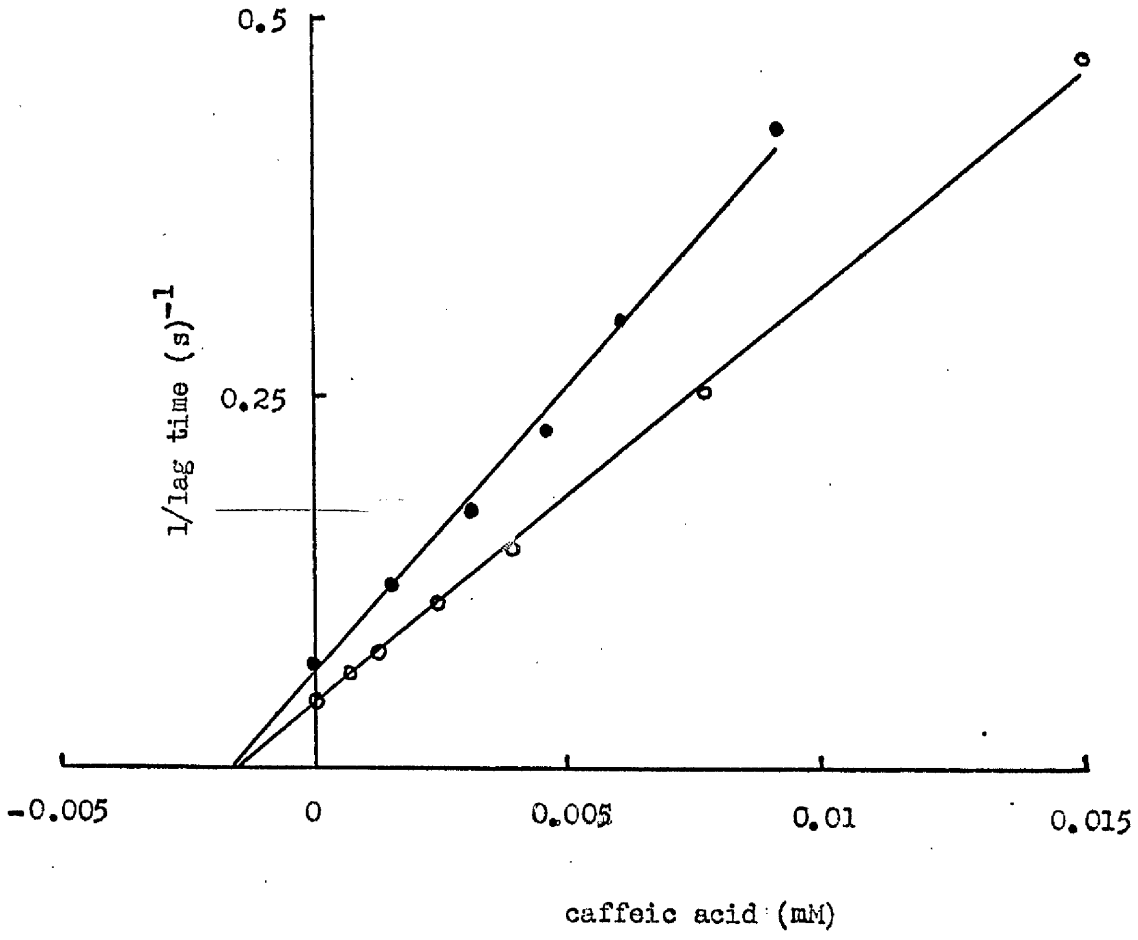

a)
200
100
lag (s)
3
2
1
0
0
2.5
5
caffeic acid (log mM)$^{-1}$
b)
15
10
5
0
lag (s)
7.5
5.0
2.5
0
0
2.5
5
caffeic acid (log mM)$^{-1}$
caffeic acid produced (uM / 5 s / 76 m units enzyme)

Fig. 20. Pomerantz and Warner plot.

The reciprocal of the lag times obtained in the presence (○) and absence of electron donor (●) were plotted against caffeic acid concentration. Experimental details are given in the legend to Fig. 19.


0.5
0.25
1/lag time (s)$^{-1}$
-0.005
0
0.005
0.01
0.015
caffeic acid (mM)

3.2. Initial velocity studies.

The initial velocity patterns obtained in the absence of electron donor, and using ascorbic acid or DMTP as electron donor, were investigated using the method of Rudolph et al, (1968), the results being analysed in three ways.

3.2.1.1. Graphical analysis.

A parallel initial velocity pattern was obtained when oxygen was varied, in the concentration range 0.056 - 0.448 mM, against different amounts of p-coumaric acid and ascorbic acid fixed in ratio of their Michaelis constants (Fig. 21a). A similar result was obtained when p-coumaric acid (0.167 - 1.67 mM : Fig 21b) was varied against different fixed ratios of oxygen and ascorbic acid. With ascorbic acid as the variable substrate (0.066 - 0.33 mM: Fig 21c), the results show that a parallel pattern is obtained at low values of changing fixed substrates (oxygen 0.056 - 0.168 mM, p-coumaric acid 0.21 mM - 0.63 mM), but that as these are increased to 0.448 mM oxygen 1.67 mM p-coumaric acid an intersecting pattern is observed.

Similar experiments were carried out with DMTP, and parallel line patterns were obtained when either oxygen (Fig. 22a) or p-coumaric acid (Fig. 22b) were varied against the other two substrates in the ratio of their Kms. In the case of varied DMTP, however, a similar result to that obtained in the presence of varied ascorbic acid (Fig. 22c) was observed, but the apparent convergence at the highest levels of oxygen and p-coumaric acid was not so marked (Fig. 21c).

In the absence of electron donor, a parallel initial velocity pattern was obtained when either oxygen (Fig. 23a) or p-coumaric acid (Fig. 23b) was the variable substrate.

Fig. 21 Initial velocity studies with ascorbic acid.

Phenolase (76 m units) was incubated under the conditions for continuous assay (2.2.5.2.) with

a) varying amounts of oxygen in the presence of

0.21 mM	p-coumaric acid		0.125 mM	ascorbic acid		(●)
0.31 mM	"	"	0.188 mM	"	"	(○)
0.42 mM	"	"	0.25 mM	"	"	(▲)
0.83 mM	"	"	0.5 mM	"	"	(△)
1.67 mM	"	"	1.0 mM	"	"	(■)

b) varying amounts of p-coumaric acid in the presence of

0.056 mM	oxygen		0.250 mM	ascorbic acid		(●)
0.084 mM	"		0.375 mM	"	"	(○)
0.112 mM	"		0.5 mM	"	"	(▲)
0.224 mM	"		1.0 mM	"	"	(△)

and c) varying amounts of ascorbic acid in the presence of

0.056 mM	oxygen		0.21 mM	P-coumaric acid		(●)
0.085 mM	"		0.31 mM	"	"	(○)
0.112 mM	"		0.42 mM	"	"	(▲)
0.168 mM	"		0.63 mM	"	"	(△)
0.224 mM	"		0.83 mM	"	"	(■)
0.448 mM	"		1.67 mM	"	"	(□)

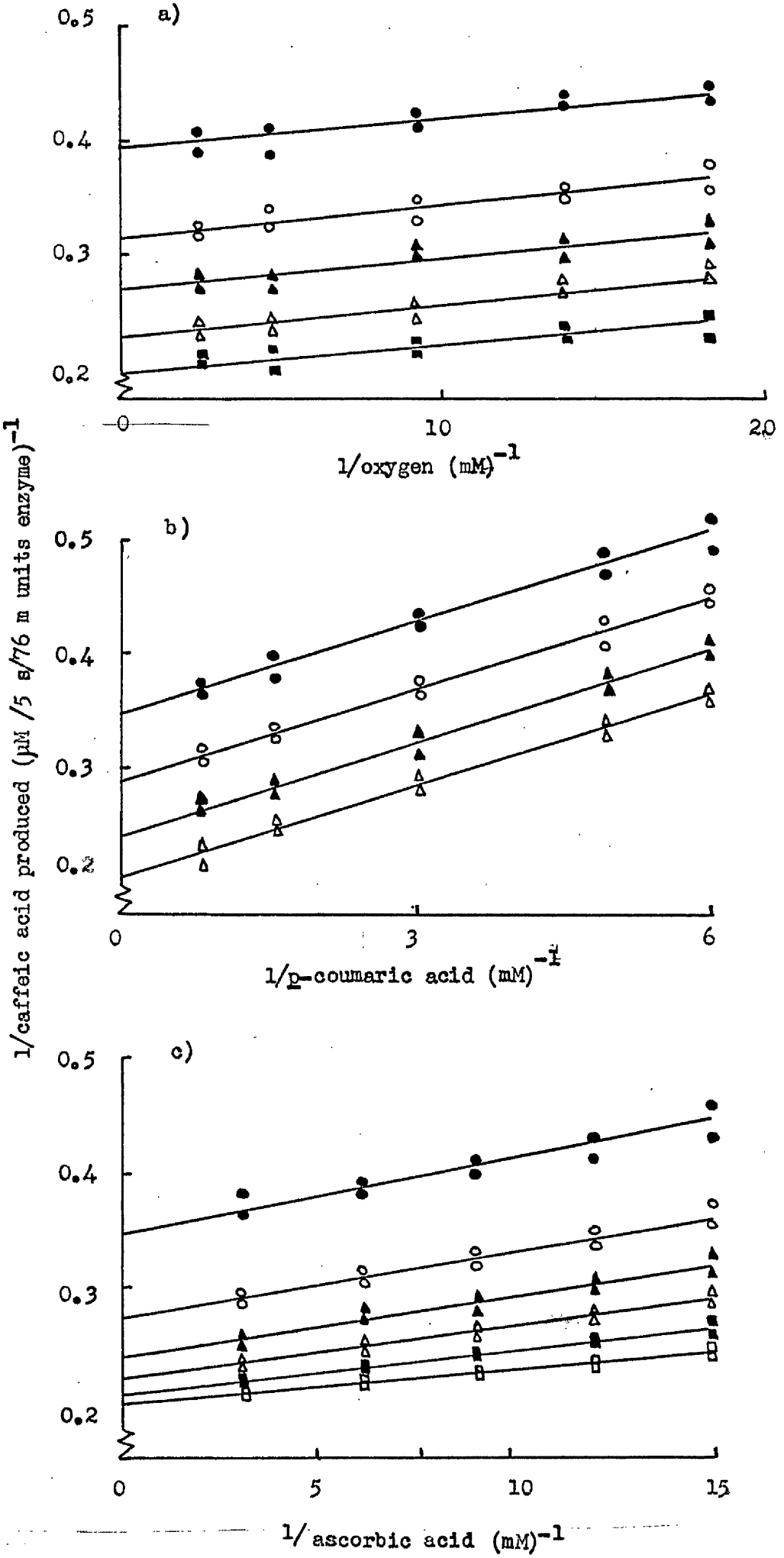

a)
0.5
0.4
0.3
0.2
0
10
20
1/oxygen (mM)$^{-1}$
b)
0.5
0.4
0.3
0.2
0
3
6
1/p-coumaric acid (mM)$^{-1}$
c)
0.5
0.4
0.3
0.2
0
5
10
15
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid produced (µM /5 s/76 m units enzyme)$^{-1}$

Fig. 22. Initial velocity studies with DMTP.

Phenolase (76 m units) was incubated under the conditions for continuous assay (2.2.5.2.) with

a) varying amounts of oxygen in the presence of

0.41	mM	p-coumaric	acid	0.21	mM DMTP	(●)
0.61	mM	"	"	0.31	mM "	(○)
0.83	mM	"	"	0.42	mM "	(▲)
1.67	mM	"	"	0.83	mM "	(△)

b) varying amounts of p-coumaric acid in the presence of

0.056	mM	oxygen	0.21	mM DMTP	(●)
0.084	mM	"	0.31	mM "	(○)
0.112	mM	"	0.42	mM "	(▲)
0.224	mM	"	0.83	mM "	(△)

and c) varying amounts of DMTP in the presence of

0.42	mM	p-coumaric	acid	0.056	mM oxygen	(●)
0.61	mM	"	"	0.084	mM "	(○)
0.83	mM	"	"	0.112	mM "	(▲)
1.67	mM	"	"	0.224	mM "	(△)

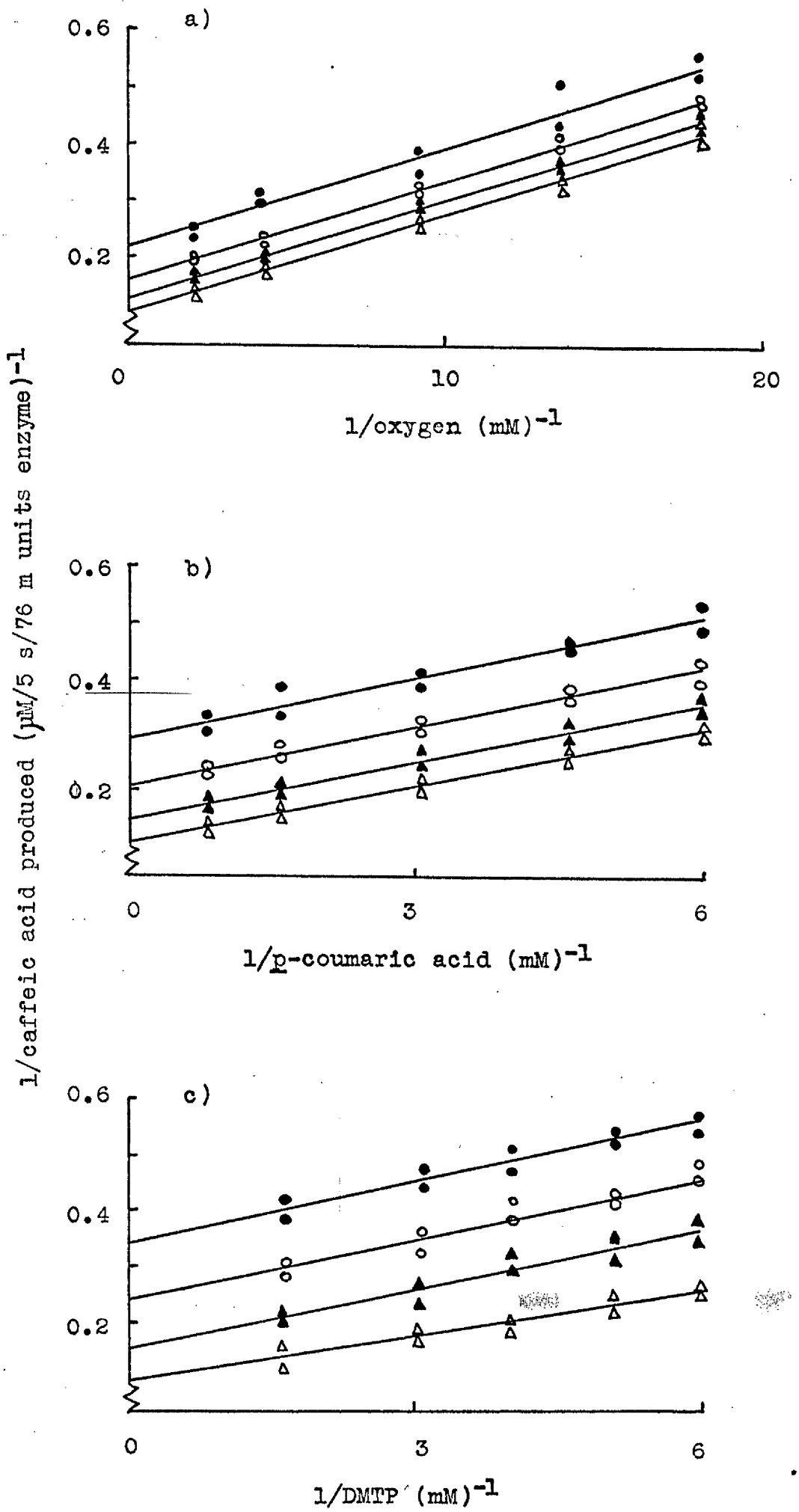

a)
0.6
0.4
0.2
0
10
20
1/oxygen (mM)$^{-1}$
b)
0.6
0.4
0.2
0
3
6
1/p-coumaric acid (mM)$^{-1}$
c)
0.6
0.4
0.2
0
3
6
1/DMTP (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units enzyme)$^{-1}$

Fig. 23. Initial velocity studies in the absence of electron donor.

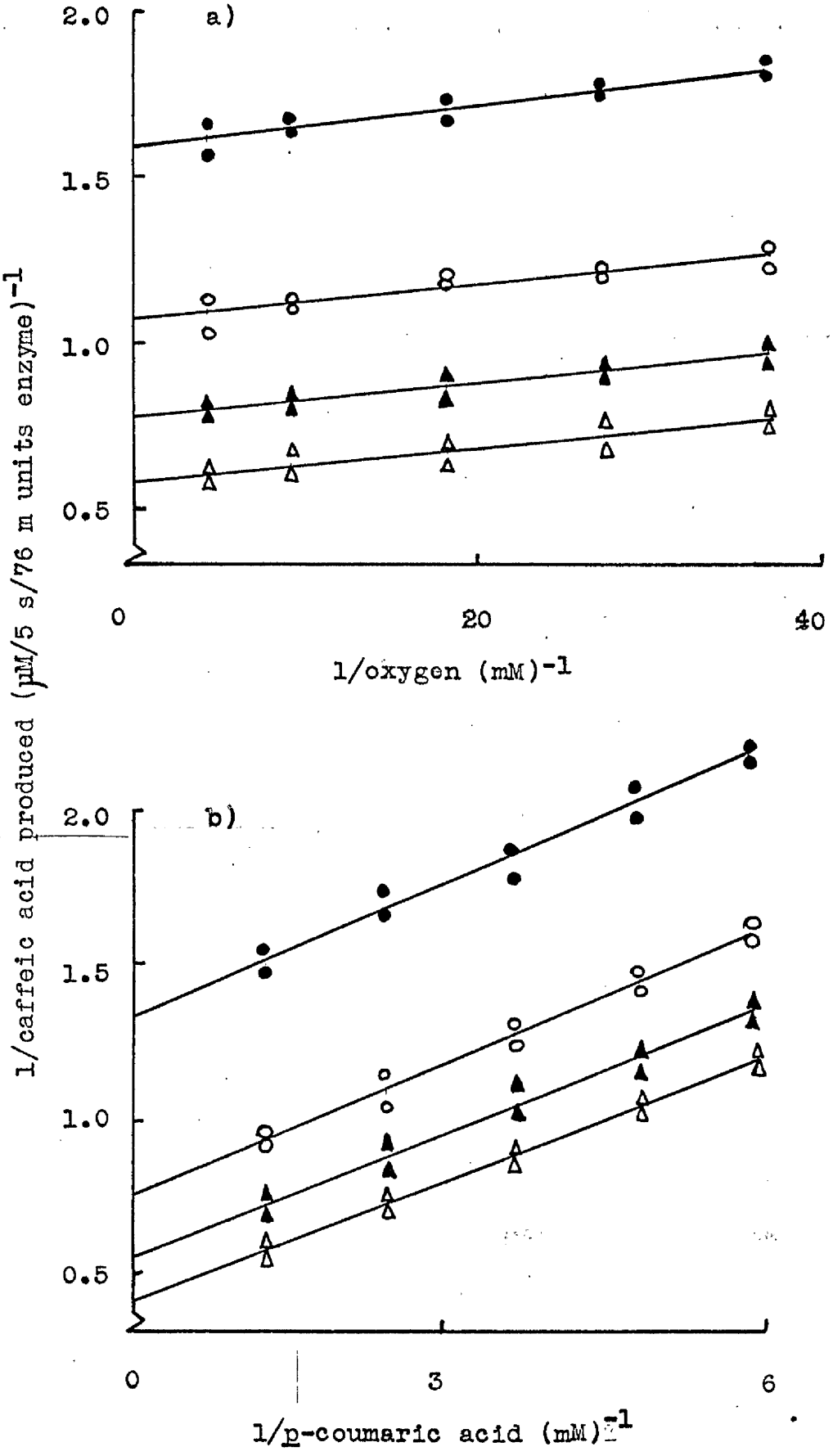
Phenolase (76 m units) was incubated under the conditions for continuous assay (2.2.5.2.) with

a) varying amounts of oxygen in the presence of

0.17 mM p-coumaric acid	(●)
0.33 mM " "	(○)
0.67 mM " "	(▲)
1.33 mM " "	(△)

and b) varying amounts of p-coumaric acid in the presence of

0.028 mM oxygen	(●)
0.056 mM "	(○)
0.112 mM "	(▲)
0.224 mM "	(△)


a)
2.0
1.5
1.0
0.5
0
20
40
1/oxygen (mM)-1
1/caffeic acid produced (µM/5 s/76 m units enzyme)-1
b)
2.0
1.5
1.0
0.5
0
3
6
1/p-coumaric acid (mM)-1

3.2.1.2. Computer analysis

The initial velocity data obtained were fitted to the equations for either ping pong (a) or sequential (b) cases, using Programs 2 and 3 in Appendix I, and the results obtained are shown in Table 8. The degree of fit of the data to a particular model was assessed using the size of the standard errors, the sign of K_{iA} and the variance as the criteria for suitability.

Table 9 demonstrates the reasons for assigning the data to one mechanism or the other. K_{iA} was considered insignificant if the error was of the same order as the value obtained for the constant. The data obtained by variation of oxygen or p-coumaric acid, i.e. those which resulted in parallel initial velocity patterns, fit the ping pong model better than the sequential, while the complex patterns found on variation of electron donor seem to fit a sequential better than a ping pong model, on the basis of the K_{iA} values.

3.2.1.3. Slope and intercept replots.

Secondary replots of slope and intercept (Cleland, 1970) are shown in Fig. 24. These are drawn from the results obtained with ascorbic acid (Fig. 24a), DMTP (Fig. 24c) and no electron donor (Fig. 24b). All experiments involving varied oxygen or p-coumaric acid result, on replotting of slopes, in a line parallel with the abscissa, showing that the initial velocity pattern obtained is truly parallel. The intercept replot, on the other hand, curves upwards. This is an example of a parabolic intercept replot (Cleland, 1970). When the experiments involving varied electron donor are considered the intercept replot is again found to be parabolic. The slope replot on the other hand, rises steadily before reaching a plateau, and

Table 8. Computer analysis of initial velocity data.

a) ping pong mechanism b) sequential mechanism

Expt. No.	Model	Experiment	K_A (mM)	K_B (mM)	V (μm/5 s/ 76 m units)	K_{iA} (mM)	Variance
1	a	p-coumaric acid v	0.168±0.008	0.0617±0.003	5.78±0.121		0.007
	b	oxygen and ascorbic acid	0.17± 0.016	0.0624±0.005	5.8 ± 0.174	-0.0039±0.019	0.0078
2	a	ascorbic acid v oxygen	0.0259±0.0022	0.065 ± 0.0028	6.19±0.116		0.017
	b	and p-coumaric acid	0.0167±0.0031	0.0502±0.0046	5.82±0.14	0.0276±0.0098	0.0144
3	a	oxygen v p-coumaric	0.0157±0.0014	0.279 ± 0.0094	5.93±0.087		0.0121
	b	acid and ascorbic acid	0.0134±0.0022	0.261 ± 0.0156	5.83±0.112	0.0056±0.0045	0.012
4	a	p-coumaric acid v	0.656 ± 0.104	0.239 ± 0.043	18.84±2.33		0.132
	b	oxygen and DMTP	0.502 ± 0.147	0.194 ± 0.049	16.67±2.54	0.0559±0.0561	0.131
5	a	DMTP v oxygen and	2.29 ± 1.13	1.605 ± 0.784	78.85±36.6		0.0436
	b	p-coumaric acid	0.422 ± 0.141	0.35 ± 0.096	23.12±4.51	0.118 ± 0.036	0.0291
6	a	oxygen v p-coumaric	0.293 ± 0.027	0.519 ± 0.06	16.2 ± 1.13		0.0436
	b	acid and DMTP	0.244 ± 0.040	0.431 ± 0.078	14.74±1.37	0.024 ± 0.02	0.043
7	a	oxygen v p-coumaric acid,	0.298 ± 0.039	0.0601±0.006	2.55 ± 0.17		0.0039
	b	no electron donor	0.212 ± 0.044	0.0441±0.008	2.26 ± 0.18	0.1 ± 0.09	0.0035
8	a	p-coumaric acid v oxygen,	0.06 ± 0.005	0.294 ± 0.07	2.77±0.15		0.0023
	b	no electron donor	0.036 ± 0.006	0.286 ± 0.06	2.47 ± 0.12	0.136±0.136	0.002

Table 9. Assignation of data to kinetic models

Expt. No.	Model	Standard errors in all parameters	K_{iA} value	Variance	Model assigned
1	a b	larger	negative	slightly lower	a
2	a b	larger	significant	lower	b
3	a b	larger	not significant	equal	a
4	a b	larger	not significant	equal	a
5	a b	larger	significant	lower	b
6	a b	larger	not significant	slightly lower	a
7	a b	larger	not significant	lower	a
8	a b	equal	not significant	lower	a

Fig. 24. Secondary replots of initial velocity data.

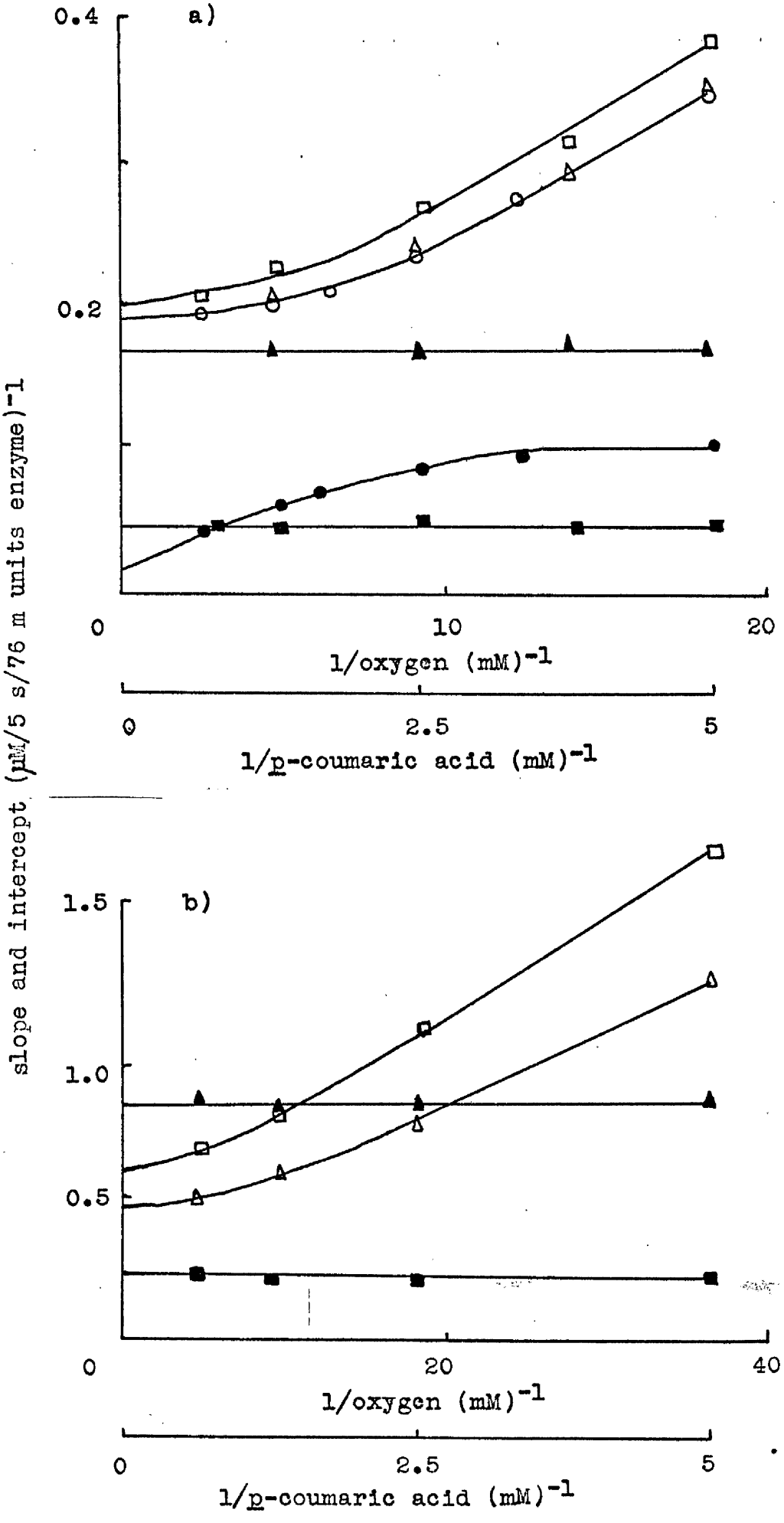
The slopes and vertical intercepts obtained from

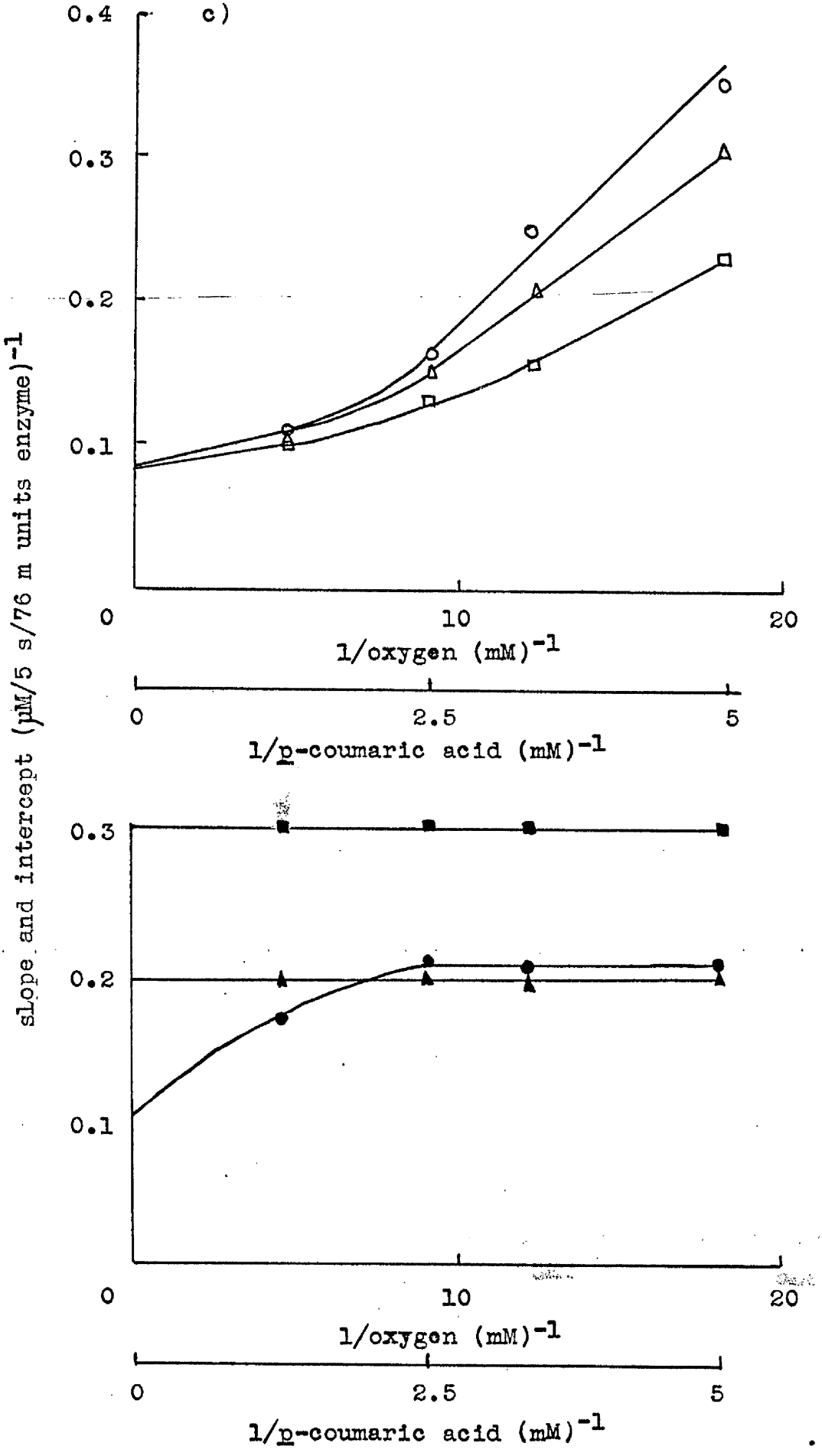
a) Fig. 21 b) Fig. 23 and c) Fig. 22

were plotted against the reciprocal of one of the changing fixed substrates. The variable substrates concerned were

	Slope	Intercept
p-coumaric acid	▲	△
electron donor	●	○
oxygen	■	□

Experimental details were as described in the legends to Figs. 21, 22 and 23.


a)
0.4
0.2
0
10
20
1/oxygen (mM)$^{-1}$
0
2.5
5
1/p-coumaric acid (mM)$^{-1}$
b)
1.5
1.0
0.5
0
20
40
1/oxygen (mM)$^{-1}$
0
2.5
5
1/p-coumaric acid (mM)$^{-1}$
slope and intercept (μM/5 s/76 m units enzyme)$^{-1}$


c)
0.4
0.3
0.2
0.1
0
10
20
1/oxygen (mM)$^{-1}$
0
2.5
5
1/p-coumaric acid (mM)$^{-1}$
slope and intercept (μM/5 s/76 m units enzyme)$^{-1}$
0.3
0.2
0.1
0
10
20
1/oxygen (mM)$^{-1}$
0
2.5
5
1/p-coumaric acid (mM)$^{-1}$

becoming parallel with the abscissa.

These results suggest that an apparently irreversible step exists after the binding of a) oxygen and b) p-coumaric acid, but that the irreversible step associated with reduction by ascorbic acid is reversed at high substrate concentrations, which lead to the formation of slightly larger amounts of caffeic acid.

The true maximum velocities for the reaction under different conditions can be obtained from a consideration of the intercept replots, the vertical intercept being a measure of the true maximum velocity. In the presence of DMTP (Fig. 24c) the maximum rate of reaction (12.5 μM/5 s/76 m units enzyme) is two and a half times that with ascorbic acid (Fig. 24a : 5 μM/5 s/76 m units enzyme) and five times that obtained in the absence of electron donor (Fig. 24b : 2.66 μM/5 s/76 m units enzyme). These results are in agreement with those obtained from substrate saturation studies (3.1.3.4.).

For simplicity of presentation of secondary replots, slope values were taken as the increase in the reciprocal of the concentration of caffeic acid produced over the concentration range of variable substrate studied.

3.2.2. Effect of product on initial velocity patterns.

The initial velocity patterns obtained in the presence of 0.066 mM caffeic acid, a non-inhibitory amount, (Fig. 19), are shown in Fig. 25. While an apparently parallel pattern is observed when oxygen (0.056 - 0.448 mM) is varied at the different fixed levels of p-coumaric acid and ascorbic acid previously described (3.2.1.) (Fig. 25a), intersecting patterns arise when either p-coumaric acid (0.167 - 1.67 mM) is varied against oxygen and ascorbic acid (Fig. 25b), or ascorbic acid is varied against oxygen and p-coumaric acid (Fig. 25c). The results of computer analysis of the data obtained are shown in Table 10. The significant K_{iA} and low variances support the choice of an apparently sequential mechanism when either p-coumaric acid or ascorbic acid are the variable substrates in the presence of added product. The data obtained at varying oxygen concentrations fit the ping pong model, as can be seen by the consistently larger errors in the sequential fit. The K_{iA} value is not significant because of the proportionally larger error. It would therefore seem that the irreversible step seen on binding of oxygen is not reversed in the presence of added product. The secondary replots show that a parabolic intercept curve is obtained when oxygen is varied (Fig. 26a) while a linear slope effect and parabolic intercept effect are seen with p-coumaric acid. Both slope and intercept replots are parabolic when ascorbic acid is the variable substrate (Fig. 26c).

The conversion of a parallel to an intersecting initial velocity pattern in the presence of product, with either p-coumaric acid or ascorbic acid as variable substrate, demonstrates the reversal of an irreversible step, and hence shows that release of caffeic acid occurs

Fig. 25. Initial velocity studies with ascorbic acid - effect of caffeic acid.

Phenolase (76 m units) was incubated in the presence of 0.067mM caffeic acid under the conditions for continuous assay (2.2.5.2.) with

a) varying amounts of oxygen in the presence of

0.21	mM	p-coumaric	acid	0.125	mM	ascorbic	acid (●)
0.42	mM	"	"	0.25	mM	"	" (○)
0.83	mM	"	"	0.5	mM	"	" (▲)
1.67	mM	"	"	1.0	mM	"	" (△)

b) varying amounts of p-coumaric acid in the presence of

0.056	mM	oxygen		0.25	mM	ascorbic	acid (●)
0.084	mM	"		0.375	mM	"	" (○)
0.112	mM	"		0.5	mM	"	" (▲)
0.224	mM	"		1.0	mM	"	" (△)

and c) varying amounts of ascorbic acid in the presence of

0.21	mM	p-coumaric	acid	0.056	mM	oxygen	(●)
0.31	mM	"	"	0.084	mM	"	(○)
0.42	mM	"	"	0.112	mM	"	(▲)
0.83	mM	"	"	0.224	mM	"	(△)

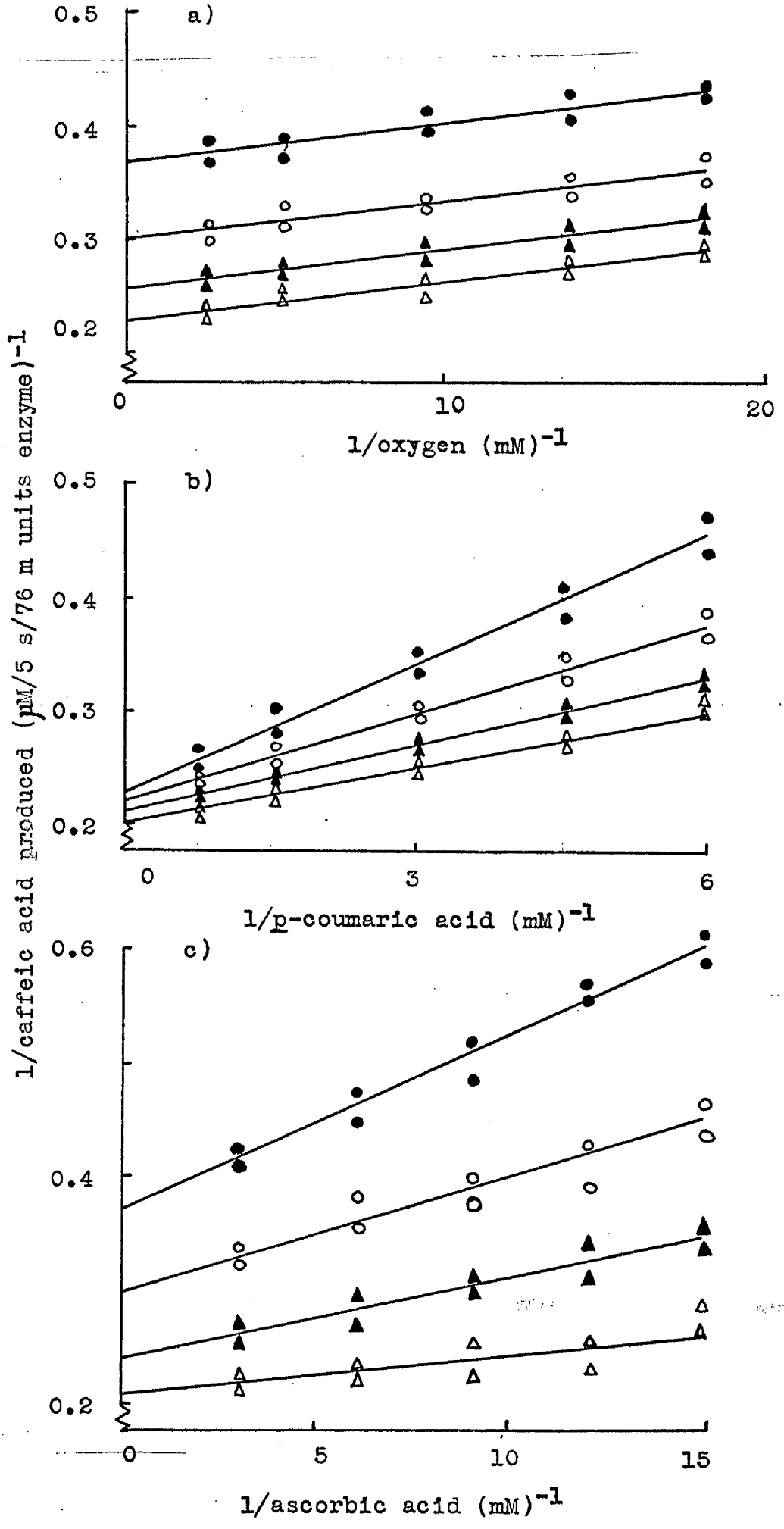

a)
b)
c)
0.5
0.4
0.3
0.2
0.6
0
10
20
3
6
5
15
1/oxygen (mM)$^{-1}$
1/p-coumaric acid (mM)$^{-1}$
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units enzyme)$^{-1}$

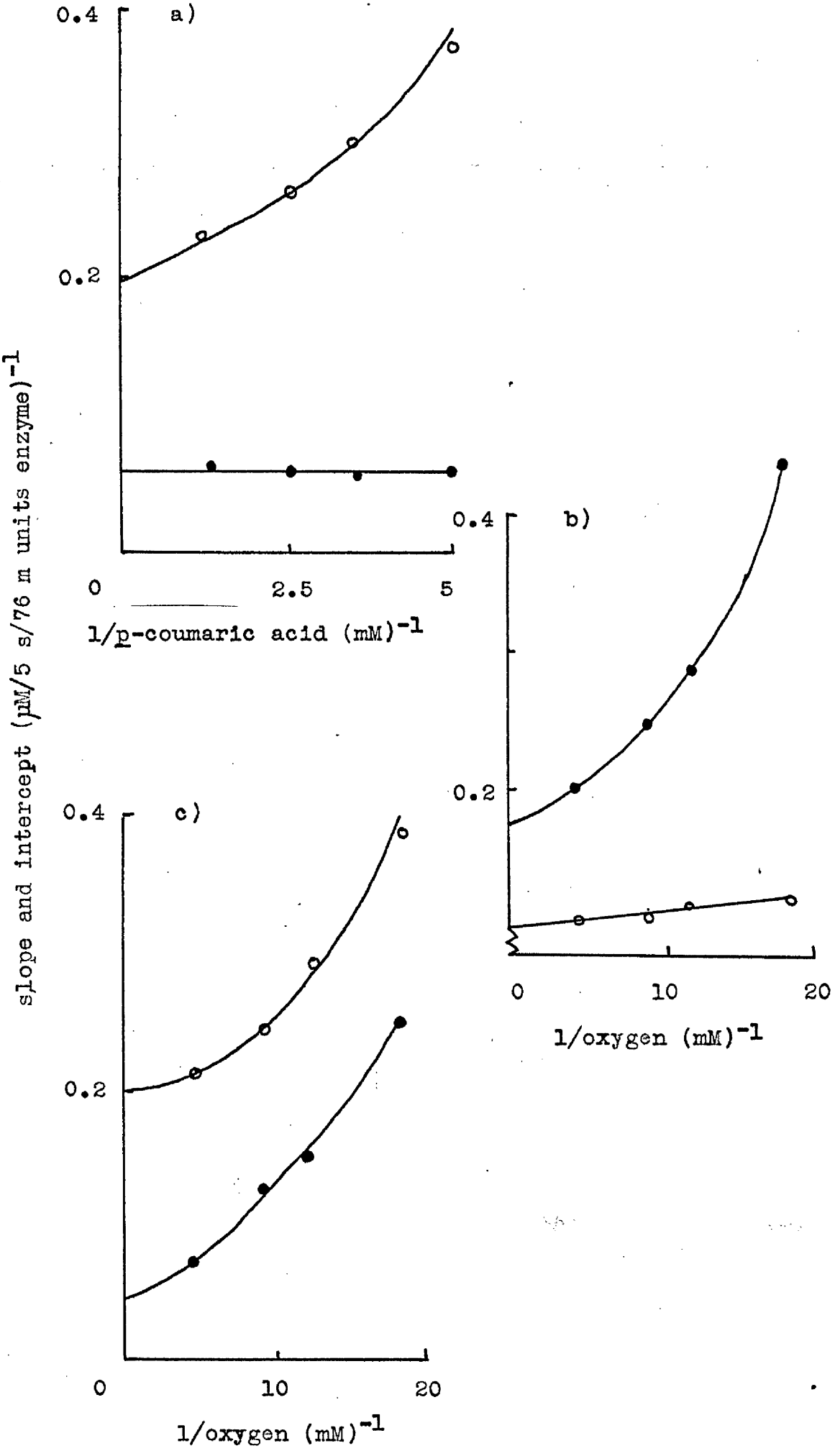
Table 10. Computer analysis of initial velocity data obtained in presence of caffeic acid.

a) ping pong mechanism b) sequential mechanism

Model	Experiment	K_A (mM)	K_B (mM)	V (μm/5 s/ 76 m units)	K_{iA} (mM)	Variance
a	p-coumaric acid v oxygen and ascorbic acid	0.174± 0.013	0.036±0.0042	6.26±0.217		0.0333
b		0.0685±0.0098	0.014±0.0024	5.33±0.114	0.629±0.149	0.0088
a	ascorbic acid v oxygen and p-coumaric acid	0.031± 0.0062	0.204±0.022	10.4±0.905		0.0232
b		0.0023±0.0045	0.082±0.018	6.84±0.58	0.0338±0.0119	0.0145
a	Oxygen v p-coumaric acid and ascorbic acid	0.0197±0.0013	0.209±0.0073	5.64±0.074		0.0104
b		0.0197±0.0022	0.209±0.0126	5.64±0.100	0.00007±0.0047	0.0106

Fig. 26. Secondary replots - effect of caffeic acid.

Slope (●) and vertical intercepts (○) obtained from Fig. 25 were plotted against the reciprocal of the concentration of one of the changing fixed substrates . Experimental details were as described in the legend to Fig. 25.


a)
0.4
0.2
0
2.5
5
1/p-coumaric acid (mM)$^{-1}$
b)
0.4
0.2
0
10
20
1/oxygen (mM)$^{-1}$
c)
0.4
0.2
0
10
20
1/oxygen (mM)$^{-1}$
slope and intercept (μM/5 s/76 m units enzyme)$^{-1}$

between the binding of p-coumaric acid and electron donor to the enzyme. The addition of a fixed amount of caffeic acid does not establish a reversible connection between oxygen and the other substrates.

It was not possible to repeat these experiments in the presence of DMTP, as a spectral shift occurred at 340 nm on the addition of caffeic acid to DMDP (Fig. 27), Calculation of results using the derived formula (2.2.4.2.) was impossible, as the molar extinction coefficient of this complex could not be determined, and hence it was decided that this method of caffeic acid estimation was not suitable for use under these conditions.

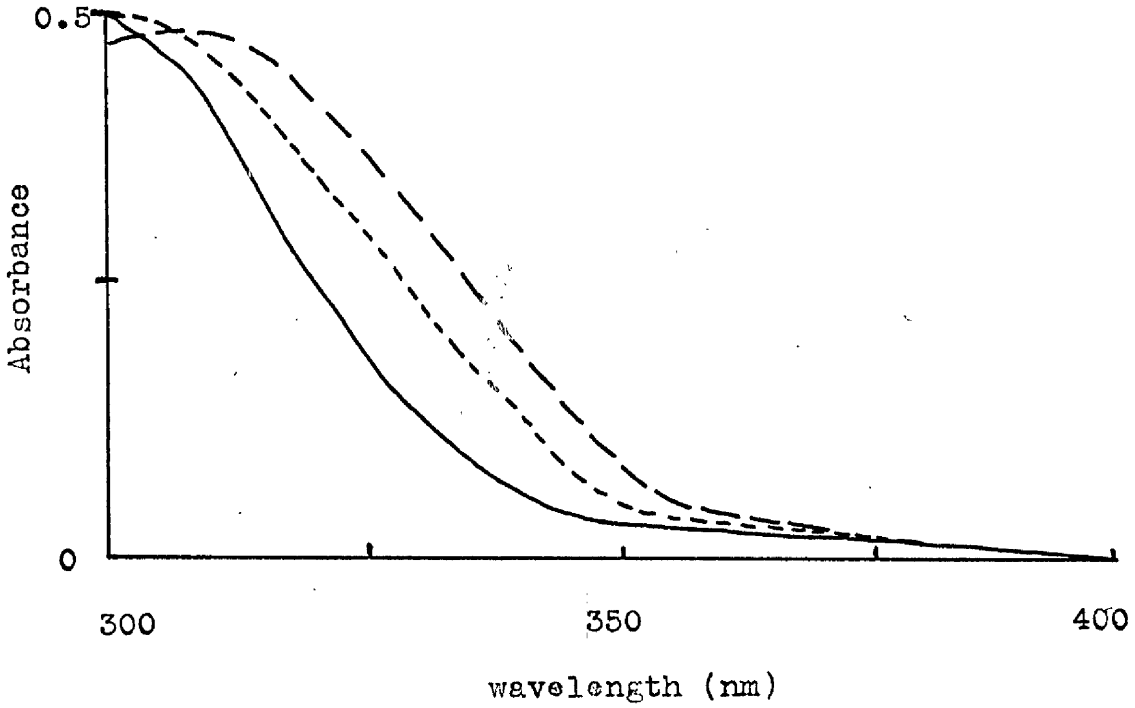
Fig. 27. Effect of caffeic acid on absorption spectrum of DMDP.

A difference spectrum of 0.11 mM DMDP in 0.1 M Na_2HPO_4-0.05M citric acid buffer pH 6.5 was obtained using a Cary 15 spectrophotometer. The absorbance was monitored after addition of various amounts of caffeic acid to both test and reference cuvettes.

——— no addition

------ 0.013 mM caffeic acid

— — — 0.067 mM caffeic acid


0.5
Absorbance
0
300
350
400
wavelength (nm)

3.3. INHIBITION STUDIES.

3.3.1. Inhibition studies with substrate analogues and alternate substrates.

Substrate analogues are commonly used in inhibition studies. These structurally similar compounds can be either catalytically inactive - substrate analogues - or catalytically active - alternate substrates.

3.3.1.1. Preliminary Studies.

3.3.1.1.1. Identification of alternate substrates.

The reactivities of p-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid were compared with those of p-coumaric acid and caffeic acid by measuring the oxygen uptake obtained in the presence of 1.67 mM ascorbic acid and air. Table 11 shows that while p-coumaric acid and caffeic acid are more efficient substrates for phenolase under these conditions than p-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid, these are also substrates for the reaction.

Benzoic acid is structurally similar to these substrates but is not hydroxylated as there is no existing hydroxyl group on the benzene ring. Thus both substrate analogues and alternate substrates are available for inhibitor studies.

3.3.1.1.2. Inhibition curves.

The effect of varying concentrations of benzoic acid, p-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid on hydroxylation in air, in the presence of saturating amounts of p-coumaric acid and ascorbic acid, is shown in Fig. 28. 50% inhibition was achieved in the presence of 0.5 mM 3,4-dihydroxy benzoic acid, 1.36 mM benzoic acid, and 2.3 mM p-hydroxybenzoic acid.

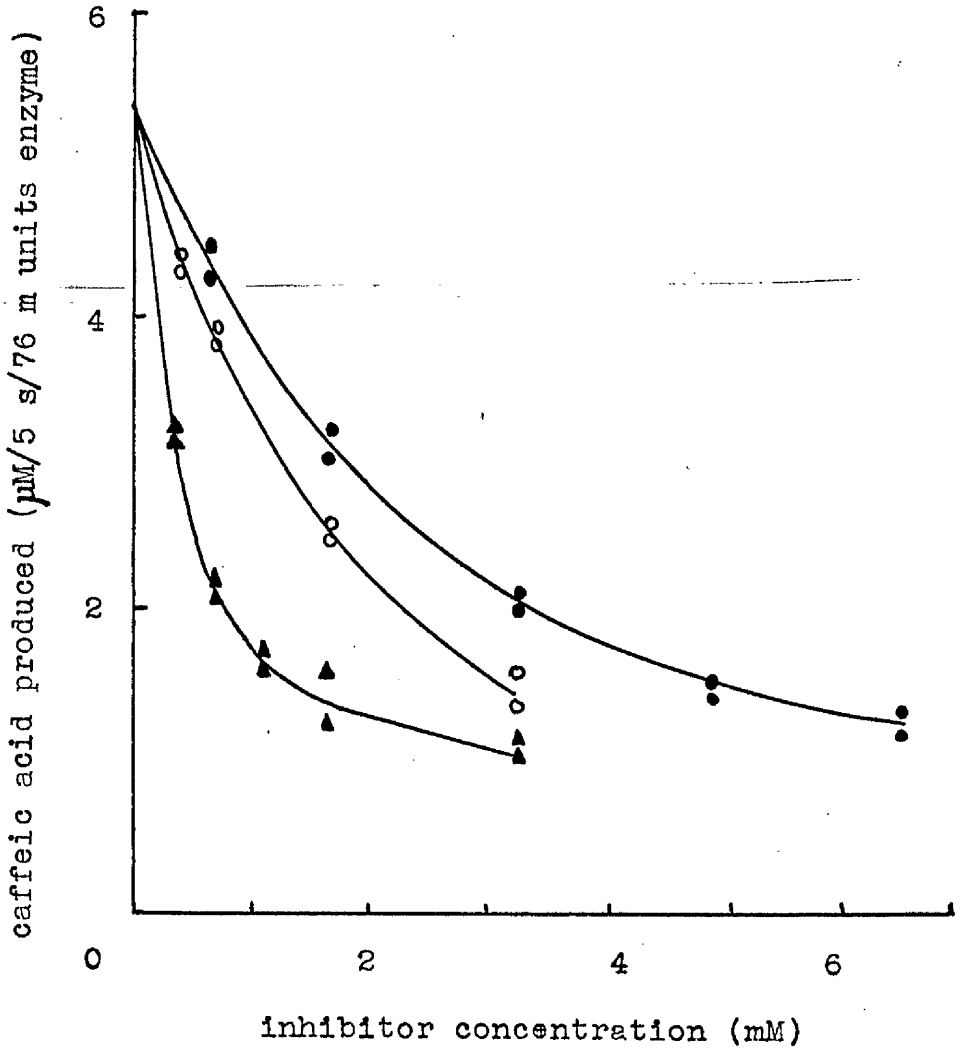
Table 11. Comparison of substrates and substrate analogues.

76 m units of enzyme were incubated with 1.66 mM ascorbic acid and the compounds tested under conditions for measurement of oxygen uptake (2.2.6).

Substrate	Concentration (mM)	Reaction rate (m mole O_2/min)
p-coumaric acid	3	0.0212
p-hydroxybenzoic acid	3	0.0017
caffeic acid	1.33	0.0140
3,4-dihydroxybenzoic acid	1.33	0.0028

Fig. 28. Inhibition curves obtained with substrate analogues and alternate substrates.

1.67 mM p -coumaric acid was incubated with 1.67 mM ascorbic acid and 76 m units phenolase, in air, in the presence of varying amounts of p-hydroxybenzoic acid (●), benzoic acid (○) and 3,4 - dihydroxybenzoic acid (▲). Assays were carried out under the conditions described for continuous assay (2.2.5.2.).


caffeic acid produced (μM/5 s/76 m units enzyme)
6
4
2
0
2
4
6
inhibitor concentration (mM)

3.3.1.2. p-hydroxybenzoic acid and benzoic acid studies.

With oxygen (0.056 - 0.448 mM : Fig. 29a) or ascorbic acid (0.0167 - 0.33 mM : Fig. 29c) as variable substrate a parallel initial velocity pattern was obtained in the presence of different concentrations of p-hydroxybenzoic acid (0.83 - 215 mM). Variation of p-coumaric acid (0.167 - 1.67 mM, Fig. 29b) resulted in a pattern intersecting on the left of the abscissa, while with caffeic acid (0.017 - 0.067mM) as variable substrate the Lineweaver-Burk plot shows a transition from an intersecting to a parallel pattern at higher inhibitor concentrations.

Secondary replots of the data showed that with oxygen as variable substrate (Fig. 30a) the slope replot was parallel with the abscissa while the intercept replot was linear. This is an example of linear uncompetitive inhibition (Cleland,1970). A similar inhibition pattern was obtained with varying amounts of ascorbic acid (Fig. 30c). Linear slope and intercept replots were observed in the presence of varied p-coumaric acid (Fig. 30b), an example of linear non-competitive inhibition. The results obtained in the presence of varied caffeic acid are more complex (Fig. 30d) and cannot be analysed in this way as the pattern observed demonstrates the transition from noncompetitive to uncompetitive inhibition.

Similar results were obtained when benzoic acid (0.05 - 0.2 mM) was added in the presence of varying amounts of oxygen, p-coumaric acid or caffeic acid (Fig. 31, 32). The inhibitor constants for p-hydroxybenzoic acid (Fig. 30b) and benzoic acid (Fig. 32b) were calculated from the linear intercept replots and were found to be 1.67 and 2.2 mM respectively.

Fig. 29. Effect of p-hydroxybenzoic acid on initial velocity patterns.

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor	(Δ)
0.84 mM p-hydroxybenzoic acid	(▲)
1.67 mM " "	(○)
or 2.5 mM " "	(●)

in the presence of

a) 1.67 mM p-coumaric acid, 1.67 mM ascorbic acid and varying amounts of oxygen.

b) 1.67 mM ascorbic acid and varying amounts of p-coumaric acid, in air.

c) 1.67 mM p-coumaric acid and varying amounts of ascorbic acid, in air.

d) 1.67 mM p-coumaric acid, 1.67 mM ascorbic acid and varying amounts of caffeic acid in air.

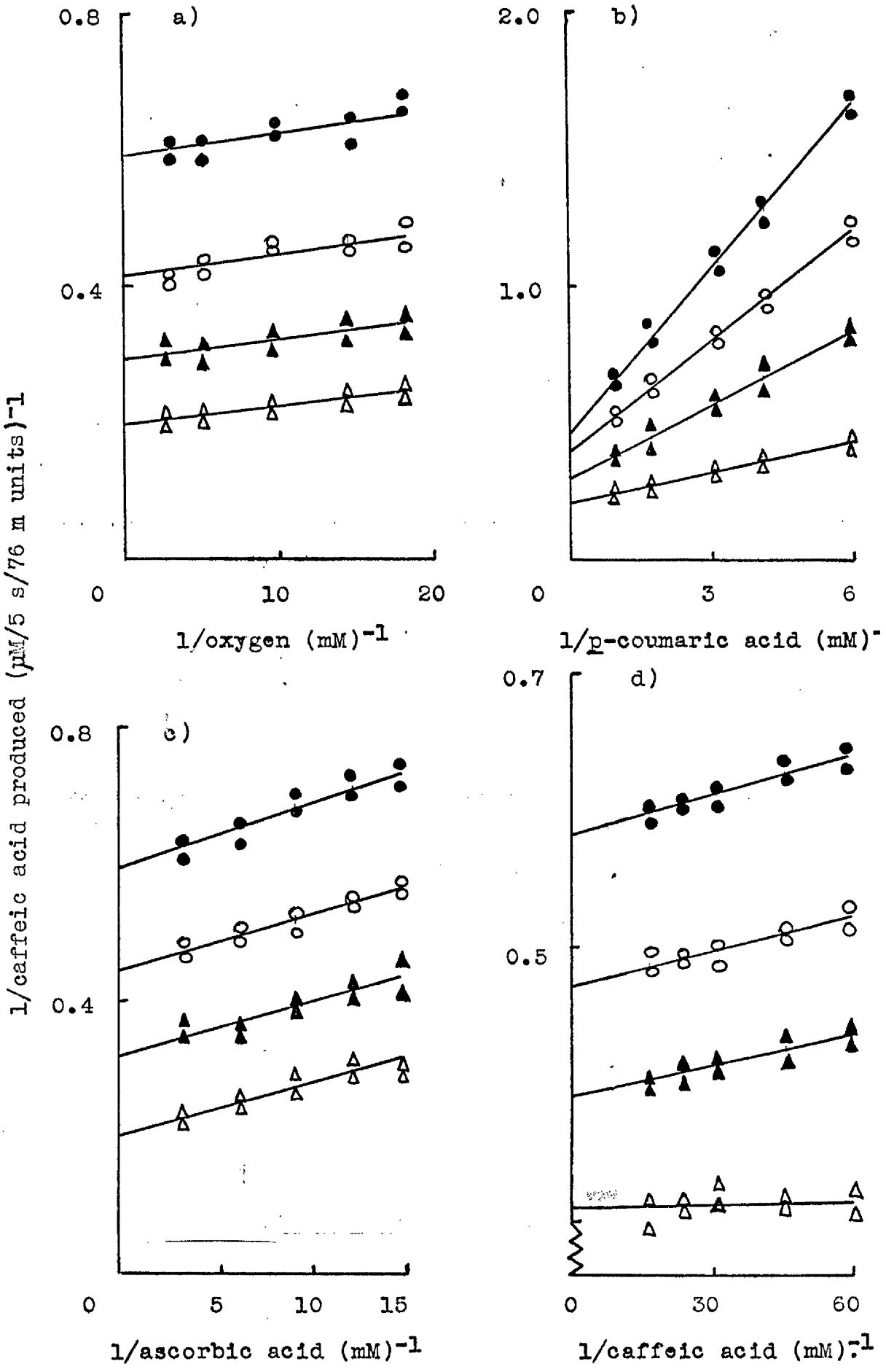

a)
b)
c)
d)
0.8
0.4
2.0
1.0
0.7
0.5
0
10
20
3
6
5
15
30
60
1/oxygen (mM)-1
1/p-coumaric acid (mM)-
1/ascorbic acid (mM)-1
1/caffeic acid (mM)-1
1/caffeic acid produced (µM/5 s/76 m units)-1

Fig. 30. Secondary replots - effect of p-hydroxybenzoic acid.

Slope (●) and vertical intercept values (○) obtained from Fig. 29 were plotted against inhibitor concentration.

Experimental details were as described in the legend to Fig. 29.

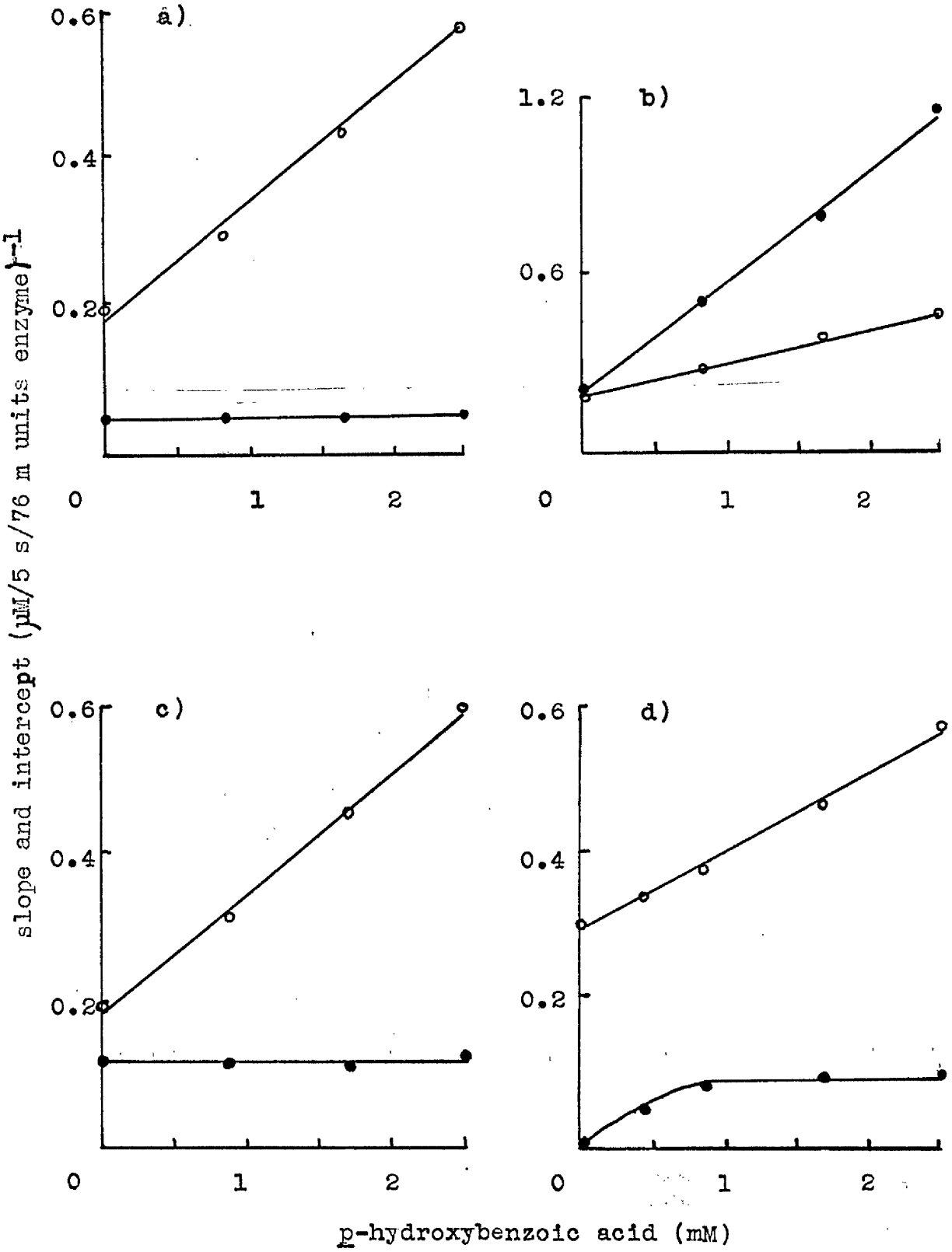

a)
b)
c)
d)
0.6
0.4
0.2
1.2
0.6
0
1
2
slope and intercept (µM/5 s/76 m units enzyme)$^{-1}$
p-hydroxybenzoic acid (mM)

Fig. 31 Effect of benzoic acid on initial velocity patterns.

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor	(Δ)
0.5 mM benzoic acid	(▲)
1.0 mM " "	(○)
1.67 mM " "	(●)

Other substrates were varied as described in the legend to Fig. 29.

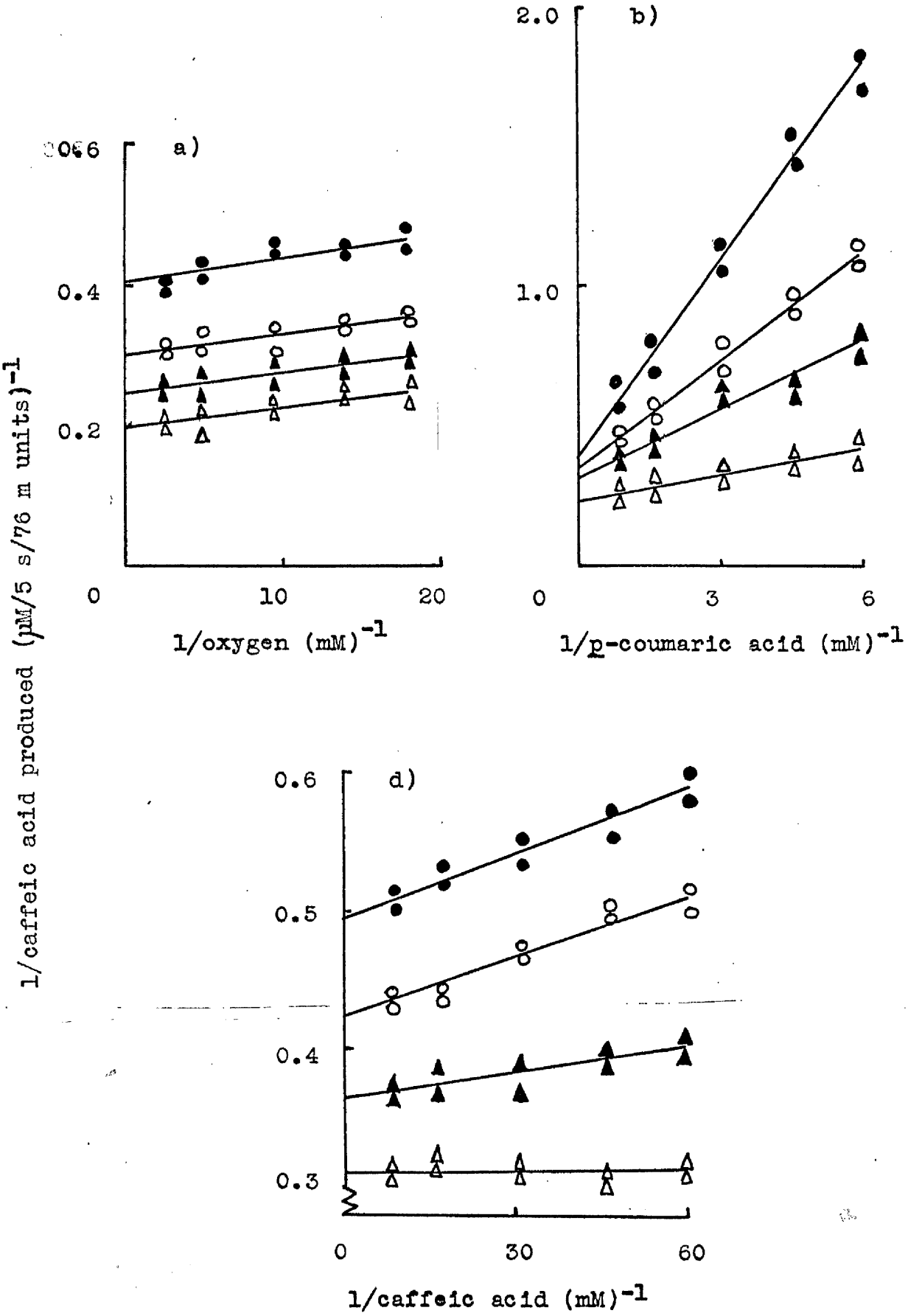
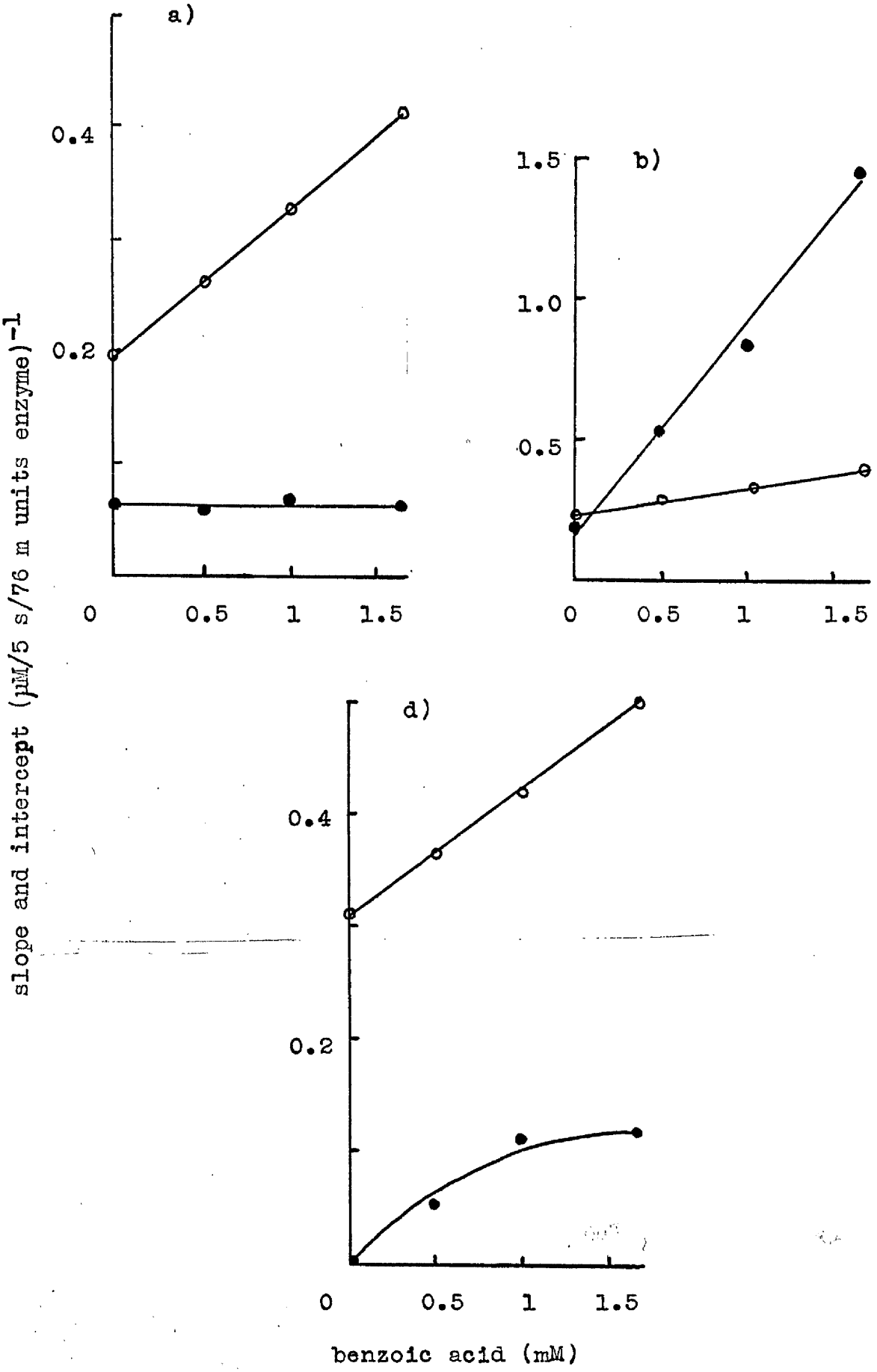

a)
b)
d)
0.6
0.4
0.2
0
10
20
2.0
1.0
3
6
0.5
0.3
30
60
1/oxygen (mM)$^{-1}$
1/p-coumaric acid (mM)$^{-1}$
1/caffeic acid (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units)$^{-1}$

Fig. 32. Secondary replots - effect of benzoic acid.

Slope (●) and vertical intercept values (○) obtained from Fig. 31 were plotted against inhibitor concentration.

Experimental details were as described in the legend to Fig. 31.


a)
b)
d)
0.4
0.2
1.5
1.0
0.5
0
0.5
1
1.5
slope and intercept (μM/5 s/76 m units enzyme)$^{-1}$
benzoic acid (mM)

3.3.1.3. 3,4- dihydroxybenzoic acid studies.

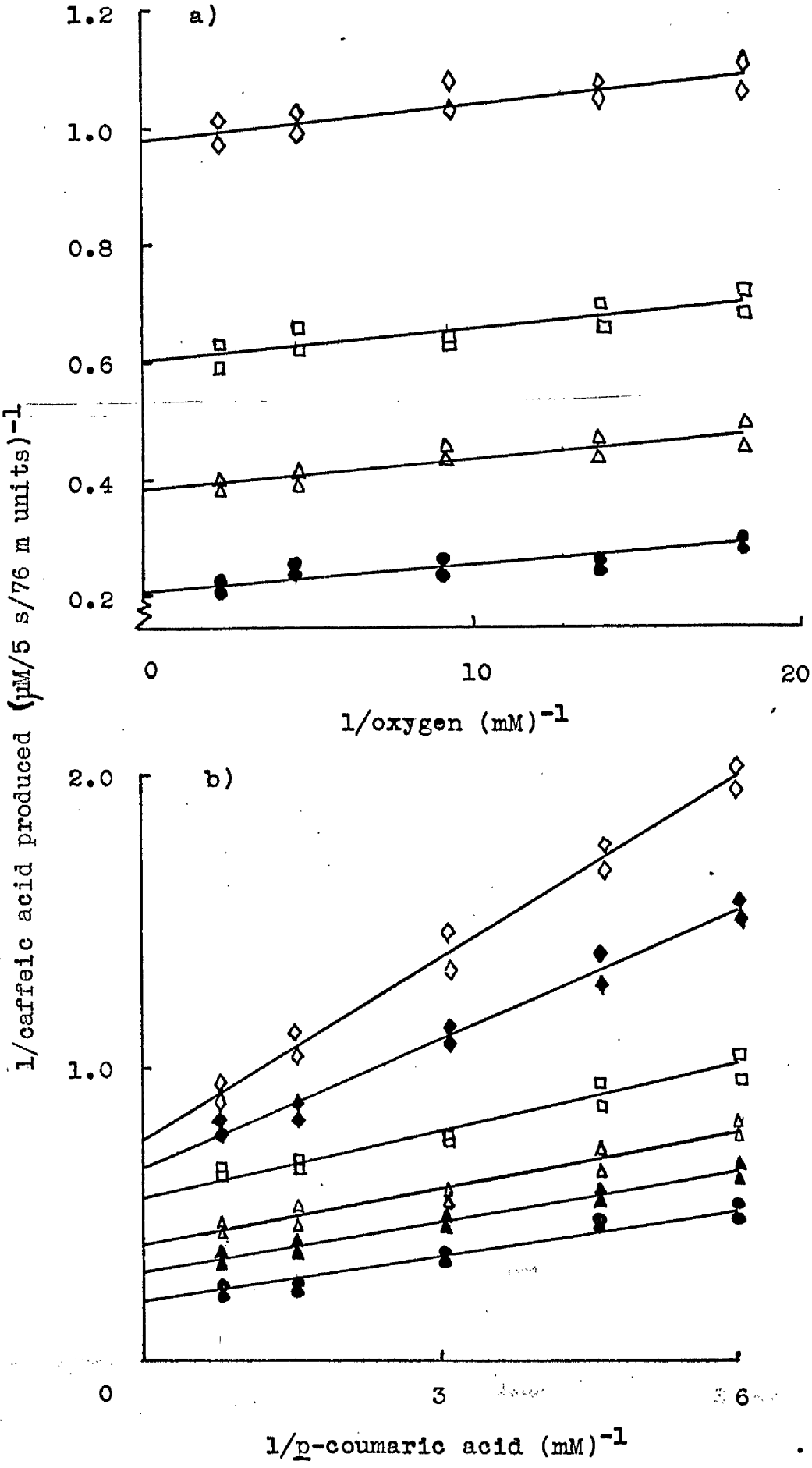
The addition of 3,4 dihydroxybenzoic acid (0.167 - 1.67 mM) to the reaction in the presence of varying amounts of oxygen (0.056 - 0.448 mM) results in a parallel initial velocity pattern (Fig. 33a). With p-coumaric acid (0.167 - 1.67 mM) as variable substrate the parallel pattern produced becomes intersecting at high inhibitor concentrations (Fig. 33b), while with varied ascorbic acid (0.167 - 2mM) the parallel plot concaves down at high concentrations of ascorbic acid (Fig. 33c). Conversion of an intersecting pattern at low inhibitor concentrations to a parallel pattern at high values is seen in the presence of varied caffeic acid (0.017 - 0.067mM : Fig. 33d). The secondary replots (Fig. 34) show a linear uncompetitive inhibition with oxygen. The results obtained with other variable substrates are more complex and cannot be further classified using secondary replots as the type of inhibition i.e. competitive, noncompetitive or uncompetitive, cannot be clearly distinguished from the Lineweaver-Burk plots.

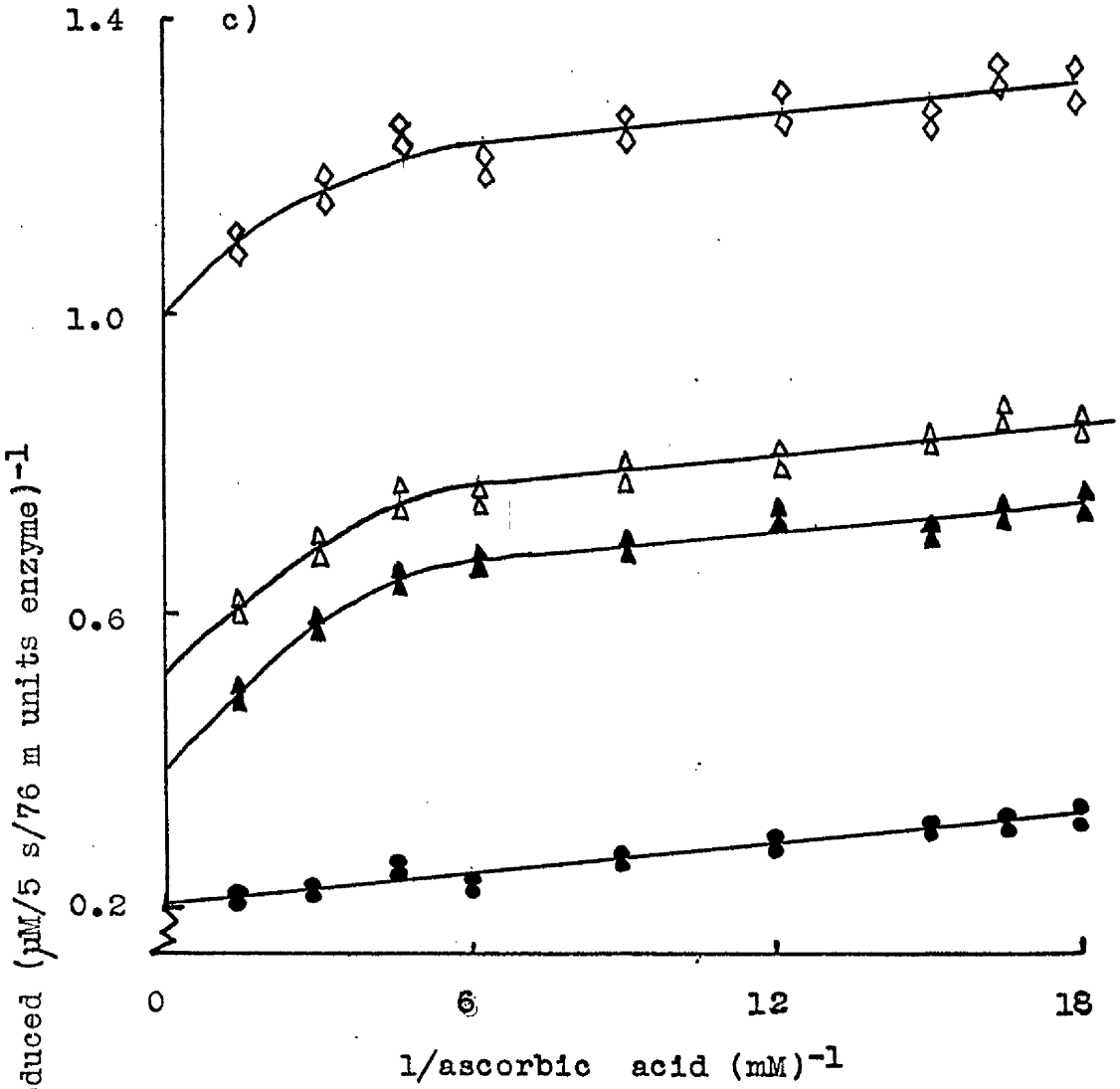
Fig. 33. Effect of 3,4 - dihydroxybenzoic acid on initial velocity patterns.

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor				(●)
0.084	mM	3,4-dihydroxybenzoic	acid	(○)
0.167	mM	"	"	(▲)
0.33	mM	"	"	(△)
0.5	mM	"	"	(■)
0.667	mM	"	"	(□)
1.167	mM	"	"	(◆)
1.67	mM	"	"	(◇)

Other substrates were varied as described in the legend to Fig. 29.


a)
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
1/oxygen (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units)$^{-1}$
b)
2.0
1.0
0
3
6
1/p-coumaric acid (mM)$^{-1}$


c)
1.4
1.0
0.6
0.2
0
6
12
18
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units enzyme)$^{-1}$

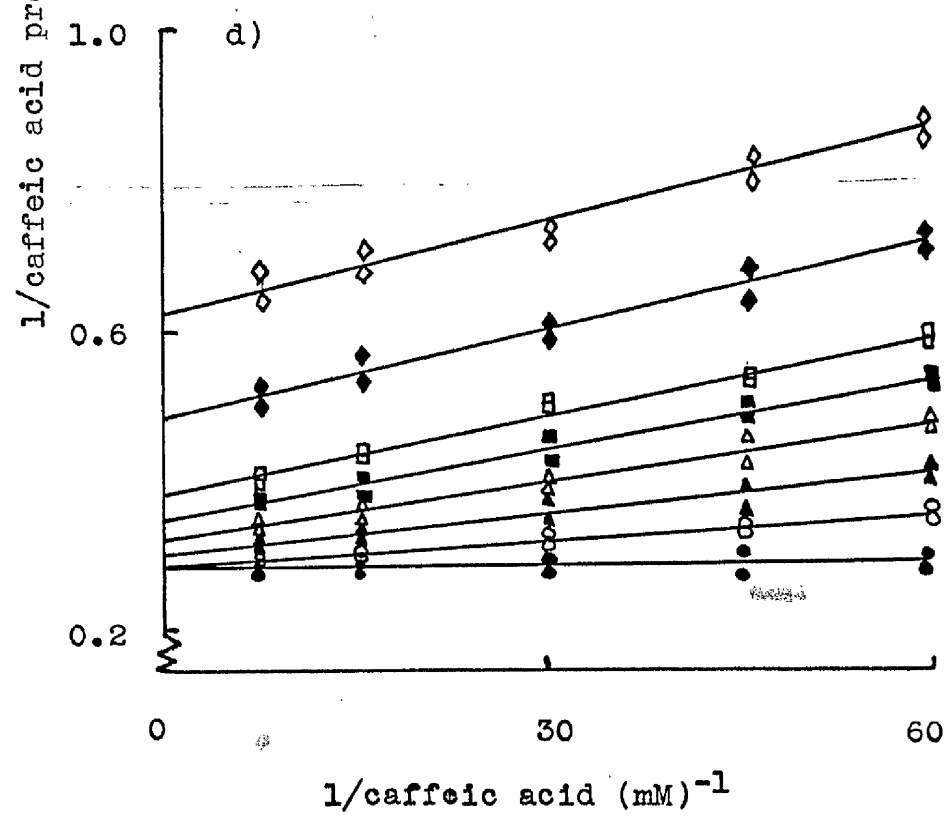
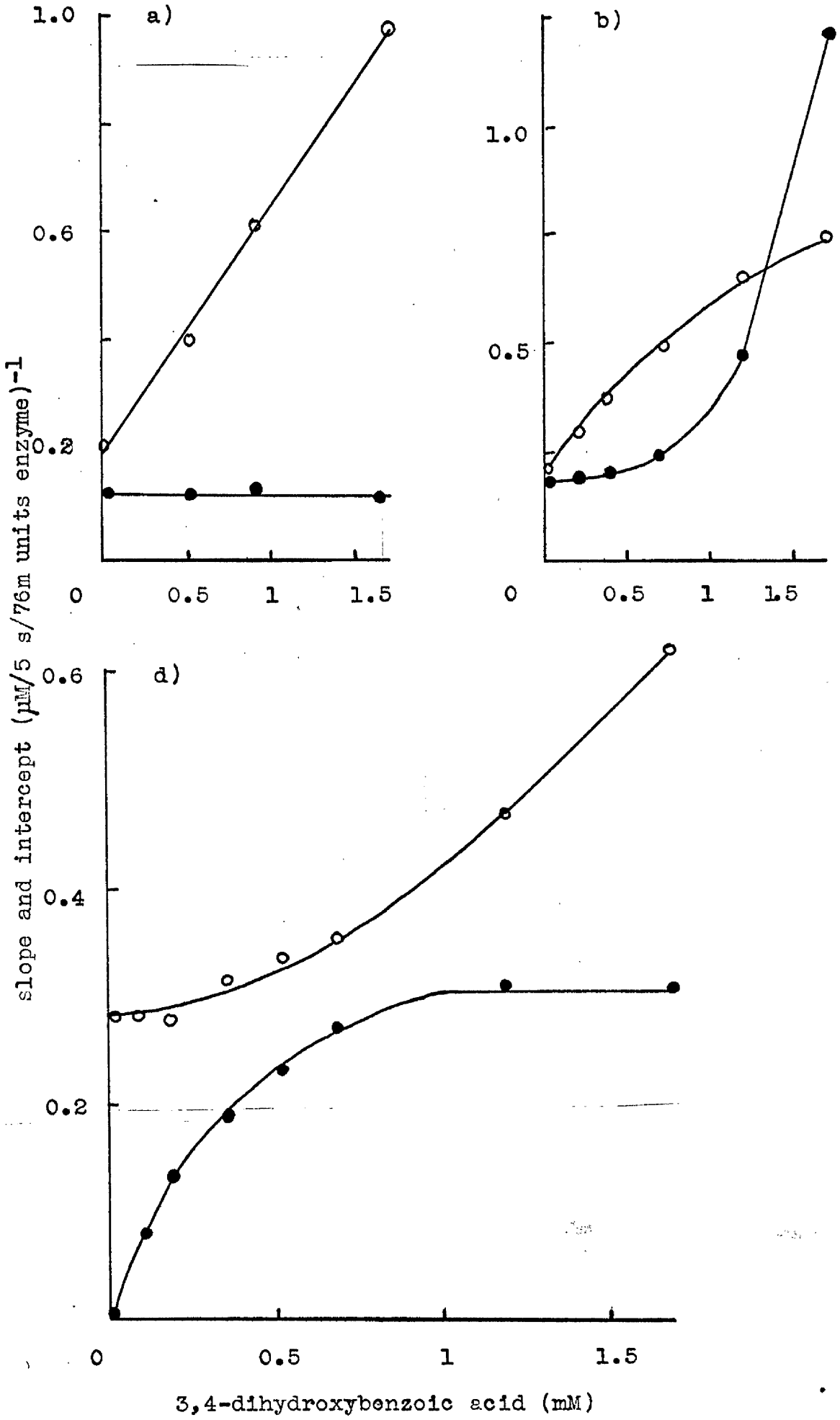

d)
1.0
0.6
0.2
0
30
60
1/caffeic acid (mM)$^{-1}$

Fig. 34. Secondary replots - effect of 3,4 - dihydroxybenzoic acid.

Slope (●) and vertical intercept values (○) obtained from Fig. 33 were plotted against inhibitor concentration.

Experimental details were as described in the legend to Fig. 33.


a)
b)
d)
1.0
0.6
0.2
0.5
0.4
0
0.5
1
1.5
slope and intercept (μM/5 s/76m units enzyme)-1
3,4-dihydroxybenzoic acid (mM)

3.3.2. Inhibition Studies with copper specific reagents.

3.3.2.1. Preliminary Studies.

3.3.2.1.1. Difference Spectra.

The difference spectra obtained for the enzyme - BCS complex in the presence and absence of dithionite (Fig. 35a) show an absorption maximum at 480 nm, as does a standard Cu+-BCS complex. Using the molar extinction coefficient determined from the standard curve (1.89×10^{4} litre $mole^{-1} cm^{-1}$) and assuming a linear relationship between concentration of the complex and absorption, it can be shown that 45% of the detectable copper (405ng) exists as Cu+ in the resting enzyme and that on addition of dithionite a further 495 ng of copper are converted to this oxidation state.

The difference spectrum of a DIECA-enzyme complex is similar to that of a standard solution (Fig. 35b : Molar extinction coefficient at 450 nm, 0.88×10^{4} litre $mole^{-1} cm^{-1}$) and it can be calculated that 505 ng of copper react with DIECA, suggesting that this copper is found as Cu++. This value is similar to that deduced from the BCS difference spectrum.

Difference spectra in the presence of carbon monoxide could not be obtained, as the amounts of enzyme apparently required were prohibitive.

3.3.2.1.2. Inhibition curves.

The effect of varying concentrations of BCS, carbon monoxide and DIECA in the presence of saturating amounts of ascorbic acid and p-coumaric acid, in air, is shown in Fig. 36. The reaction is almost totally inhibited by the addition of 0.01 mM DIECA. Much higher concentrations of BCS were required for inhibition; 0.33 mM BCS giving a 30% loss in activity. Higher concentrations of BCS could

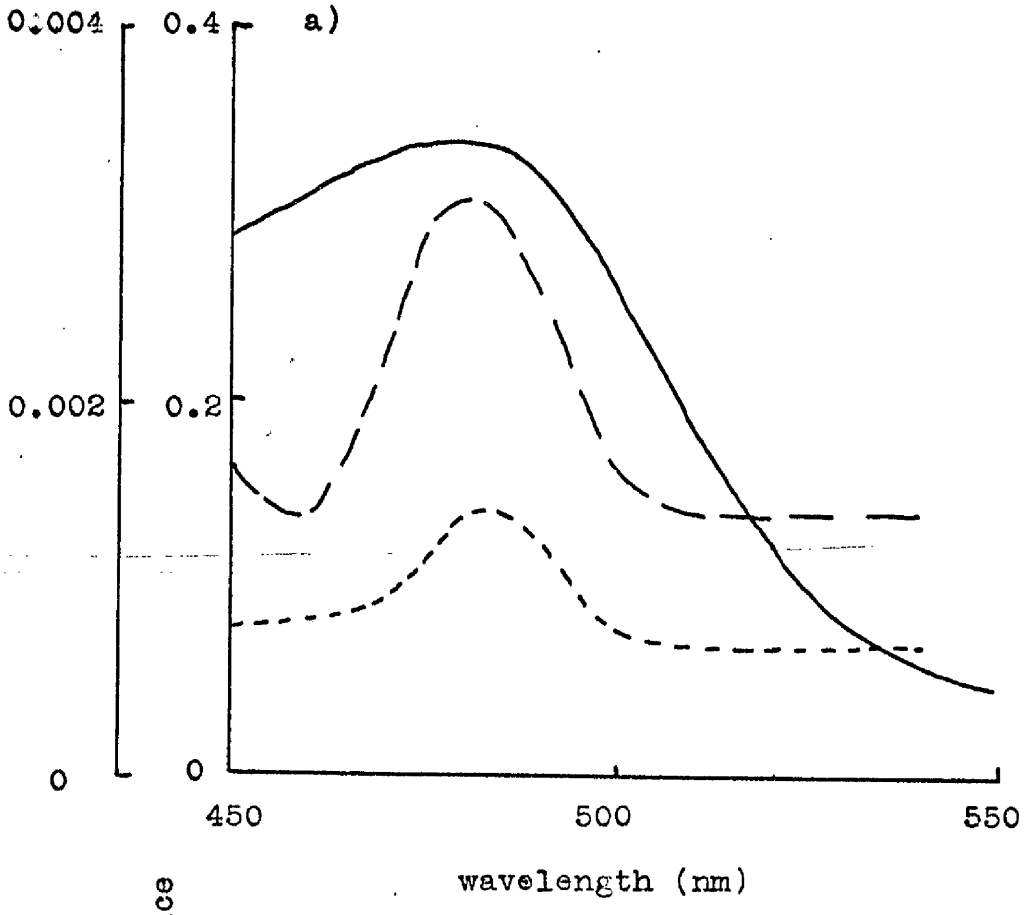
Fig. 35. Difference spectra obtained on addition of copper specific reagents to phenolase.

a) BCS.

0.003 mg copper was added to 1.67 mM BCS in 0.1 M Na_2HPO_4 - 0.05 M citric acid buffer, pH 6.5, and 0.5 M $(NH_4)_2 SO_4$ in 3 ml. The absorbance was measured after the addition of a pinch of dithionite (———). 1.67 mM BCS was added to 0.1 M Na_2HPO_4 - 0.05 M citric acid buffer, pH 6.5, and 0.5M $(NH_4)_2SO_4$ containing 1,900 m units of enzyme in 3 ml. The absorbance spectrum was measured against a reference cell containing all components except BCS in the presence (— — —), and absence (-----) of dithionite.

b) DIECA.

0.003 mg copper was added to 3.3 mM DIECA in 0.1 M Na_2HPO_4 - 0.05 M citric acid buffer, pH 6.5, and 0.5 M $(NH_4)_2SO_4$ in 3 ml, and the absorbance measured (———). 3.3 mM DIECA was added to 0.1M Na_2HPO_4 - 0.05 M citric acid buffer, pH 6.5, and 0.5 M $(NH_4)_2SO_4$ containing 1,900 m units enzyme in 3 ml. The absorbance spectrum was measured against a reference cell containing all components except DIECA (-----).


a)
0.004
0.4
0.002
0.2
0
0
450
500
550
wavelength (nm)
Absorbance

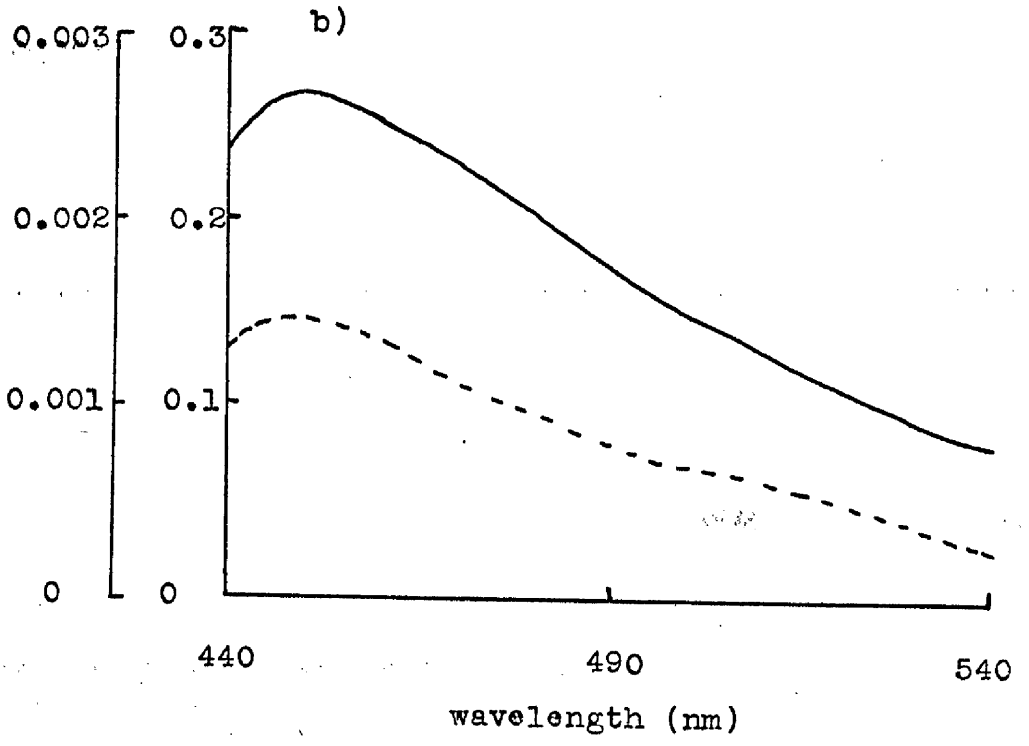
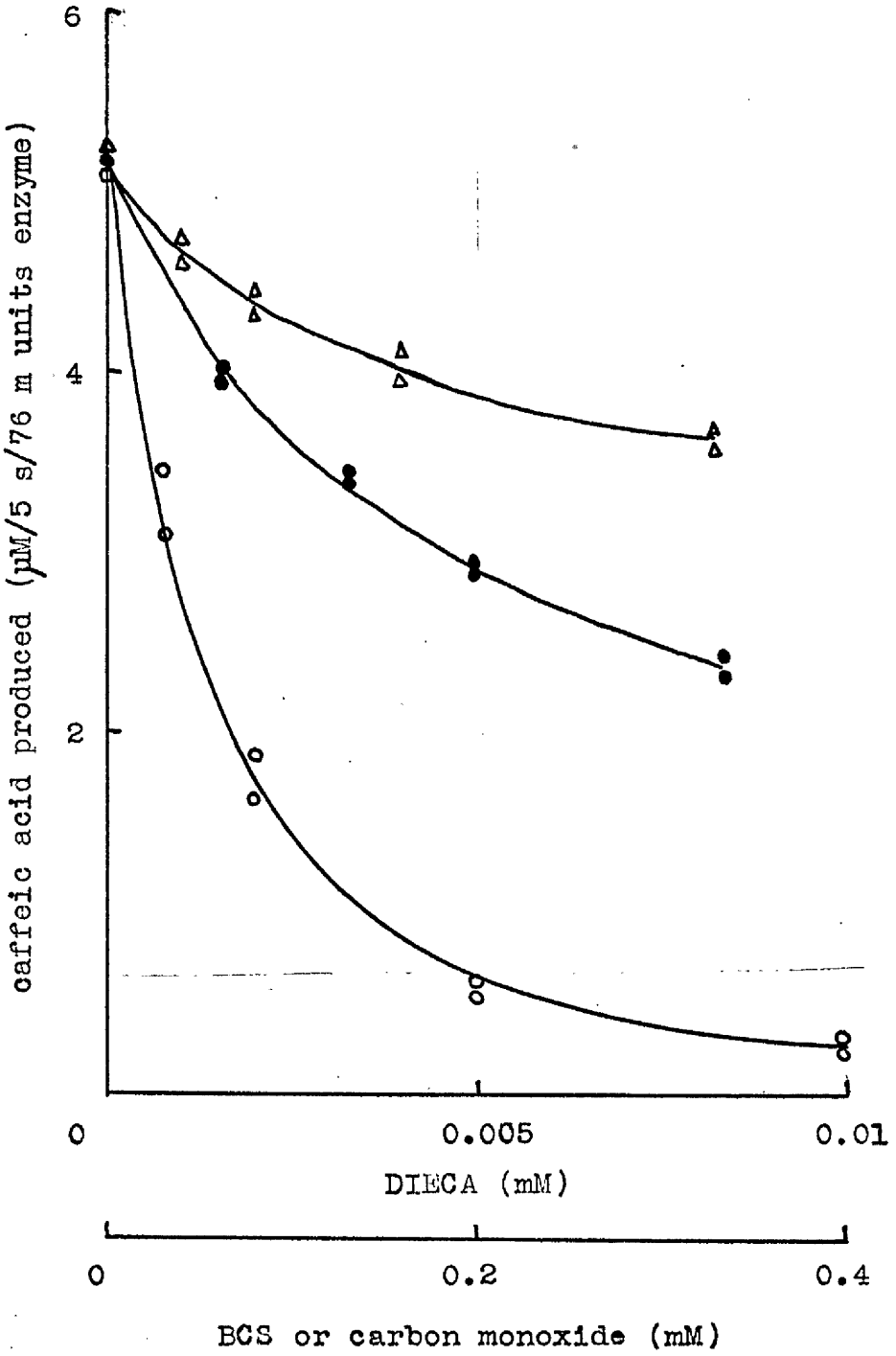

b)
0.003
0.3
0.002
0.2
0.001
0.1
0
0
440
490
540
wavelength (nm)

Fig. 36. Inhibition curves obtained with copper inhibitors.

1.67 mM p-coumaric acid was incubated with 1.67 mM ascorbic acid and 76 m units phenolase, in air, in the presence of varying amounts of DIECA (○) carbon monoxide (●) and BCS (Δ). Assays were carried out under the conditions described for continuous assay (2.2.5.2.).


6
4
2
caffeic acid produced (μM/5 s/76 m units enzyme)
0
0.005
0.01
DIECA (mM)
0
0.2
0.4
BCS or carbon monoxide (mM)

not be used owing to its absorption at 340 nm (Fig. 37) which resulted in highly absorbing solutions. 30% inhibition was obtained using 0.16 mM carbon monoxide.

3.3.2.2. Carbon monoxide studies.

Parallel initial velocity patterns were obtained when p-coumaric acid (0.05 - 1.6 mM:Fig. 38b), ascorbic acid (0.067 - 0.33 mM : Fig. 38c) and caffeic acid (0.017 - 0.067 mM : Fig. 38d) were varied in the presence of carbon monoxide (0.044 - 0.133 mM). With oxygen as variable substrate, however, the initial velocity pattern obtained converges on the abscissa.

Secondary replots of these data (Fig. 39) show that carbon monoxide is a linear uncompetitive inhibitor with respect to p- coumaric acid, ascorbic acid and caffeic acid, but a linear competitive inhibitor with oxygen.

3.3.2.3. DIECA Studies.

In the presence of varying amounts of DIECA (0.5 - 1.5 uM) parallel patterns were obtained when oxygen (0.056 - 0.448 mM : Fig. 40a) or ascorbic acid (0.067 - 0.33 mM : Fig. 40c) were varied. With varied p-coumaric acid (0.167 - 1.67 mM : Fig. 40b), however, a pattern intersecting to the left of the abscissa was found, while with varied caffeic acid (0.017 - 0.067mM : Fig. 40d) the pattern intersected on the abscissa. A consideration of the secondary replots (Fig. 41) shows that linear uncompetitive inhibition is observed with varied oxygen (Fig. 41a) and ascorbic acid (Fig. 41c), S-parabolic, I-linear inhibition with p-coumaric acid (Fig. 41b) and parabolic competitive inhibition with caffeic acid as variable substrate.(Fig. 41d).

Fig. 37. Absorption spectrum of BCS.

The absorbance of 0.067 mM BCS in 0.1M Na_2HPO_4 - 0.05M citric acid buffer, pH6.5, and 0.5M $(NH_4)_2SO_4$ was determined using a Cary 15 spectrophotometer.

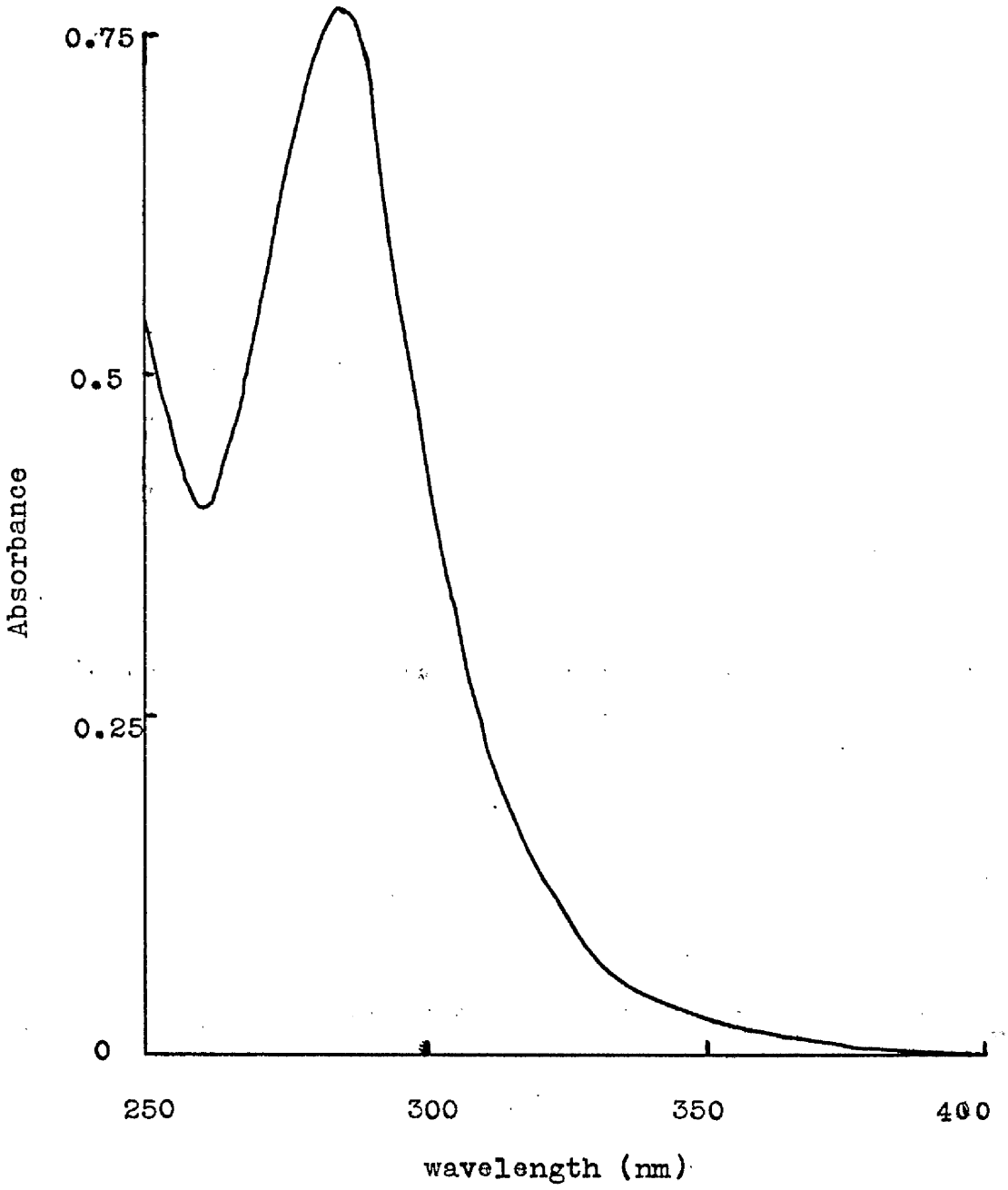


Fig. 38. Effect of carbon monoxide on initial velocity patterns.

---

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor	(Δ)
0.033 mM carbon monoxide	(▲)
0.067 mM " "	(○)
0.133 mM " "	(●)

Other substrates were varied as described in the legend to Fig. 29.

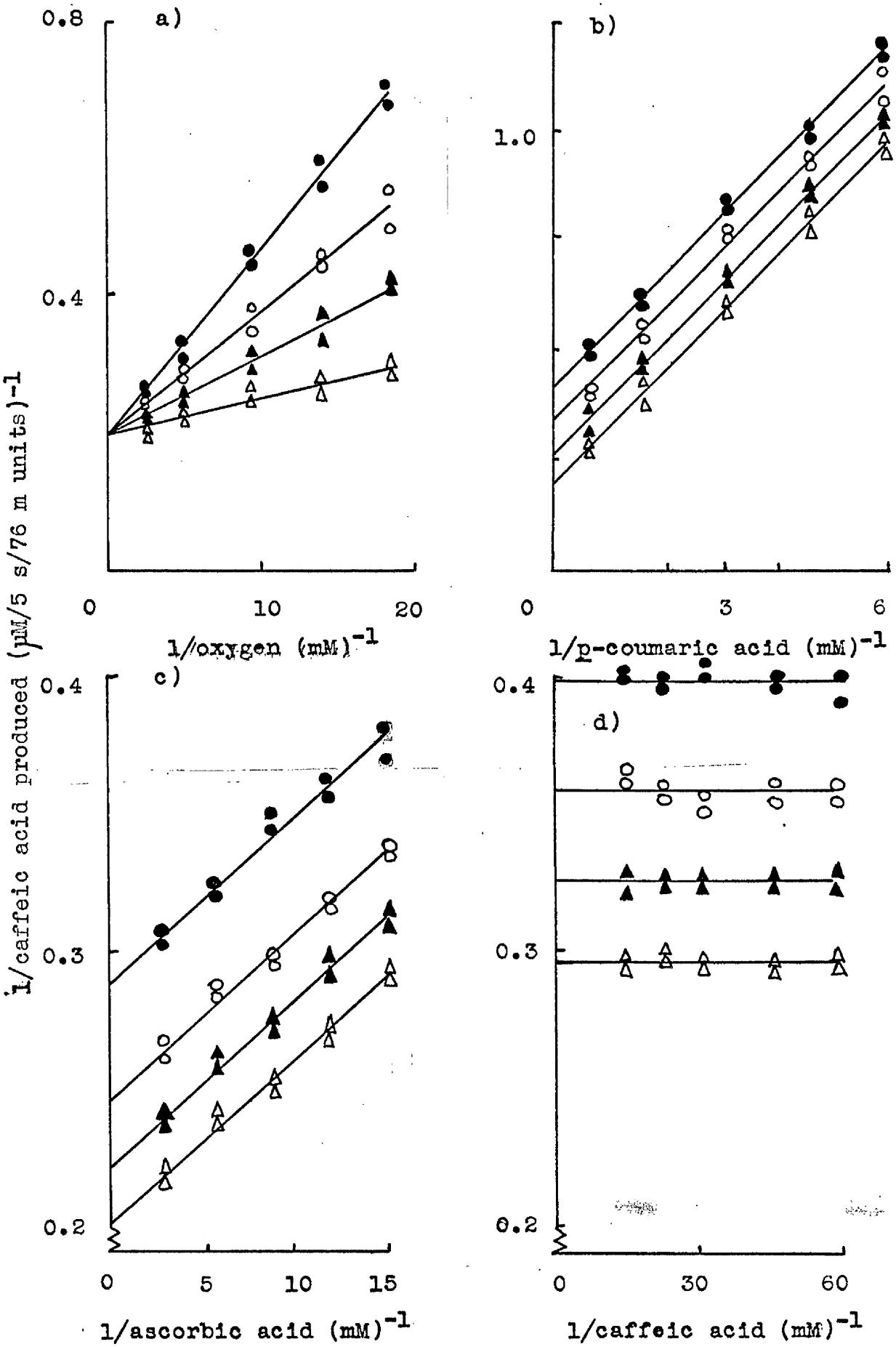

a)
b)
c)
d)
0.8
0.4
0
10
20
1.0
3
6
0.3
0.2
5
15
30
60
1/oxygen (mM)$^{-1}$
1/p-coumaric acid (mM)$^{-1}$
1/caffeic acid produced (μM/5 s/76 m units)$^{-1}$
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid (mM)$^{-1}$

Fig. 39. Secondary replots - effect of carbon monoxide.

Slope (●) and vertical intercept values (O) obtained from Fig. 38 were plotted against inhibitor concentration.

Experimental details were as described in the legend to Fig. 38.

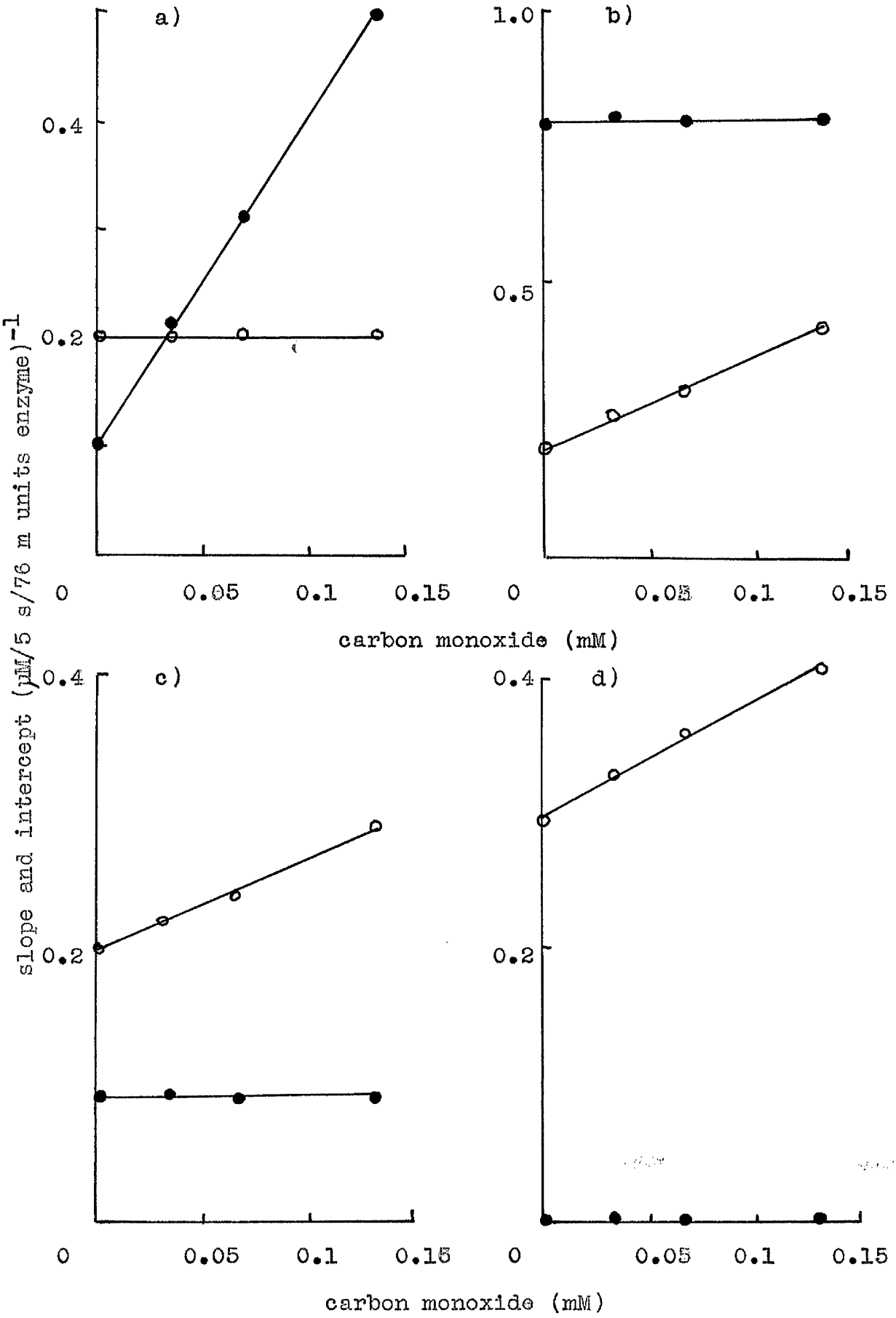

a)
b)
c)
d)
0.4
0.2
1.0
0.5
0
0.05
0.1
0.15
slope and intercept (μM/5 s/76 m units enzyme)$^{-1}$
carbon monoxide (mM)
carbon monoxide (mM)

Fig. 40. Effect of DIECA on initial velocity patterns.

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor	(Δ)
0.5 uM DIECA	(▲)
1.0 uM "	(○)
1.5 uM "	(●)

Other substrates were varied as described in the legend to Fig. 29.

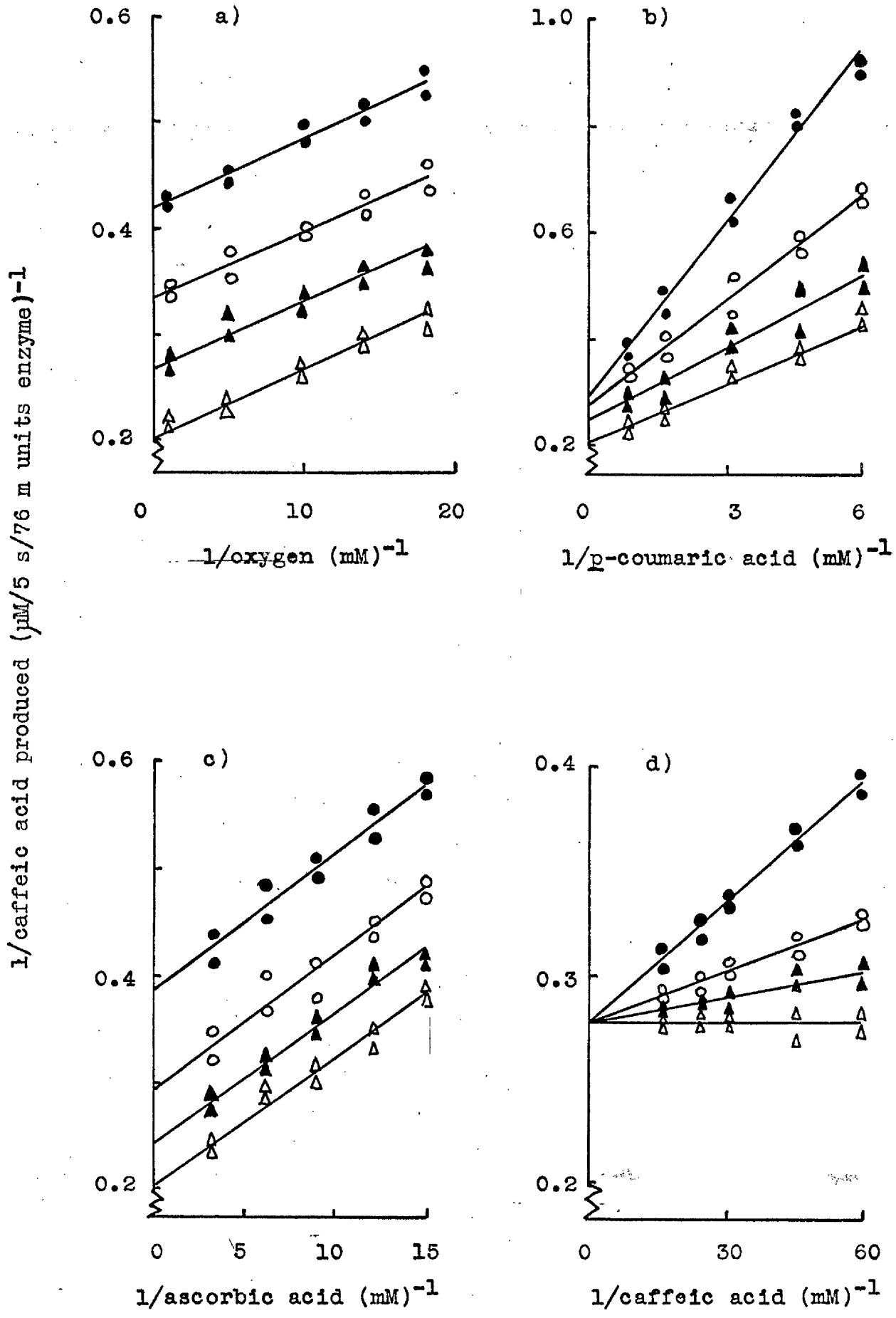
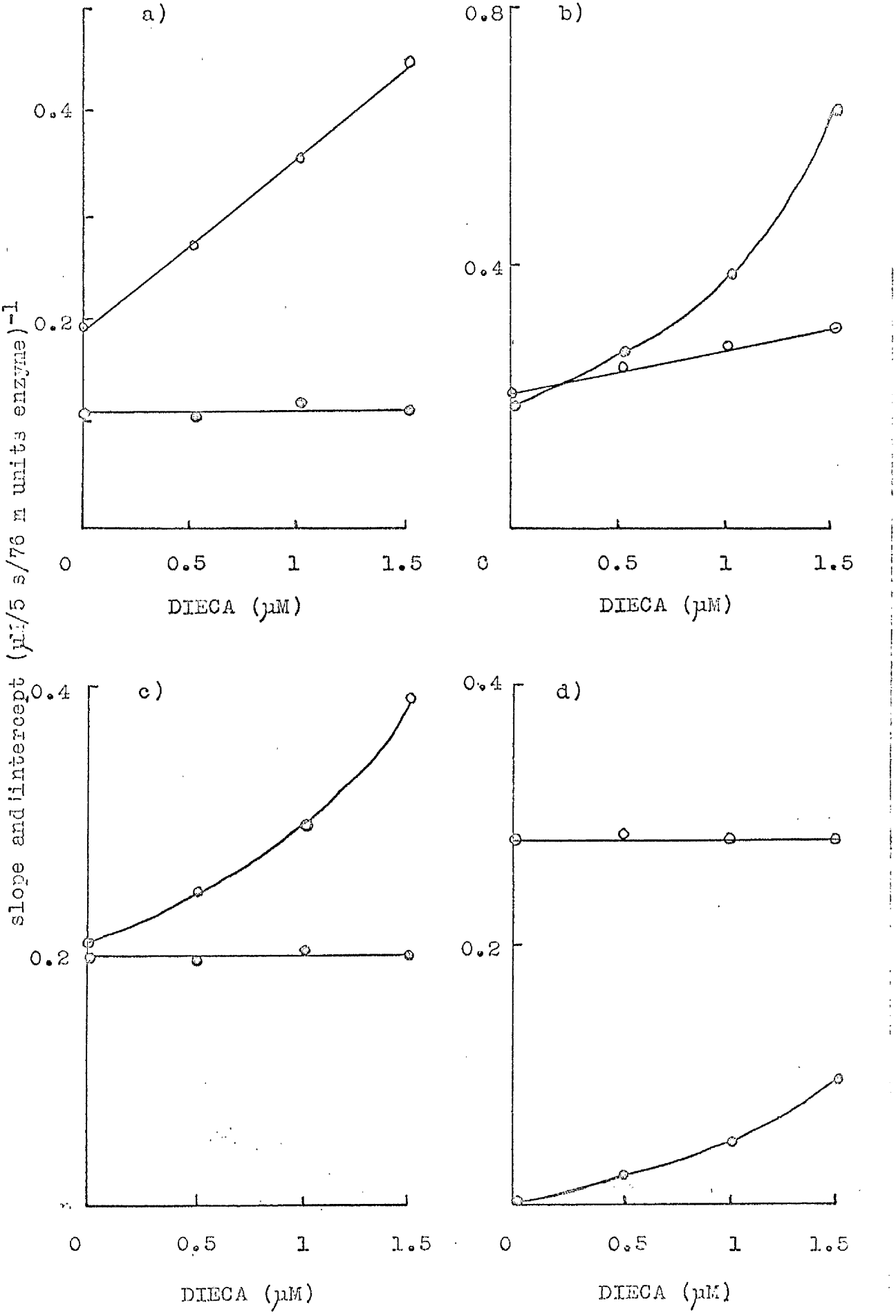

a)
b)
c)
d)
0.6
0.4
0.2
1.0
0.6
0.2
0.6
0.4
0.2
0.4
0.3
0.2
0
10
20
0
3
6
0
5
10
15
0
30
60
1/oxygen (mM)$^{-1}$
1/p-coumaric acid (mM)$^{-1}$
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid (mM)$^{-1}$
1/caffeic acid produced (µM/5 s/76 m units enzyme)$^{-1}$

Fig. 41. Secondary replots - effect of DIECA.

Slope (●) and vertical intercept values (O) obtained from Fig. 40 were plotted against inhibitor concentration.

Experimental details were as described in the legend to Fig. 40.


a)
b)
c)
d)
0.4
0.2
0.8
0
0.5
1
1.5
DIECA (μM)
slope and intercept (μl/5 s/76 m units enzyme)$^{-1}$

Thus DIECA would only seem to compete directly with caffeic acid. The inhibitor constant for DIECA was calculated from Fig. 41b, to be 5.15 µM.

3.3.2.4. BCS studies

In this case, parallel patterns were obtained with p-coumaric acid (0.167 - 1.67 mM : Fig. 42b), ascorbic acid (0.067 - 0.33 mM : Fig. 42c) or caffeic acid (0.017 - 0.067 mM : Fig. 42d) as variable substrate, in the presence of varying amounts of BCS (0.083 - 0.33 mM). When oxygen was varied (0.056 - 0.448 mM : Fig. 42a), however, an intersecting pattern was obtained, as no intercept effect occurred. Scrutiny of the secondary replots (Fig. 43) suggests that BCS is a linear uncompetetive inhibitor with p-coumaric acid, ascorbic acid and caffeic acid. It is a linear competetive inhibitor against oxygen, this being the only direct competition observed. The inhibitor constant was calculated in this case from the intercept replot of Fig. 43d and was found to be 0.62 mM for BCS.

Fig. 42. Effect of BCS on initial velocity patterns.

76 m units of phenolase were incubated under conditions for continuous assay (2.2.5.2.) with

no added inhibitor	(Δ)
0.084 mM BCS	(Δ)
0.167 mM "	(○)
0.33 mM "	(◎)

Other substrates were varied as described in the legend to Fig. 29.

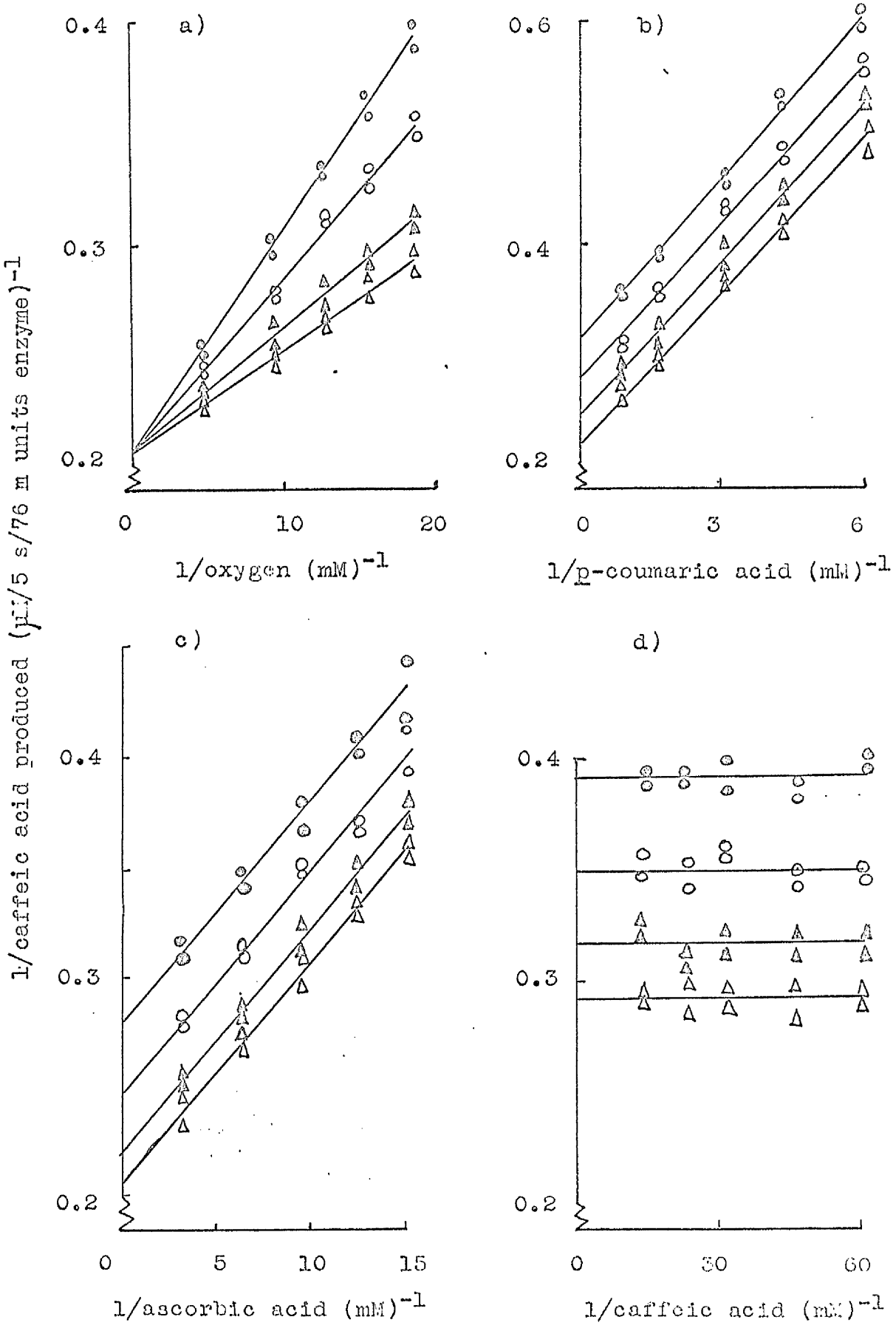
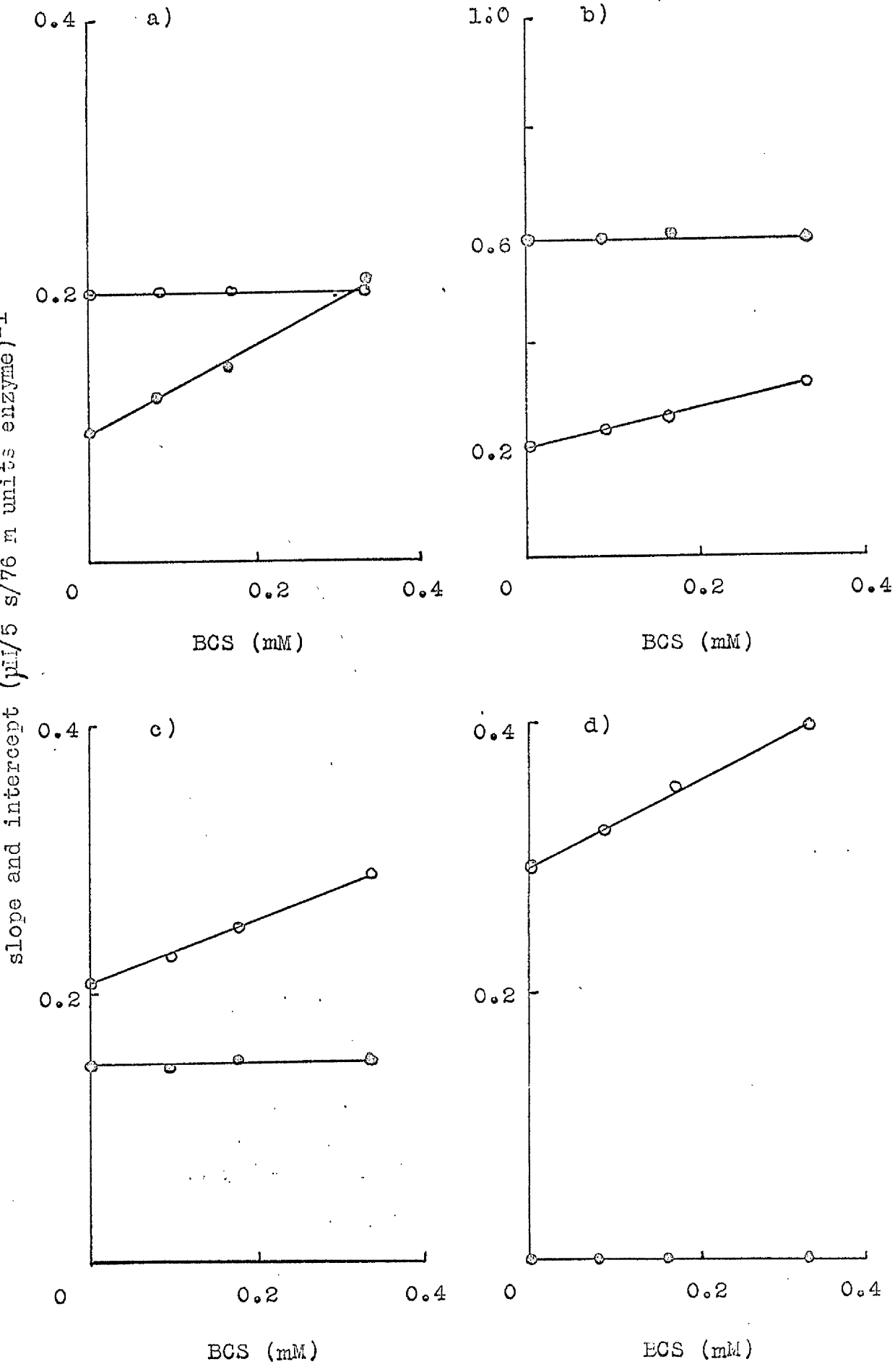

a)
b)
c)
d)
0.4
0.3
0.2
0.6
0.4
0.2
0.4
0.3
0.2
0
10
20
0
3
6
0
5
10
15
0
30
60
1/oxygen (mM)$^{-1}$
1/p-coumaric acid (mM)$^{-1}$
1/ascorbic acid (mM)$^{-1}$
1/caffeic acid (mM)$^{-1}$
1/caffeic acid produced (µl/5 s/76 m units enzyme)$^{-1}$

Fig. 43. Secondary replots - effect of BCS.

Slope (○) and vertical intercept values (○) obtained from Fig. 42 were plotted against inhibitor concentration. Experimental details were as described in the legend to Fig. 42.


a)
b)
c)
d)
0.4
0.2
0
1.0
0.6
slope and intercept (µl/5 s/76 m units enzyme)-1
BCS (mM)

IV. DISCUSSION

4.1. Preliminary work.

The close agreement between results obtained using the colorimetric and spectrophotometric methods (Table 5) has led to the routine use of the spectrophotometric assay for determination of initial rates of caffeic acid production. The method is satisfactory, with ascorbic acid and DMTP as electron donors, or in the absence of reducing agent, for p-coumaric acid concentrations up to 1.67 mM in 10 mm cuvettes, or 3.33 mM in 5 mm cells. The high absorbance of NADH and p-coumaric acid at 240 nm limits this method to relatively low, about 0.5 mM, amounts of these substrates.

Before a kinetic investigation can be attempted the basic properties of the enzymic reaction must be elucidated. The occurrence of two pH optima observed during the hydroxylation of p-coumaric acid by spinach phenolase (Fig. 13) has also been noted on reaction of chlorogenic acid with potato phenolase (Patil and Zucker, 1965 : pH 5 and 7, Alberghina 1964 : pH 4 and 5.5) A similar result was obtained by Harel et al (1964) for apple phenolase with 4 - methylcatechol as substrate, but Walker and Hulme (1965) detected only one pH maximum during the catalysis of chlorogenic acid or 4 - methylcatechol by the same enzyme. Although two pH optima have been found in several systems, their exact positions vary. The high salt concentrations employed in the assay of spinach phenolase (Vaughan and Butt, 1969) possibly determines the alkaline trend of the pH curve obtained.

The inhibition of enzyme activity by catalase, in the presence of ascorbic acid or DMTP (Fig. 14), may be due to the removal of hydrogen peroxide from the assay mixture, as a stimulation of the hydroxylase activity of phenolase has been demonstrated (P.F.T. Vaughan, personal communication). Nielsen (1969) has observed the formation

of hydrogen peroxide on auto-oxidation of DMTP. The inhibition observed with EDTA in the presence of ascorbic acid could, on the other hand, result from the prevention of copper catalysed auto-oxidation (Butt and Hallaway, 1961) thus restricting the liberation of hydrogen peroxide.

In order to compensate for this effect and determine the true rate of hydroxylation, 3.33 mM EDTA was included in all ascorbic acid assays and 200 units/ml catalase were added to all assays involving DMTP.

The purity of the enzyme preparation used in kinetic work is also important in the determination of true initial rates. An impure preparation may be used if no side reactions occur which affect the assay, but results obtained with a homogeneous enzyme sample are less open to question. The phenolase used throughout this work gives only one band on gel electrophoresis (Fig. 12) and is therefore relatively free of protein contamination. The catechol oxidase activity of phenolase might be considered as a side reaction, but, under the conditions normally used, does not seem to interfere with the hydroxylase assay. The removal of naturally occurring inhibitors from the enzyme during preparation is also necessary if meaningful data are to be obtained. During the isolation of phenolase (Table 4) this does not seem to occur, suggesting that such inhibitors are not associated with this enzyme, or that they co-purify with it.

The rate of reaction observed in the presence of electron donor and small amounts of caffeic acid was equal to that attained after the short lag period found with reducing agent alone (Fig. 19b). This suggests that the addition of caffeic acid is not necessary for

the measurement of initial velocity. Consequently all assays, unless stated otherwise, were carried out in the absence of added o-dihydricphenol, which might have further complicated the kinetic analysis by functioning as a reaction product.

Long et al (1971) demonstrated that the effect of catechol on p-cresol oxidation by mushroom tyrosinase could be described by Michaelis-Menten rather than cofactor-activation kinetics (Alben, 1966), as the concentration of enzyme bound copper is small relative to diphenol concentration. Taking the specific activity of 30,000 m units/mg protein for phenolase and a copper content of 0.25% (Vaughan and Butt, 1969) the copper content / assay can be calculated as 0.033 nM. As 30 µM caffeic acid is sufficient to eliminate the lag, it would seem, using the criteria adopted by Long et al (1971), that Michaelis Menten kinetics can be applied to spinach leaf phenolase.

The detection of a linear relationship between rate of reaction and enzyme concentration (Fig. 15) suggests that over the range studied- up to 250 m units enzyme / assay - the errors inherent in the steady state assumption are within experimental error (Wong, 1965), and hence steady state analysis can be applied to phenolase.

A necessary preliminary in a more detailed kinetic study is the determination of the Michaelis constant for each substrate in the presence of saturating amounts of the other substrates. The linear Lineweaver - Burk plots obtained (Fig. 18) are further evidence of the Michaelis-Menten behaviour of the enzyme with all the substrates concerned. No substrate, or product, inhibition or activation occurs over the range considered, as p-coumaric acid concentrations greater than 2 mM were not used, and hence kinetic analysis will not be further complicated by deviations of this kind.

The Michaelis constant of spinach phenolase for oxygen, 0.053 mM or 4.8%, is independent of electron donor in the presence of saturating amounts of p-coumaric acid (Table 7). A similar value, 5.3% has been obtained for grape phenolase in the presence of p-coumaric acid and absence of electron donor (Harel and Mayer, 1971), while the K_M for oxygen of apple phenolase catalysing the hydroxylation of p-cresol has been calculated as 2.25% under comparable conditions of assay. (Harel et al, 1964). Robb et al, (1966) have estimated the Michaelis constant of broad bean tyrosinase for oxygen to be 0.585 mM in the presence of 7.7 mM p-cresol, with no added reductant.

These values are in agreement with those obtained for similar enzymes, the K_M for oxygen being 0.053 mM for phenol hydroxylase (Neujahr and Gaal, 1973) in the presence of phenol and NADH and 8.5% for phenylalanine hydroxylase in the presence of dimethylpterine and phenylalanine (Kaufman, 1971). A calculated value of 0.52mM, however, has been reported for p-hydroxybenzoate hydroxylase (Nakamura et al, 1970) in the presence of NADPH.

Although the Michaelis constant of spinach phenolase for oxygen is not affected by the electron donor present, the same is not true for p-coumaric acid, a value of 0.268 mM being obtained with ascorbic acid and 0.192 mM with DMTP, in air. In the absence of electron donor 1.1 mM p-coumaric acid gives half maximum velocity with spinach phenolase, compared with 2.7 mM for mushroom phenolase with p-cresol (Robb et al, 1966) and 0.6 mM p-coumaric acid with grape phenolase (Harel and Meyer, 1971), all assays being carried out in air.

The Km for caffeic acid was measured under two distinct conditions.

1) Where caffeic acid was acting as a substrate for catechol oxidase activity (Fig. 17) the value obtained with spinach phenolase

was 3.33×10^{-4}M. This agrees with the lower values obtained for o-dihydricphenols - 1.7×10^{-4}M for chlorogenic acid with potato phenolase (Alberghina, 1964) and 2.2×10^{-6}M and 6.7×10^{-5}m for the two fractions arising from DEAE chromatography of the potato enzyme (Patil and Zucker, 1965).

2) where caffeic acid is reducing the length of the lag observed in the absence of electron donor (Fig. 19a).

The values obtained for the Michaelis constants for caffeic acid were 1) 3.33×10^{-4}M and 2) 1.8×10^{-6}M. These are comparable to those determined for dihydroxyphenylalanine, in the absence of electron donor, with melanoma tyrosinase 1) 5×10^{-4}M and 2) 2×10^{-6}M. (Pomerantz and Warner, 1967). These results suggest the existence of two binding sites for caffeic acid, one of which, the lag reducing site, binds o-diphenol much more strongly than the other. The reduction of the lag by increasing amounts of caffeic acid, suggests a function for this compound as internal reducing agent for the reaction.

If possible, kinetic experiments should be carried out over a wide concentration range, preferably 0.20 Km to 5 Km (Plowman, 1972), if a meaningful spread of results are to be obtained. The severe restrictions on the concentrations of NADH (0.5 mM) and p-coumaric acid (0.5 mM) limit this range, and hence it was decided that the NADH assay was not suitable for further study. Initial velocity studies were therefore carried out, in the presence of ascorbic acid or DMTP, or in the absence of electron donor, as the required range of concentrations could be covered under these conditions.

4.2. Initial Velocity studies.

The parallel initial velocity patterns obtained on variation of either oxygen or p-coumaric acid in the absence of electron donor suggest a ping pong type of mechanism under the defined conditions (Fig. 3). Thus some form of irreversible step occurs after the binding of oxygen or p-coumaric acid to the enzyme. A similar result was obtained using broad bean phenolase with oxygen and p-cresol as the variable substrates (Robb et al, 1966). The existence of these apparently irreversible steps is supported by the parallel patterns obtained with either oxygen or p-coumaric acid as the variable substrate in the presence of ascorbic acid (Fig. 21) or DMTP (Fig. 22).

The conversion of a parallel to an intersecting pattern on variation of electron donor at high concentrations of the changing fixed substrates, oxygen and p-coumaric acid, is probably due to the production of sufficient amounts of caffeic acid to affect the slope of the initial velocity pattern. The reversal of an apparently irreversible step by a product is characteristic of a classical ping pong mechanism (Cleland, 1970). In order to clarify the situation further, experiments of this type were performed. The Vmax values obtained in the presence (Table 10 ; 5.73 μM/5 s/76 m units) and absence (Table 8 ; 5.83 μM/5 s/76 m units) of caffeic acid show that the amount of added product was non-inhibitory.

Experiments with added caffeic acid show that the binding of oxygen to phenolase is not reversibly connected to the binding of p-coumaric acid or electron donor, as the parallel pattern originally observed remains unchanged (Fig. 25). Addition of caffeic acid, however, does alter the Lineweaver-Burk plots found in the presence of varied concentrations of p-coumaric acid and ascorbic acid.

Both these substrates gave rise to intersecting patterns under these conditions. On analysis by the Fromm method, this is characteristic of the existence of a reversible step between either 1) p-coumaric acid and oxygen or ascorbic acid or 2) ascorbic acid and oxygen or p-coumaric acid. As the results with oxygen confirm the existence of an irreversible step between the binding of oxygen and the other substrates, then it is apparent that the irreversible step between the binding of p-coumaric acid and ascorbic acid is removed by the addition of caffeic acid. As monophenol hydroxylation results in the formation of caffeic acid, then p-coumaric acid must bind to the enzyme before electron donor.

Several criteria (Plowman and Cleland, 1967) were used to assign the best fit for the computer analysed data to either a ping pong or a sequential type of mechanism. (Tables 8,9,10). Generally, the variances calculated did not differ greatly. Thus the degree of fit of the data to a particular model was largely decided on the basis of the validity of K_{iA}, although the standard errors of all the parameters influenced this decision.

Similar experiments with the other products of the reaction were not attempted, as these are water and oxidised electron donor, which is probably unstable o-quinone.

On the basis of the above results, p-coumaric acid has been shown to bind to the enzyme before electron donor. For hydroxylation to be possible under these circumstances, oxygen must bind before p-coumaric acid. Consequently the suggested order of substrate addition is first, oxygen, second, p-coumaric acid, and third, electron donor. Some form of irreversible step occurs after addition of oxygen, and product is released after binding of

p-coumaric acid. A rate equation based on the above mechanism has been evaluated by the method of Orsi (1971) as shown in Appendix II. This mechanism can be classified as bi uni uni ping pong.

As the results from initial velocity studies with ascorbic acid and DMTP supported the same basic mechanism, all further work was carried out using ascorbic acid as electron donor as this assay was simpler. This decision was also influenced by the high rate of autocatalysis observed with DMTP, which might have led to errors in substrate concentration values at low amounts of reducing agent, resulting in inaccurate initial velocity patterns.

4.3. Inhibition studies.

The use of inhibition studies to support the results of initial velocity work is widespread. Richter (1934) observed the competitive inhibition of potato phenolase by resorcinol and since then a wide range of phenolic compounds have been shown to inhibit phenolase (Webb, 1966).

Using Dalziel's nomenclature, the velocity expression for the derived mechanism is

$$\frac{v}{[E_o]} = \alpha_0 + \frac{\alpha_1}{[A]} + \frac{\alpha_2}{[B]} + \frac{\alpha_3}{[C]} \qquad \text{eqn 4.1}$$

where A is oxygen, B p-coumaric acid and C electron donor.

An inhibitor I, which competes with A for the same enzymic form will react as follows (Fromm 1967)

$$\frac{v}{[E_o]} = \alpha_0 + \frac{\alpha_1}{[A]}\left[1 + \frac{I}{K_I}\right] + \frac{\alpha_2}{[B]} + \frac{\alpha_3}{[C]} \qquad \text{eqn 4.2}$$

where K_I is the dissociation constant for the reaction $E + I \rightleftharpoons EI$. On the basis of eqn 4.2., the inhibition observed will be competitive with respect to A and uncompetitive with the other substrates. Similar analysis with competitive inhibitors for B and C should result in a permutation of the above conclusions.

Studies with the caffeic acid analogue, 3,4 -dihydroxybenzoic acid (Fig.33) resulted in an uncompetitive pattern with oxygen, while the parallel pattern observed in the presence of varying amounts of ascorbic acid became concave at high concentrations of electron donor. On the other hand, the uncompetitive inhibition recorded at low concentrations with varied amounts of p-coumaric acid became non-competitive as the amount of 3,4 - dihydroxybenzoic acid was increased. The explanation for these phenomena may lie in the results obtained with varied caffeic acid. These demonstrated that 3,4 - dihydroxy-benzoic acid is competitive at low concentrations but uncompetitive with increasing amounts of inhibitor. This implies the existence of a distinct site for the binding of caffeic acid and suggests that at high concentrations, inhibitor can bind at the p-coumaric acid site. The concentrations of caffeic acid used (0.017-0.067mM) show that this range is non-inhibitory (Fig. 19) and is sufficient to completely remove the lag, implying that caffeic acid binds to the low K_M site already detected ($K = 1.8 \times 10^{-6}$M)

The parallel curved pattern obtained in the presence of varied ascorbic acid, can perhaps be explained in terms of the larger amounts of caffeic acid produced at higher concentrations of electron donor competing against 3,4 - dihydroxybenzoic acid, while the inhibition remains uncompetitive.

p-hydroxybenzoic acid was chosen as a possible competitive

inhibitor against p-coumaric acid. On addition of this inhibitor to assay mixtures, the expected uncompetitive patterns were obtained with oxygen or ascorbic acid as variable substrate. A noncompetitive pattern, however, was found in the presence of varying amounts of p-coumaric acid and an intersecting - parallel pattern conversion was seen in the presence of caffeic acid (Fig. 29). This unexpected result might be due to the production of a small amount of 3,4-dihydroxybenzoic acid, and subsequent binding of this at the caffeic acid site. Similar experiments in the presence of benzoic acid (Fig. 31) which is not a substrate for phenolase produce comparable results which show that this is not the case. This suggests that the deviation from the expected results is due to the ring structure of the molecule occupying both phenolic binding sites on the enzyme.

Table 12 summarises the expected inhibitor patterns and those observed on analysis of results with the inhibitors discussed. Discrepancies in the predicted patterns have been explained on the basis of structural similarities between substrates and inhibitors.

4.4. Copper specific inhibitor studies.

The oxidation state of the copper in the resting enzyme has been the subject of considerable investigation. Kertesz (1957) showed, using the Cu^{+} specific reagent, biquinoline, that all the copper in resting mushroom tyrosinase was in the reduced form. This method is open to criticism as a technique to determine the valency state of a metal *in situ* (Malmstrom, 1965), as the strong acid required to liberate the metal from the protein. may affect the oxidation state of the copper. ESR studies on mushroom tyrosinase give a Cu^{++} signal which corresponds to 10 - 20% of the total copper,

Table 12. Comparison of predicted and observed inhibitor patterns.

Inhibitor	Variable Substrate	Predicted	Observed
p-hydroxy-benzoic acid	oxygen	uncompetitive	uncompetitive
	p-coumaric acid	competitive	noncompetitive
	ascorbic acid	uncompetitive	uncompetitive
	caffeic acid	uncompetitive	non→uncompetitive
benzoic acid	oxygen	uncompetitive	uncompetitive
	p-coumaric acid	competitive	noncompetitive
	caffeic acid	uncompetitive	non→uncompetitive
3'4-dihydroxy-benzoic acid	oxygen	uncompetitive	uncompetitive
	p-coumaric acid	uncompetitive	un→noncompetitive
	ascorbic acid	uncompetitive	curved uncompetitive
	caffeic acid	competitive	competitive → uncompetitive

(Bouchilloux et al, 1963) while only 1% of the metal is detectable in Neurospora tyrosinase(Fling et al, 1963). The absence of a Cu^{++} signal does not preclude the existence of a paramagnetic ion, as a pair of Cu^{++} ions may result in a broad undetectable signal. The Neurospora enzyme is thought to have only one copper atom per molecule, thus the possibility of signal broadening can be ruled out, suggesting that in the resting state the metal is in the reduced form. The occurrence of a small amount of Cu^{++} is explained by the work of Heyneman (1965), using copper specific reagents, which demonstrated the oxidation of copper on ageing of mushroom phenolase.

Assuming a molecular weight for spinach phenolase of 40,000 (P.F.T. Vaughan, personal communication) and taking the copper content as 0.3% (Vaughan and Butt, 1969) for the high specific activity preparation used throughout, it can be deduced that 2 moles of metal are associated with one mole of protein. Dihydroxphenylethylamine β-hydroxylase (Friedman and Kaufman, 1965) has been shown to contain similar amounts of copper, but although both Neurospora and mushroom phenolase are thought to contain one copper per sub unit (Fling et al, 1963 ; Bouchilloux et al, 1963), these enzymes require 2 and 4 subunits respectively for activity. Studies with copper specific inhibitors were undertaken in an attempt to define the role of the metal in substrate binding.

Difference spectra with phenolase were carried out in the presence of BCS and DIECA. BCS is supposedly a Cu^{+} specific compound and has been widely used in this capacity (e.g. Heyneman, 1965: Brady et al, 1972). The specificity of DIECA is not so clearcut, but it has been used as a Cu^{++} chelator (Brady et al, 1972). Although it is impossible to definitely allocate such a role for either of these compounds,

coloured complexes have been observed with Cu^{+} and BCS (Diehl and Smith, 1958) and Cu^{++} and DIECA (McFarlane, 1932). Both of these complexes, BCS - Cu^{+} and DIECA - Cu^{++}, can be detected in the presence of enzyme (Fig. 35), suggesting that both the valency states of copper are present in the phenolase sample used, and that the protein bound metal can be complexed by these chelators. The observation that 55% of the detectable copper is in the Cu^{++} form supports the suggestion that oxidation occurs on ageing (Heyneman, 1965) as the enzyme preparation used was over one year old.

The amounts of DIECA and BCS required for 30% inhibition are considerably different (Fig. 36), 0.001 mM and 0.33 mM respectively. This discrepancy may be due to steric hindrance, as BCS is a bulky three ringed molecule, or to a difference in affinity for the enzyme copper between these two chelators.

BCS was found to be a competitive inhibitor with respect to oxygen and an uncompetitive inhibitor in the presence of p-coumaric acid, ascorbic acid and caffeic acid (Fig. 42). DIECA on the other hand, was uncompetitive when either oxygen or ascorbic acid was used as variable substrate, but competitive against caffeic acid (Fig. 40). With varied amounts of p-coumaric acid, however, the results showed increasing convergence with rising inhibitor concentration. This might suggest that the caffeic acid produced at high substrate concentrations was competing with DIECA, or that DIECA was in some way interfering with the binding of p-coumaric acid. Copper has been implicated in the binding of phenolic compounds to enzymes, as copper-benzene complexes have been identified by crystallographic studies (Turner and Amma, 1963), but the meaning of this observed inhibition is not clear.

It is apparent that BCS and DIECA are competing for a different form of copper in the enzyme, if it is assumed that these compounds only affect the metal ion. In view of the difference spectra obtained (Fig. 35) it is tempting to suggest that these are the two valencies of copper, Cu^{+} and Cu^{++}.

Kubowitz (1938) showed that two molecules of enzymic copper bound one molecule of carbon monoxide. Warburg (1949) demonstrated that this compound had a higher affinity for Cu^{+} than Cu^{++} while Cu^{+} - carbon monoxide complexes have been crystallised (Colton and Wilkinson, 1967). The carbon monoxide inhibition of phenolase is unusual among the copper oxidases, (Kertesz and Zito, 1962) and is almost certainly due to competition with oxygen for the reduced copper of the active site. In view of this, carbon monoxide would be expected to be competitive with respect to oxygen, and uncompetitive with all other substrates. This is found to be the case (Fig. 38). The lack of effect of the internal electron donor, caffeic acid, on the inhibition by carbon monoxide is surprising in view of the low K_M of this compound for the lag reducing site. (1.8×10^{-6}M).

The similarity of the results obtained with carbon monoxide and BCS suggests that both these compounds compete for the same type of copper, Cu^{+}. This may eliminate the possibility that the differences observed are caused by steric effects on accessibility to the active site, owing to the difference in molecular sizes. DIECA, although a small molecule like carbon monoxide, has its effect on another type of copper ion, probably Cu^{++}.

It is impossible to tell from these results whether more than one copper atom in phenolase is being affected at any time, or indeed whether the differences observed are due to changes in the environment rather than changes in valency state.

4.5 Proposed Model for hydroxylation.

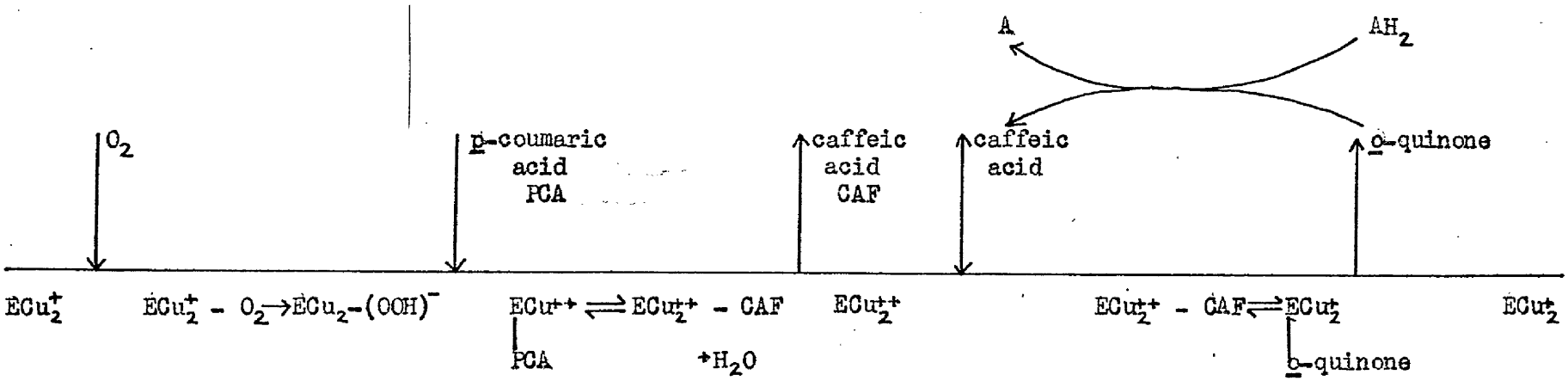
A model for the hydroxylation of p-coumaric acid can be proposed on the basis of the results obtained (Fig. 44). Inhibition experiments suggest that caffeic acid is the electron donor at the active site, and that it is non-enzymically maintained in the reduced state by external reducing agent, ascorbic acid, DMTP or NADH. Classical ping pong steps are observed on release of the products, caffeic acid and o-quinone - the oxidised electron donor (Cleland, 1970). The order of addition of p-coumaric acid and electron donor, caffeic acid, has been established by experiments in the presence of added product.

Oxygen has been proposed as the first substrate binding to the enzyme since it would be impossible for hydroxylation to occur otherwise. The maintenance of copper in the Cu^+ form is of interest here, as combination of this with oxygen, and the ensuing formation of caffeic acid, suggests a possible mechanism for removal of the lag observed during hydroxylation in the absence of added product.

The lack of dependence of the Michaelis constant for oxygen on the nature of the electron donor is further evidence to suggest that oxygen is the first substrate bound to the enzyme. Ingraham (1957) showed that low concentrations of electron donor did not affect the K_M for oxygen of mushroom tyrosinase, and proposed that oxygen bound first during catechol oxidase activity. Duckworth and Coleman (1970), however, found that the K_M for oxygen with mushroom tyrosinase varied with the catechol used as substrate, suggesting that oxygen was not the first substrate bound.

The ping pong pattern observed for spinach phenolase, with oxygen as varied substrate, is also found during hydroxylation of

Fig. 44 Suggested model for phenolase action.



where AH_2 = ascorbic acid, DMTP or NADH.

p-hydroxybenzoic acid by the specific hydroxylase from Pseudomonas desmolytica (Nakamura et al, 1970). When monophenol is replaced by 4 - acetyl or 4 - formylcatechol, a sequential addition of oxygen and substrate is found with mushroom tyrosinase (Duckworth and Coleman, 1970). Similar results have been obtained with broad bean phenolase catalysing the oxidation of dihydroxyphenylalanine (Robb et al, 1966). This may indicate a difference between the catechol oxidase and hydroxylase activities of phenolase. The parabolic intercept replots obtained with ascorbic acid, DMTP or no electron donor (Fig. 24) suggest that the mechanism of the reaction may be more complex than supposed, as a hexa uni ping pong mechanism should result in a linear intercept replot. This discrepancy may be attributable to the dual role of caffeic acid in hydroxylation, small amounts of this compound being produced and partially reversing the irreversible step caused by release of the o-dihydricphenol. Although the nature of the irreversible step on binding of oxygen has not been fully clarified, several explanations are possible. The classic cause of a parallel initial velocity pattern is the release of a product. As water is not considered as a reactant in this type of kinetic analysis, owing to its high concentration, and as it is the only possible product which could be released on binding of oxygen, another cause for the observed pattern must be sought. A possible explanation which is invalid in this case, is saturation by one of the reactants as no step can be reversed in the face of an infinite concentration of a reactant. An infinite amount of substrate is required for saturation and this is not found under the conditions used. This method can be used to study a sequential addition of A, B and C. In the presence of saturating B, a ping

pong step will be observed between the binding of A and C.

Henderson et al (1968) and Deleo et al (1973) have described another system where parallel initial velocity patterns are determined erroneously. Mathematically, the term introducing the nonparallel character into a double reciprocal plot is $(1 + K_{iA}/[A])$ where K_{iA} is the true dissociation constant for the enzyme-substrate-complex (eqn. 1.11.). Unless the ratio $K_{iA}/[A]$ is greater than 0.1 it is almost impossible to detect any deviation from parallel. This has been shown on the basis of product inhibition studies to give rise to initial velocity patterns which are incompatible with known enzymic mechanisms for hypoxanthine-guanine and adenine-phosphoribosyltransferase (Henderson et al, 1968) and 3 - Deoxy - D-arabino-heptulosonic acid 7 - phosphate (DAHP) synthetase (Deleo et al, 1973). If initial velocities are measured at very low substrate concentrations, intersecting double reciprocal plots will be obtained as the ratio of $K_{iA}/[A]$ is increased. This has been shown to occur with DAHP synthetase, (Deleo et al, 1973) but cannot be investigated with phenolase as at concentrations of oxygen below 0.028 mM, the rate of reaction becomes too slow for accurate measurement by the assays used.

Cases involving the erroneous production of an intersecting pattern when a parallel pattern would have been expected on the basis of other results are also known. Initial velocity studies with succinyl coenzyme A synthetase (Moffat and Bridger, 1970) suggest a sequential mechanism for the reaction, but this catalysis has been shown to involve a covalent intermediate (Kreil and Boyer, 1964). Thus great care must be taken in the interpretation of insubstantiated initial velocity patterns.

An alternative explanation for the parallel velocity pattern observed is the existence of an irreversible step simply because the equilibrium constant for the reaction is extremely high, and as a result free energy is lost from the system. This is unusual, but a good example is the oxidation of 3-hydroxyanthranilate by oxygen (Ogasawara et al, 1966) in which parallel patterns result with varied oxygen or 3-hydroxyanthranilate. This reaction must be sequential as both the atoms of oxygen are found in the product. It appears that the binding of the first substrate is separated from the binding of the second by a very slow or irreversible reaction. This has been described by Dalziel (1957) as a type IV mechanism.

Duckworth and Coleman (1970) have interpreted this in terms of a much faster rate of turnover of the enzyme than rate of dissociation of the first enzyme-substrate complex. Thus in the presence of a slowly oxidised substrate an intersecting initial velocity pattern should occur. Such studies should be possible with phenolase and p-hydroxybenzoic acid, although changes in the method of assay might be required.

Since the discovery of copper as the prosthetic group of phenolases (Kubowitz, 1938), it has been assumed the catalytic activity of the enzyme is based on the cuprous-cupric valence change. Isaka (1957) demonstrated this, using copper chelating compounds with melanin tyrosinase, while Goldstein et al (1965) and Friedman and Kaufman (1965) found a similar redox system in 3,4 dihydroxyl-phenylethylamine -B- hydroxylase.

Several possible oxygenated copper complexes have been proposed as the reactive species for hydroxylation, among them the Enzyme

Cu^{++} - $(OOH)^-$ form proposed by Orgel (1956). Model studies have shown that the systems Cu^{++} - H_2O_2 (Konecy, 1954) or Cu^{++} - amine - H_2O_2 (Colton and Wilkinson, 1967) can effect the hydroxylation of phenols. Mechanisms involving peroxide intermediates have also been proposed by Hamilton (1969), and Ingraham (1962), who has stressed the observation that in this reaction Cu^{++} performs the same role as two protons in other acid-catalysed hydrogen peroxide oxidations.

The implication of peroxide intermediates is interesting, suggesting a possible reaction where a large free energy change could occur. A consideration of the energy loss on reduction of oxygen shows that, at pH 7.0, the following hold.

$$O_2 + e^- = O_2^- \qquad G = +10.38 \text{ K cal/mole}$$

$$O_2^- + 2H^+ + e^- = H_2O_2 \qquad G = -22.60 \text{ K cal/mole}$$

(Latimer, 1952)

Hence the net change in free energy is a loss of 12.22 K cal/mole, suggesting that this might cause the apparently irreversible step on binding of oxygen to phenolase at pH 6.5, assuming a negligible pH effect between the two values considered.

Various models for the hydroxylation of monophenols by phenolase have been proposed. Mason et al (1955) envisaged the formation of an oxygenated enzyme species which would react with monophenol to produce o-dihydricphenol and an enzyme (Cu^{++}) complex which could be reduced by external electron donor (Fig. 45a). Such a system has been shown to operate for dihydroxyphenylethylamine-β-hydroxylase (Friedman and Kaufman, 1965) on the basis of ESR and BCS studies.

Frieden et al (1965) have put forward a more detailed scheme

(Fig.25) but have not clarified the exact nature of the reduction step involved in reactivation of the enzyme.

Both these suggestions on the role of copper in phenolase are included in the proposed mechanism for hydroxylation. Further work on the exact nature of the irreversible step on binding of oxygen and the number and valency state of the copper atoms is required before the mechanism of hydroxylation of p-coumaric acid by phenolase can be fully understood.

Fig. 45a. Role of copper in hydroxlation of monophenols

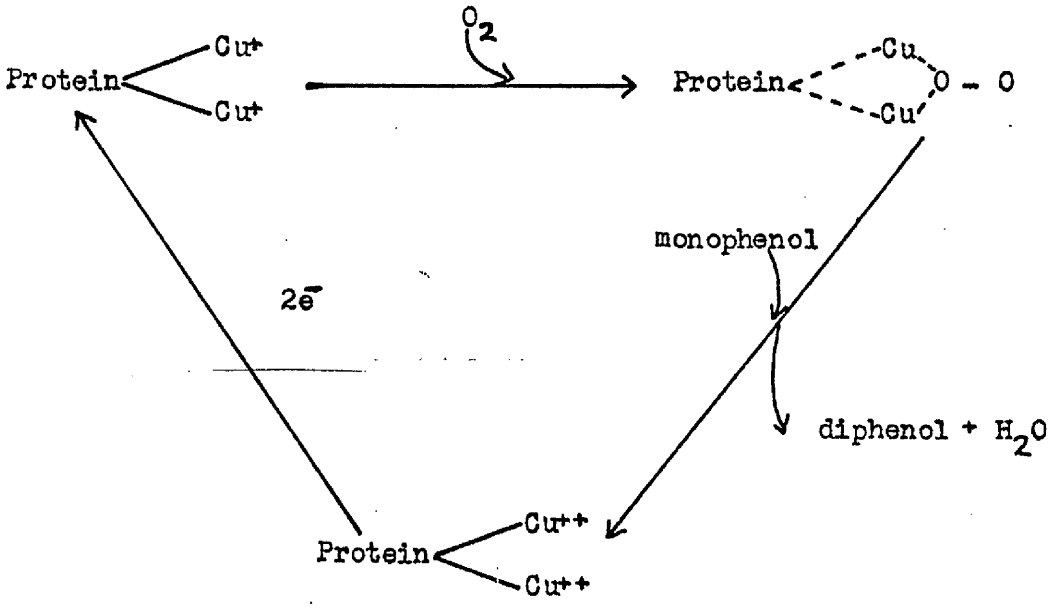


Fig. 45b.

$$En\ Cu_2^{+} + O_2 \rightleftharpoons En\ Cu_2^{+} - O_2 \rightleftharpoons En\ Cu_2^{++}\ (O_2)^{-}\ H^{+} \rightleftharpoons En\ Cu_2^{++} - (OOH)^{-}$$

$$En\ Cu_2^{++} - (OOH)^{-} + R - OH \rightleftharpoons R - (OH)_2 + En\ Cu_2^{++} - (O)$$

R = cinnamic acid.

APPENDICES

Appendix I - Computer programs.

All programs were writtem in FORTRAN IV.

Program I

```
      C     PROGRAM HYPER
ØØØ1        DIMENSION V(100), A(100), W(100), S(3,4), Q(3),
                                      SM(3), SS(3)
ØØØ2          WRITE (6,100)
ØØØ3   100 FORMAT ('1','FIT TO HYPERBOLA V=VMAX*A/(K+A)')
ØØØ4    11 FORMAT (13,17X,'ANYTHING HERE WILL BE PRINTED
                                                 DURING OUTPUT')
ØØØ5     1 FORMAT (3F1Ø,5)
ØØØ6          JJ=Ø
ØØØ7    14 READ (5,11) NP
ØØØ8          IF (NP) 99,99,12
ØØØ9    12 M=1
ØØ1Ø          N=2
ØØ11          P=NP-N
ØØ12          N1=N+1
ØØ13          N2=N+2
ØØ14          GO TO 2
ØØ15    15 READ (5,1) V(I), A(I), W(I)
ØØ16          IF (W(I)) 19,19,2Ø
ØØ17    19 W(I)=1
ØØ18    2Ø Q(1)=V(I)**2/A(I)
ØØ19          Q(2)=V(I)**2
ØØ2Ø          Q(3)=V(I)
ØØ21          GO TO 13
ØØ22    16 CK=S(1,1)/S(2,1)
ØØ23          JJ=JJ+1
ØØ24          WRITE (6,11) JJ
ØØ25          NT=Ø
ØØ26          M=2
ØØ27          GO TO 2
ØØ28    17 D=CK-A(I)
ØØ29          Q(1)=A(I)/D
ØØ3Ø          Q(2)=Q(1)/D
ØØ31          Q(3)=V(I)
ØØ32          GO TO 13
ØØ33    18 CK=CK-S(2,1)/S(1,1)
ØØ34          NT=NT+1
ØØ35          IF (NT-3) 2,21,21
ØØ36    21 S2=Ø
ØØ37          DO 22 I 1,NP
ØØ38    22 S2=S2+(V(I)-S(1,1)*A(I)/(CK+A(I)))**2*W(I)
ØØ39          S2=S2/P
ØØ4Ø          S1=SQRT (S2)
ØØ41          SL=CK/S(1,1)
ØØ42          VINT=1./S(1,1)
ØØ43          VK=1./SL
```

```
ØØ44          DO 1Ø J = 2,N1
ØØ45          DO 1Ø K = 1,N
ØØ46       1Ø S(K,J)=S(K,J)*SM(K)*SM(J-1)
ØØ47          SEV=S1*SQRT(S(1.2))
ØØ48          SECK=S1*SQRT(S(2,3))/S(1,1)
ØØ49          SEVI=SEV/S(1,1)**2
ØØ5Ø          S(1,3)=S1 SQRT(CK**2*S(1,2)+S(2,3)+2.*CK*S(1,3))
ØØ51          SESL=S(1,3)/S(1,1)**2
ØØ52          SEVK=S(1,3)/CK**2
ØØ53          WCK=1./SECK**2
ØØ54          WV=1./SEV**2
ØØ55          WSL=1./SESL**2
ØØ56          WVI=1./SEVI**2
ØØ57          WVK=1./SEVK**2
ØØ58          WRITE (6,3Ø) CK, SECK, WCK
ØØ59          WRITE (6,31) S(1,1), SEV, WV
ØØ6Ø          WRITE (6,32) SL, SESL, WSL
ØØ61          WRITE (6,33) VINT, SEVI, WVI
ØØ62          WRITE (6,34) VK, SEVK, WVK
ØØ63          WRITE (6,35) S2, S1
ØØ64       3Ø FORMAT ('','K=',E12,6,'S.E.(K)=',E11,6,'W=',E14,5)
ØØ65       31 FORMAT ('','V=',E12,6,'S.E.(V)=',E11,6,'W=',E14,5)
ØØ66       32 FORMAT ('','K/V=',E12,6,'S.E.(K/V)=',E11,6,'W=',E14,5
ØØ67       33 FORMAT ('','1/V=',E12,6,'S.E.(1/V)=',E11,6,'W=',E14,5
ØØ68       34 FORMAT ('','V/K=',E12,6,'S.E.(V/K)=',E11,6,'W=',E14,5
ØØ69       35 FORMAT ('','VARIANCE=',E14,5,'SIGMA=',E12,7)
ØØ7Ø          GO TO 14
         C     MATRIX SOLUTION SUBROUTINE
ØØ71        2 DO 3 J=1,N2
ØØ72          DO 3 K=1,N1
ØØ73        3 S(K,J)=Ø
ØØ74          DO 4 I=1,NP
ØØ75          GO TO (15,17),M
ØØ76       13 DO 4 J=1,N1
ØØ77          DO 4 K=1,N
ØØ78        4 S(K,J)=S(K,J)+Q(K)*Q(J)*W(I)
ØØ79          DO 5 K=1,N
ØØ8Ø        5 SM(K)=1./SQRT(S(K,K))
ØØ81          SM(N1)=1.
ØØ82          DO 6 J=1,N1
ØØ83          DO 6 K=1,N
ØØ84        6 S(K,J)=S(K,J)*SM(K)*SM(J)
ØØ85          SS(N1)=-1.
ØØ86          S(1,N2)=1.
ØØ87          DO 8 L=1,N
ØØ88          DO 7 K=1,N
ØØ89        7 SS(K)=S(K,1)
ØØ9Ø          DO 8 J=1,N1
ØØ91          DO 8 K=1,N
ØØ92        8 SS(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
ØØ93          DO 9 K=1,N
ØØ94        9 S(K,1)=S(K,1)*SM(K)
ØØ95          GO TO (16,18),M
ØØ96       36 FORMAT('PROGRAM COMPLETED FOR',14,'LINES')
ØØ97       99 WRITE (6,36) JJ
ØØ98          STOP
ØØ99          END
```

Program II.

```
     C      PROGRAM PING PONG (EQ: 1Ø CLELAND 17/6/62)
ØØØ1        DIMENSION V(1ØØ), A(1ØØ), B(1ØØ), W(1ØØ), S(5,6),
                                      Q(5,1ØØ), SM(5), SS(5)
ØØØ2        WRITE (6,1ØØ)
ØØØ3    1ØØ FORMAT ('1','FIT TO Y=V*A*B /(KA*B+KB*A+A*B)')
ØØØ4     11 FORMAT (I3,17X,'ANYTHING HERE WILL BE PRINTED
                                                 DURING OUTPUT')
ØØØ5      1 FORMAT (4 F1Ø,5)
ØØØ6        JJ=Ø
ØØØ7     14 READ (5,11) NP
ØØØ8        IF (NP) 99,99,12
ØØØ9     12 M=1
ØØ1Ø        N=3
ØØ11        P=NP-N
ØØ12        N1=N+1
ØØ13        N2=N+2
ØØ14        GO TO 2
ØØ15     15 READ (5,1) V(I), A(I), B(I), W(I)
ØØ16        IF (W(I)) 19,19,2Ø
ØØ17     19 W(I)=1.
ØØ18     2Ø Q(1,I)=V(I)**2/B(I)
ØØ19        Q(2,I)=V(I)**2/A(I)
ØØ2Ø        Q(3,I)=V(I)**2
ØØ21        Q(4,I)=V(I)
ØØ22        GO TO 13
ØØ23     16 CKA=S(2,1)/S(3,1)
ØØ24        CKB=S(1,1)/S(3,1)
ØØ25        JJ=JJ+1
ØØ26        WRITE (6,11) JJ
ØØ27        NT=Ø
ØØ28        M=2
ØØ29        GO TO 2
ØØ3Ø     17 D=CKA/A(I)+CKB/B(I)+1.
ØØ31        Q(1,I)=1./D
ØØ32        Q(2,I)=1./A(I)/D**2
ØØ33        Q(3,I)=1./B(I)/D**2
ØØ34        Q(4,I)=V(I)
ØØ35        GO TO 13
ØØ36     18 CV=S(1,1)
ØØ37        CKA=CKA-S(2,1)/S(1,1)
ØØ38        CKB=CKB-S(3,1)/S(1,1)
ØØ39        NT=NT+1
ØØ4Ø        IF (NT-5) 2,21,21
ØØ41     21 S2=Ø
ØØ42        DO 22 I=1,NP
ØØ43     22 S2=S2+V(I)-CV/(CKA/A(I)+CKB/B(I)+1.))**2*W(I)
ØØ44        S2=S2/P
ØØ45        S1=SQRT (S2)
ØØ46        DO 1ØJ=2,N1
ØØ47        DO 1ØK=1,N
ØØ48     1Ø S(K,J)=S(K,J)*SM(K)*SM(J-1)
ØØ49        SEKA=S1*SQRT(S(2,3))/S(1,1)
ØØ5Ø        SEKB=S1*SQRT(S(3,4))/S(1,1)
```

```
ØØ51          SEV=S1*SQRT(S(1,2))
ØØ52          WCKA=1./SEKA**2
ØØ53          WCKB=1./SEKB**2
ØØ54          WCV=1./SEV**2
ØØ55          WRITE (6,3Ø) CKA, SEKA, WCKA
ØØ56          WRITE (6,31) CKB, SEKB, WCKB
ØØ57          WRITE (6,32) CV, SEV, WCV
ØØ58          WRITE (6,34) S2,S1
ØØ59       3Ø FORMAT ('','KA=',E12,6,'S.E.(KA)=',E11,6,'W=',E14,5)
ØØ6Ø       31 FORMAT ('','KB=',E12,6,'S.E.(KB)=',E11,6,'W=',E14,5)
ØØ61       32 FORMAT ('','V=',E12,6,'S.E.(V)=',E11,6,'W=',E14,5)
ØØ62       34 FORMAT ('','VARIANCE=',E14,5,'SIGMA=',E12,7)
ØØ63          GO TO 14
        C     MATRIX SOLUTION SUBROUTINE
ØØ64        2 DO 3 J=1,N2
ØØ65          DO 3 K=1,N1
ØØ66        3 S(K,J)=Ø
ØØ67          DO 4 I=1,NP
ØØ68          GO TO (15,17),M
ØØ69       13 DO 4 J=1,N1
ØØ7Ø          DO 4 K=1,N
ØØ71        4 S(K,J)=S(K,J)+Q(K,I)*Q(J,I)*W(I)
ØØ72          DO 5 K=1,N
ØØ72        5 SM(K)=1./SQRT(S(K,K))
ØØ74          SM(N1)=1.
ØØ75          DO 6 J=1,N1
ØØ76          DO 6 K=1,N
ØØ77        6 S(K,J)=S(K,J)*SM(K)*SM(J)
ØØ78          SS(N1)=-1.
ØØ79          S(1,N2)=1.
ØØ8Ø          DO 8 L=1,N
ØØ81          DO 7 K=1,N
ØØ82        7 SS(K)=S(K,1)
ØØ83          DO 8 J=1,N1
ØØ84          DO 8 K=1,N
ØØ85        8 S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
ØØ86          DO 9 K=1,N
ØØ87        9 S(K,1)=S(K,1)*SM(K)
ØØ88          GO TO (16,18),M
ØØ89       36 FORMAT ('PROGRAM COMPLETED FOR',14,'LINES')
ØØ9Ø       99 WRITE (6,36) JJ
ØØ91          STOP
ØØ92          END
```

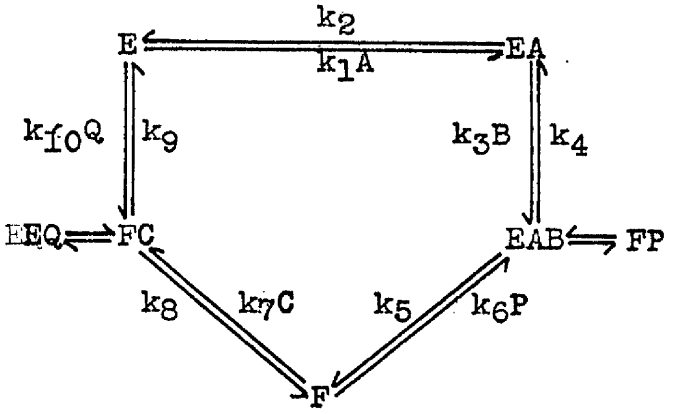
Program III.

```
      C     PROGRAM SEQEN ( EQ: 7 CLELAND 17/6/62)
ØØØ1        DIMENSION V(1ØØ), A(1ØØ), B(1ØØ), W(1ØØ), S(5,6),
                      Q(5,1ØØ), SM(5), SS(5)
ØØØ2        WRITE(6,1ØØ)
ØØØ3    1ØØ FORMAT ('1','FIT TO Y=V*A*B/(KA*B+KB*A+A*B+KIA*KB')
ØØØ4     11 FORMAT (I3,17X,'ANYTHING HERE WILL BE PRINTED
                                                  DURING OUTPUT')
ØØØ5      1 FORMAT (4 F1Ø,5)
ØØØ6        JJ=Ø
ØØØ7     14 READ(5,11) NP
ØØØ8        IF (NP) 99,99,12
ØØØ9     12 M=1
ØØ1Ø        N=4
ØØ11        P=NP-N
ØØ12        N1=N+1
ØØ13        N2=N+2
ØØ14        GO TO 2
ØØ15     15 READ (5,1- V(I), A(I), B(I), W(I)
ØØ16        IF (W(I)) 19,19,2Ø
ØØ17     19 W(I)=1
ØØ18     2Ø Q(1,I)=V(I)**2/B(I)
ØØ19        Q(2,I)=V(I)**2/A(I)
ØØ2Ø        Q(3,I)=V(I)**2
ØØ21        Q(4,I)=V(I)**2/A(I)/B(I)
ØØ22        Q(5,I)=V(I)
ØØ23        GO TO 13
ØØ24     16 CKA=S(2,1)/S(3,1)
ØØ25        CKB=S(1,1)/S(3,1)
ØØ26        CKIA=S(4,1)/S(1,1)
ØØ27        JJ=JJ+1
ØØ28        WRITE (6,11) JJ
ØØ29        NT=Ø
ØØ3Ø        M=2
ØØ31        GO TO 2
ØØ32     17 D=CKA/A(I)+CKB/B(I)+1.+CKIA*CKB/A(I)/B(I)
ØØ33        Q(1.I)=1./D
ØØ34        Q(2,I)=1./A(I)/D**2
ØØ35        Q(3,I)=(1.+CKIA/A(I))/B(I)/D**2
ØØ36        Q(4,I)=1./A(I)/B(I)/D**2
ØØ37        Q(5,I)=V(I)
ØØ38        GO TO 13
ØØ39     18 CV=S(1,1)
ØØ4Ø        CKA=CKA-S(2,1)/S(1,1)
ØØ41        CKB=CKB-S(3,1)/S(1,1)
ØØ42        CKIA=CKIA-S(4,1)/S(1,1)/CKB
ØØ43        NT=NT+1
ØØ44        IF (NT-5) 2,21,21
ØØ45     21 S2=Ø
ØØ46        DO 22 I=1,NP
ØØ47     22 S2=S2+V(I)-CV/(CKA/A(I)+CKB/B(I)+1.+CKIA*CKB/B(I)))
                                                  **2*W(I)
ØØ48        S2=S2/P
ØØ49        S1=SQRT (S2)
ØØ5Ø        DO 1ØJ=2,N1
ØØ51        DO 1ØK=1,N
```

```
ØØ52     1Ø S(K,J)=S(K,J)*SM(K)*SM(J-1)
ØØ53        SEKA=S1*SQRT(S(2,3))/S(1,1)
ØØ54        SEKB=S1*SQRT(S(3,4))/S(1,1)
ØØ55        SEV=S1*SQRT(S(1,2))
ØØ56        SEKIA=S1*SQRT(S(4,5))/CKB/S(1,1)
ØØ57        WCKA=1./SEKA**2
ØØ58        WCKB=1./SEKB**2
ØØ59        WCV=1./SEV**2
ØØ6Ø        WCKIA=1./SEKIA**2
ØØ61        WRITE (6,3Ø) CKA, SEKA, WCKA
ØØ62        WRITE (6,31) CKB, SEKB, WCKB
ØØ63        WRITE (6,32) CV, SEV, WCV
ØØ64        WRITE (6,33) CKIA, SEKIA, WCKIA
ØØ65        WRITE (6,34) S2, S1
ØØ66     3Ø FORMAT ('','KA=',E12,6,'S.E.(KA)=',E11,6,'W=',E14,5)
ØØ67     31 FORMAT ('','KB=',E12,6,'S.E.(KB)=',E11,6,'W=',E14,5)
ØØ68     32 FORMAT ('','V=',E12,6, 'S.E.(V)=',E11,6,'W=',E14,5)
ØØ69     33 FORMAT ('','KIA=',E12,6,'S.E.(KIA)=',E11,6,'W=',
                                                        E14,5)
ØØ7Ø     34 FORMAT ('','VARIANCE=',E14,5,'SIGMA=',E12,7)
ØØ71        GO TO 14
         C  MATRIX SOLUTION SUBROUTINE
ØØ72      2 DO 3 J=1,N2
ØØ73        DO 3 K=1,N1
ØØ74      3 S(K,J)=Ø
ØØ75        DO 4 I=1,NP
ØØ76        GO TO (15,17),M
ØØ77     13 DO 4 J=1,N1
ØØ78        DO 4 K=1,N
ØØ79      4 S(K,J)=S(K,J)+Q(K,I)*Q(J,I)*W(I)
ØØ8Ø        DO 5 K=1,N
ØØ81      5 SM(K)=1./SQRT(S(K,K))
ØØ82        SM(N1)=1.
ØØ83        DO 6 J=1,N1
ØØ84        DO 6 K=1,N
ØØ85      6 S(K,J)=S(K,J)*SM(K)*SM(J)
ØØ86        SS(N1)=-1.
ØØ87        S(1,N2)=1.
ØØ88        DO 8 L=1,N
ØØ89        DO 7 K=1,N
ØØ9Ø      7 SS(K)=S(K,1)
ØØ91        DO 8 J=1,N1
ØØ92        DO 8 K=1,N
ØØ93      8 S(K,J)=S(K+1,J+1)-SS(K+1)*S(1.J+1)/SS(1)
ØØ94        DO 9 K=1,N
ØØ95      9 S(K,1)=S(K,1)*SM(K)
ØØ96        GO TO (16,18),M
ØØ97     36 FORMAT ('PROGRAM COMPLETED FOR',14,'LINES')
ØØ98     99 WRITE (6,36) JJ
ØØ99        STOP
Ø1ØØ        END
```

Appendix II - Rate equation for proposed mechanism.

The rate equation for the proposed mechanism, involving three irreversible steps, can be deduced by the method of Orsi (1971). The King-Altman pattern for this reaction is



where A is oxygen, B p-coumaric acid, Q o-quinone and P and C are caffeic acid. E, EA and F are stable enzyme forms. Distribution terms in E, EA, EAB, F and FC can be evaluated, and hence the rate equation for the reaction can be shown to be

$$v = \frac{V_1V_2\,(\,ABC - PQ/K_{eq}\,)}{\begin{aligned}&K_{1A}K_B\,V_2C + K_BV_2AC + K_CV_2AB + K_AV_2BC + V_2ABC + \\ &K_QV_1P/K_{eq} + K_PV_1Q/K_{eq} + V_1PQ/K_{eq} + K_QV_1AP/K_{1A}K_{eq} + \\ &K_{1C}K_AV_2QB/K_{1Q} + K_{1A}K_BV_2QC/K_{1Q} + K_AV_2BCQ/K_{1Q} + \\ &K_{1C}K_AV_2BPQ/K_{1P}K_{1Q} + K_QV_1ABP/K_{1A}K_{1B}K_{eq}\end{aligned}}$$

where $K_{eq} - k_1k_3k_5k_7k_9/k_2k_4k_6k_8k_{10}$

$V_1 - k_5k_9/(k_5 + k_9)$

$V_2 - k_2k_4k_8/k_2k_4 + k_2k_8 + k_4k_8$

$K_A = k_5k_9/k_1(k_5 + k_9)$

$K_B = k_9(k_4 + k_5)/k_3(k_5 + k_9)$

$K_C = k_5(k_8 + k_9)/k_7(k_5 + k_9)$

$K_P = k_2(k_4 + k_5)/k_6(k_2k_4 + k_2k_8 + k_4k_8)$

$K_Q = k_2k_4(k_8 + k_9)/k_{10}(k_2k_4 + k_2k_8 + k_4k_8)$

$K_{1A} = k_2/k_1$

$K_{1B} = k_4/k_3$

$K_{1C} = k_8/k_7$

$K_{1P} = k_5/k_6$

$K_{1Q} = k_9/k_{10}$

Under conditions for determination of initial velocities, product concentrations are assumed to be negligible and hence all terms in P and Q can be disregarded. The equation is then simplified to

$$v = \frac{V_1V_2ABC}{K_{1A}K_BV_2C + K_BV_2AC + K_CV_2AB + K_AV_2BC + V_2ABC}$$

The rate of the back reaction (V_2) is also negligible, leading to further simplification.

$$v = \frac{V_1ABC}{K_{1A}K_BC + K_BAC + K_CAB + K_ABC + ABC}$$

B I B L I O G R A P H Y

Alben J.O. (1967) J.Biol.Chem. 242, 1827 - 1832

Alberghina F.A.M. (1964) Phytochemistry, 3, 65 - 72

Alberty R.A. (1953) J.Amer.Chem.Soc. 75, 1928 - 1932

Alberty R.A. (1958) J.Amer.Chem.Soc. 80, 1777 - 1782

Arnon D.L. (1949) Plant Physiol. 24, 1 - 15

Bailey J.L. in Techniques in Protein Chemistry, Chapter 11, p.293, Elsevier Publishing Co., Amsterdam (1962)

Bartlett D.J., Poulton J.E.,and Butt V.S. (1972) Febs Letters, 23, 265 - 267

Beevers H. in Respiratory Metabolism in Plants, pp.65 - 118, Row,Peterson and Co., New York.(1961)

Bertrand G. (1895) C.R.Acad.Sci.Paris, 121, 166 - 168

Bonner J. and Wildman S.G. (1946) Arch. Biochem. 10, 497 - 518

Bonner W.D. (1957) An.Rev.Plant Physiol. 8, 427 - 452 (Crafts A.S. Ed.) An.Rev.Inc., Palo Alto,U.S.A.

Bordner C.A. and Nelson J.M. (1939) J.Amer.Chem.Soc. 61, 1507 - 1513

Bouchilloux S., McMahill P. and Mason H.S. (1963) J.Biol. Chem., 238, 1699 - 1707

Brady F.O., Monaco M.E., Förman H.J. Schutz G. and Fiegelson P. (1972) J.Biol.Chem. 247, 7915 - 7922

Briggs G.E. and Haldane J.B.S. (1925) Biochem.J. 19, 338 - 345

Brown A.J. (1902) Trans. Chem. Soc. 81, 373 - 388

Brown S.A. (1966) An.Rev.Plant Physiol. 17, 223 - 244 (Machlis L. Ed.) An.Rev.Inc., Palo Alto, U.S.A.

Brown S.A. and Neish A.C. (1956) Can.J.Biochem.Biophys. 34, 769 - 778

Butt V.S. and Hallaway M. (1961) Arch.Biochem.Biophys. 92, 24 - 32

Cleland W.W. (1963a) Biochem.Biophys.Acta 67, 104 - 137

Cleland W.W. (1963b) Biochem.Biophys,Acta 67, 173 - 187

Cleland W.W. (1963c) Biochem.Biophys.Acta 67, 188 - 196

Cleland W.W. Adv. in Enzymol. 29, 1 - 32 (1967)

Cleland W.W. (1970) in The Enzymes,Vol.2,3rd Edition, (Boyer P.D. Ed.) Academic Press, New York.

Colton F.A. and Wilkinson G. (1967) in Advanced Inorganic Chemistry, 2nd Edition, pp.893 - 908, Wiley - Interscience

Dalziel K. (1957) Acta Chem.Scand. 11, 1706 - 1722

Dalziel K. (1958) Trans. Faraday Soc. 54, 1247 - 1253

Dalziel K. (1969) Biochem.J. 114, 547 - 556

Data for Biochemical Research, 2nd Edition, Clarendon Press, Oxford.

Dawson C.R. and Magee R.J. (1955) in Methods in Enzymology, Vol 2, 817 - 827 (Colowick S.P.and Kaplan N.O. Eds.) Academic Press, New York.

Deleo A.B., Dayan J. and Sprinson D.B. (1973) J.Biol.Chem. 248, 2344 - 2353

Diehl H. and Smith G.F. (1958) in The Copper Reagents, Cuproine, Neocuproine and Bathocuproine pp. 39 - 44 G.F.Smith Chem.Co. Columbus, Ohio.

Duckworth H.W. and Coleman J.E. (1970) J.Biol.Chem. 245, 1613 - 1625

El Basyouni S.Z., Neish A.C. and Towers G.H.N. (1964) Phytochemistry 3, 627 - 639

Finkle B.J. and Nelson R.T. (1963) Biochem.Biophys.Acta 78, 747 - 749

Fling M., Horowitz N.H. and Heinemann S.F. (1963) J.Biol.Chem, 238, 2045 - 2053

Frieden E., Osaki S. and Kobayashi H. (1965) J.Gen.Physiol. 49, 213 - 255

Friedman S. and Kaufman S. (1965) J.Biol.Chem. 240, 4763 - 4773

Fromm H.J. (1967) Biochem.Biophys,Acta 139, 221 - 230

Goldstein M., Lauber E. and McKereghan M.R. (1965) J.Biol.Chem. 240, 2066 - 2072

Hallaway M. (1959) Biochem.Biophys.Acta 36, 538 - 540

Hamilton G.A. (1969) Adv. in Enzymol. 32, 55 - 96

Handbook of Chemistry and Physics (1948) 30th Edition, Chemical Rubber Publishing Co., Cleveland, Ohio.

Harel E. and Meyer A.M. (1971) Phytochemistry 10, 17 - 22

Harel E., Meyer A.M. and Shain Y. (1964) Physiol. Plant 17, 921 - 930

Hayaishi O., Rotheburg S. and Mehler A.A. (1956) Abst. 130th Meeting Amer.Chem.Soc. p.53c

Heineken F.G., Tsuchiya H.M. and Aris R. (1967) Mathematical Biosciences 1, 95 - 113

Henderson J.F., Brox L.W., Kelley W.N., Rosenbloom F.M. and Seegmiller J.E. (1968) J.Biol.Chem. 243,2514 -252

Henri V. (1901) C.R.Acad.Sci.Paris 133, 891- 894

Henri V. C.R.Acad.Sci.Paris 135, 916 - 919

Heyneman R.A. (1965) Biochem.Biophys,Res.Com. 21, 162 - 169

Hoepfner W. (1932) Chemikerzeitung 56, 991

Ingraham L.L. (1957) J.Amer.Chem.Soc. 79, 666 - 669

Ingraham L.L. (1962) in Biochemical Mechanisms p.71, Wiley, New York.

Isaka S. (1957) Nature(Lond.) 179, 578 - 579

Johansen G. and Lumry R. (1961) Compt Rend. Trav. Lab. Carlsberg 32, 185 - 214

John V.T. and Weintraub M. (1967) Phytopathology 57, 154 - 15

Kaufman S. Adv. in Enzymol. 35, 245 - 317(1971)

Keilin D. and Mann T. (1938) Proc.Roy.Soc.B 125, 187 - 204

Kelley W.N., Rosenbloom F.M., Henderson J.F. and Seegmiller J. (1967) Biochem.Biophys.Res.Com. 28, 340 - 345

Kertesz D. (1957) Nature(Lond.) 180, 506 - 507

* King E.L. and Altman C. (1956) J.Phys.Chem. 60, 1375 - 1378

Konechy J.O. (1954) J.Amer.Chem.Soc. 76, 4993

Koukol J. and Conn E.E. (1961) J.Biol.Chem. 236, 2692 - 2697

Kreil G. and Boyer P.D. (1964) Biochem.Biophys.Res.Com. 16, 551 - 555

Krueger R.C. (1958) Arch.Biochem.Biophys. 76, 87 - 96

Krueger R.C. (1950) J.Amer.Chem.Soc. 72, 5582 - 5587

Kubowitz F. (1937) Biochem.Z. 292, 221 - 229

Kubowitz F. (1938) Biochem.Z. 299, 32 - 57

** Laidler K.J. Can. J.Chem. 33, 1614 - 1624 (1955)

Lerner A.B. and Fitzpatrick T.B. (1950) Physiol. Rev. 30, 91 - 126

Levy C.C. and Zucker M. (1960) J.Biol.Chem. 235, 2418 - 2425

Lineweaver H. and Burk D. (1934) J.Amer.Chem.Soc. 56, 658 - 66

Long T.J., Och F.F. and Alben J.O. (1971) Arch.Biochem. Biophys. 146, 64 - 70

Lowry O.H., Rosebrough N.J. Farr A.L. and Randall R.J. (1951) J.Biol.Chem. 193, 265 - 275

Mallette M.F. and Dawson C.R. (1947) J.Amer.Chem.Soc. 69, 466 - 467

Malmstrom B.G. (1965) in Oxidases and Related Redox Systems p. 216 ,(King T.E., Masen H.S. and Morrison M. Eds.) Wiley, New York.

Mason H.S. (1955) Adv. in Enzymol. 16, 105 - 184

Mason H.S. (1957) Adv. in Enzymol. 19, 79 - 234

Mason H.S., Foulks W.L. and Peterson E. (1955) J.Amer.Chem. Soc. 77, 2914 - 2915

Maurer H.R. (1971) in Disc Electrophoresis and Related Techniques 2nd Edition, De Gruyter, Berlin.

* * *

Michaelis L. and Menten M.L. (1913) Biochem.Z. 49, 333 - 369

Moffet F.J. and Bridger W.A. (1970) J.Biol.Chem. 245, 2758 - 2762

M^{c}Calla D.R. and Neish A.C. (1959) Can.J.Biochem.Biophys. 37, 537 - 547

M^{c}Farlane W.D. (1932) Biochem.J. 26, 1022 - 1033

Nakamura S., Ogura Y., Yano K., Higashi N. and Arima K. (1970) Biochemistry 9, 3235 - 3242

Nair P.M. and Vining L.C. (1965) Phytochemistry 4, 161 - 168

Neish A.C. (1961) Phytochemistry 1, 1 - 24

Nelson J.M. and Dawson C.R. Adv. in Enzymol. 4, 99 - 152 (1944

Neujahr H.Y. and Gaal A. (1973) Eur.J.Biochem. 35, 386 - 400

Nielsen K.H. (1969) Eur.J.Biochem. 7, 560 - 369

Ogasawara N., Gander J.E. and Henderson L.M. (1966) J.Biol.Chem. 241, 613 - 619

Orgel L.E. (1956) in Biochemical Society Symposium no. 15 p.8, (Crook E.M. Ed.), Cambridge University Press.

Orsi B.A. ~~(1971)~~ Biochem.Biophys.Acta 258, 4 - 8

Parish R.W. (1972) Z.Pflanzenphysiol. 66, 176 - 188

Patil S.S. and Zucker M. (1965) J.Biol.Chem. 240, 3938 - 3943

Plowman K.M. (1972) in Enzyme Kinetics M^{c}Graw - Hill, New York

Plowman K.M. and Cleland W.W. (1967) J.Biol.Chem. 242, 4239 - 4244

Pomerantz S.H. and Warner M.C. (1967) J.Biol.Chem. 242, 5308 - 5314

Pshenova K.V. (1956) Biokhimiya 21, 279 - 289

Purich F.B. and Fromm H.J. (1971) Arch. Biochem.Biophys. 147, 515 - 526

Richter D. (1934) Biochem.J. 28, 901 - 908

Ritchie G.A. (1972) Honours Dissertation, Biochemistry Dept., Glasgow University.

Robb D.A., Swain T. and Mapson L.M. (1966) Phytochemistry 5, 665 - 675

Robinson J. and Cooper J.M. (1970) Anal. Biochem. 33, 390 - 399

Rudolph F.B., Purich D.L. and Fromm H.J. (1968) J.Biol. Chem. 243, 5539 - 5545

Russell D.W. (1971) J.Biol.Chem. 246, 3870 - 3878

Sato M. (1966) Phytochemistry 5, 385 - 389

Sato M. (1969) Phytochemistry 8, 353 - 362

Turner R.W. and Amma E.L. (1963) J.Amer.Chem.Soc. 85, 4046 - 4047

Uritani . and Hyodo (1967) Arch.Biochem.Biophys. 122, 299 - 309

Vaughan P.F.T. and Butt V.S. (1969) Biochem.J. 113, 109 - 115

Vaughan P.F.T. and Butt V.S. (1970) Biochem.J. 119, 89 - 94

Volkenstein M.V. and Goldstein B.N. (1966) Biochem.Biophys. Acta 115, 471 - 477

Wagreich H. and Nelson J.M. (1938) J.Amer.Chem.Soc. 60, 1545 - 1548

Walker J.R.L. and Hulme A.C. (1965) Phytochemistry 4, 677-685

Walter C. (1966) J.Theoret.Biol. 11, 181 - 206

Warburg O. (1949) in Heavy Metal Prosthetic Groups, p.181, Clarendon Press, Oxford.

Webb J.L. (1963) in Enzyme and Metabolic Inhibitors, Vol.1, Academic Press, New York.

Webb J.L. (1966) in Enzyme and Metabolic Inhibitors, Vol. 2, pp. 296 - 302, Academic Press, New York.

Weber K. and Osborne M. (1969) J.Biol.Chem. 244, 4406 - 4412

Williams A.H. (1963) in Enzyme Chemistry of Phenolic Compounds (Pridham J.B. Ed.) Pergamon Press, Oxford.

Wilkinson G.N. (1961) Biochem.J. 86, 324 - 332

Wong J.T-F. (1965) J.Amer.Chem.Soc. 87, 1788 - 1793

Wong J.T-F. and Hanes C.S. (1962) Can.J.Biochem.Biophys. 40, 763 - 804

Wong J.T-F. and Hanes C.S. (1969) Arch.Biochem.Biophys. 135, 50 -59

* Kertesz D. and ZitoR. in Oxygenases, chapter 8, (1962) (Hayaishi O. Ed.) Academic Press, New York.

** Latimer W.M. (1952) The Oxidation states of The Elements. 2nd edition, Prentice Hall, New York.

*** Mayer A.M. and Friend J. (1960) J. Exp. Bot. 11, 143

S U M M A R Y

The reaction catalysed by spinach leaf phenolase has been shown to be complex, but little is known about the mechanism of action of this enzyme. The present investigation was undertaken in an attempt to clarify the molecular interactions involved, using the techniques of steady state kinetics.

Phenolase (EC: 1.3.10.1., oxygen o-diphenol oxidoreductase) was prepared in bulk by a modification of the method in use in the laboratory. A continuous spectrophotometric assay for the determination of the reaction product, caffeic acid, was devised. Product formation could then be monitored at 340 nm in the absence of electron donor, or in the presence of ascorbic acid as reducing agent. With dimethyltetrahydropteridine (DMTP) or NADH as electron donor, absorbance changes were monitored at 340 and 370 nm, and 240 and 370 nm respectively. The changes in absorbance were related to caffeic acid concentration.

Assay conditions were investigated, the pH of the reaction mixture and enzyme concentration being adjusted to give suitable reaction rates. The Michaelis constants for the substrates, oxygen, p-coumaric acid and electron donor were determined in the presence of saturating amounts of the other two substrates. The nature of the electron donor did not seem to affect the K_m for oxygen, but did affect that of p-coumaric acid. The determination of these constants was a necessary preliminary to a steady state kinetic analysis of the enzyme.

Initial velocity studies were carried out by varying one substrate in the presence of fixed amounts of the other two substrates, fixed in the ratio of their Michaelis constants. The initial velocity patterns thus obtained demonstrated the existence of irreversible steps on binding of each of the three substrates, and suggested that

product formation led to the reversal of one of these steps. Experiments carried out in the presence of a fixed amount of caffeic aci acid suggested that a classical ping pong step occurs on the release of caffeic acid after p-coumaric acid hydroxylation, and before electron donor has bound to the enzyme. No effect was observed on the apparently irreversible step on binding of oxygen. These results were assessed both graphically and by computer analysis in an attempt to aid the interpretation of the observed patterns. On the basis of these results a model for the reaction mechanism is proposed, involving binding of oxygen followed by p-coumaric acid and then electron donor.

Inhibitor studies with substrate analogues and alternate substrates were used to confirm the proposed order of substrate addition. Studies with the caffeic acid analogue, 3, 4-dihydroxybenzoic acid, suggested the existence of a distinct site for caffeic acid. It is proposed that this is the site occupied by caffeic acid in the role of internal electron donor to the enzyme, the o-dihydricphenol being maintained in the reduced state by the external electron donor, ascorbic acid, NADH or DMTP. This result may explain the removal of the lag observed during hydroxylation, as caffeic acid production can occur in the presence of oxygen and p-coumaric acid. Studies with benzoic acid and p-hydroxybenzoic acid support the proposed mechanism, although complex results are obtained owing to the structural similarity of these compounds to caffeic acid, and consequent binding to the o-dihydricphenol site on the enzyme.

The implication of copper in the hydroxylation of monophenols provided another area for study. Copper specific reagents were used in an attempt to elucidate the role of the metal in substrate

binding. Carbon monoxide, a Cu+ specific chelator, was found to compete directly with oxygen for the enzyme. Similar results were obtained with bathocuproine sulphonate (BCS) which has been thought to be Cu+ specific. Diethyldithiocarbamate (DIECA), however, competed with caffeic acid, and would therefore seem to be acting on a different form of copper. This compound has been thought to be more specific for Cu++. In view of the difference spectra obtained with DIECA and BCS in the presence of enzyme, it is suggested that both Cu+ and Cu++ may be implicated in the mechanism of action of phenolase. A scheme for the mechanism of the reaction of the enzyme is proposed and its validity discussed.