THE FORMATION OF ISOVALERALDEHYDE FROM ℓ-LEUCINE AND ISOBUTYRALDEHYDE FROM ℓ-VALINE CATALYZED BY BANANA POLYPHENOLOXIDASE AND PEROXIDASE

by

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I. INTRODUCTION

As many as 200 chromatographic peaks have been obtained in gas chromatographic analysis of banana volatiles (Wick et al., 1969). Branched-chain alcohols and esters have been identified in these volatiles and have been shown to be important contributors to typical banana flavor and aroma. However, the mechanisms of production of these compounds have not been studied thoroughly. Knowledge of how these flavor volatiles are produced during ripening could lead to development of better varieties; regulation of the formation of flavor during transportation and storage; and preservation or development of fresh flavor in processed banana products.

Tressl et al. (1970a,b,c,d and 1973) investigated the biogenesis of aroma substances in bananas and proposed that the formation of branched-chain flavor compounds is via the Ehrlich mechanism involving transamination of branched-chain amino acids to form α-keto acids followed by decarboxylation of the α-keto acids to form aldehydes. These aldehydes could then undergo reduction to alcohol or oxidation to acid, which then would react further to form the esters.

These same branched-chain aldehydes in the flavor volatiles of black tea are proposed to be formed via Strecker degradation (Co and Sanderson, 1970; Saijo and Takeo, 1970a,b). This mechanism involves oxidation of tea flavonols by polyphenoloxidase or peroxidase to form quinones. These active quinones were postulated to react further with the amino acids and form the aldehydes via Strecker degradation.

Since bananas possess a highly active quinone-producing system involving banana polyphenoloxidase and dopamine, the mechanism proposed for tea may also exist in bananas.

The purpose of this study was to determine if aldehydes in bananas could be produced from amino acids in a reaction with quinones formed by the enzyme catalyzed oxidation of dopamine.

II. REVIEW OF LITERATURE

Many of the prominent flavor compounds in ripe bananas are branched-chain alcohols and esters (Issenberg and Wick, 1963; McCarthy et al., 1963,1964; Wick et al., 1966; Murray et al., 1968; Tressl et al., 1970a,b). These compounds include isobutyl (2-methyl propyl) and isoamyl (3-methyl butyl) alcohols, acetates and butyrates. Wick et al. (1966,1969) suggested the presence of 2-methyl propanoate and 3-methyl butyrate esters, which was confirmed by Tressl et al. (1970a,b). In earlier sensory evaluations, McCarthy et al. (1963) found an excellent correlation between the above flavor compounds and the aroma of the banana. Isoamyl acetate and isoamyl butyrate were reported to have a "banana-like" aroma, and isobutyl alcohol and isobutyl acetate were reported to generate "fruity" notes. As the banana fruit ripened, "sour", "green", "woody" or "musty" notes developed into more typical ripe banana flavor, accompanied by increasing complexity of the gas chromatograms of the flavor volatiles. Later, Tressl and Jennings (1972) collected sequential samples of air swept through a chamber containing ripening bananas and reported

that the isoamyl and isobutyl acetates and butyrates increased during ripening.

Further investigations were performed to reveal the precursors and the mechanism of biosynthesis of these volatiles. It was presumed that the biosynthesis of the branched-chain volatile components in bananas was similar to that occurring in the formation of fusel oil during fermentation. The formation of fusel oil involves branched-chain amino acids valine, leucine and isoleucine as the precursor of branched-chain alcohols, isobutyl alcohol, isoamyl alcohol and 2-methyl butyl alcohol,respectively (Sentheshanmuganathan, 1959). Buckley and Sullivan (reported by Wick et al., 1966 and Palmer, 1971) determined the free amino acid content during the ripening of banana fruit. They reported a marked increase in the content of valine and leucine during ripening (table 1), which correlated with the rapid increase in the formation of isobutyl and isoamyl alcohols and esters. Similar findings were reported by Tressl et al. (1973). Therefore, leucine and valine appeared to be potential precursors of banana volatiles.

Preliminary labeling experiments involving incubation of ripe banana slices with ^{14}C-labeled amino acids and measurement of radioactivity in the evolved volatiles

Table 1 Amino Acids in Banana Pulp[1]

(μmoles per gram fresh weight)

Stage	Days After Cutting	Valine	Leucine	Isoleucine
Green	3	0.01	0.13	0.06
	7	0.37	0.51	0.28
	10	0.33	0.49	0.22
Ripening	11	0.37	0.61	0.23
	12	0.59	0.86	0.20
Flavor development	13	0.95	1.60	0.21
	14	1.22	2.17	0.23
Maximum flavor	15	1.62	2.68	0.24
	16	0.72	1.86	0.17
	17	1.44	2.41	0.18
Overripe	21	0.97	1.52	0.11

[1]From Wick et al., 1966

suggested that valine is a precursor of isobutyl alcohol and isobutyl acetate, and leucine is a precursor of isoamyl alcohol and isoamyl acetate (Wyman et al., 1964). This finding was further confirmed by Myers et al. (1969, 1970) and Tressl et al., (1970d). The latter investigators also found ^{14}C label in free branched-chain acids and, upon hydrolysis of the banana aroma extract, also found distribution of radioactive label in both the alcohol and the acid moiety of the esters. Similarly, Yabumoto et al. (1977), using radioactive tracer experiments with ^{14}C-isoleucine, indicated that in muskmelon isoleucine was converted to esters in which the branched-chain skeleton was found either as the acyl or alkyl moiety.

Tressl et al. (1970c,d) also found radioactivity in the corresponding branched-chain aldehyde and α-keto acid. They postulated that the biosynthesis follows Ehrlich's mechanism and proposed a reaction scheme (figure 1). This mechanism involves enzymic transamination of leucine to the corresponding α-keto acid followed by enzymic oxidative decarboxylation of the keto acid to the aldehyde. At this point the path splits, some of the aldehyde being further reduced to the alcohol, which is the alkyl component of the ester and some being oxidized to the acid, which is the acyl component of the ester. Significantly, Palmer (1971)

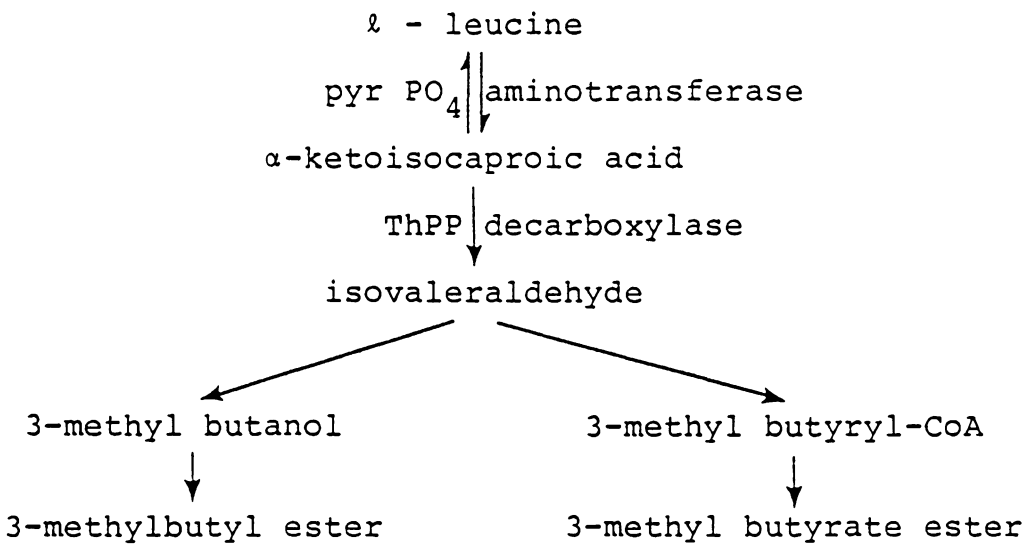

Figure 1 Proposed mechanism for formation of aldehyde and esters from leucine in bananas (Tressl et al., 1970c,d)

Pyr PO_4 = pyridoxal phosphate
ThPP = thiamine pyrophosphate

reported that α-ketoisovaleric and α-ketoisocaproic acids, the intermediates from valine and leucine, respectively, were only detected in fully-ripe banana fruits.

Similar mechanisms were proposed by other workers. Yu and Spencer (1968) reported that an enzyme preparation from fresh tomato fruit catalyzed the conversion of ℓ-leucine to α-ketoisocaproic acid via transamination. The conversion was confirmed by using ^{14}C-leucine. Yamashita et al. (1978) reported that α-keto acids were converted to aldehydes when added to a strawberry homogenate. The reaction involved pyruvate decarboxylase, since the addition of ThPP (thiamine pyro phosphate) and Mg^{2+} enhanced the formation of aldehyde. When NADH (reduced nicotinamide adenine dinucleotide) and alcohol dehydrogenase from strawberry seeds were included in the reaction mixture, aldehyde and alcohol were simultaneously produced.

The ^{14}C data of Tressl (1970d) clearly indicates that aldehydes are intermediates in the production of flavor compounds from amino acids in bananas. However, aldehydes can be formed from amino acids by mechanisms other than transamination-decarboxylation. Aldehydes, which occur as aroma constituent of many foods, such as tea, cocoa, tobacco and wine, are also known to be formed from amino acids in a reaction between the amino acids and quinone formed from a

polyphenoloxidase (PPO) catalyzed oxidation of polyphenols. Two mechanisms have been proposed for the formation of aldehyde from the reaction between amino acid and quinone.

The first mechanism is the amino acid oxidation proposed by Trautner and Roberts (1950) as shown in figure 2. It involves an oxidative deamination of the amino acid resulting in the release of an α-keto acid. The keto acid may subsequently undergo decarboxylation to form an aldehyde with one carbon atom less than the amino acid. The first step of the scheme in figure 2 involves the substitution of a nitrogen of an amino acid at the 4 position of the catechol ring. This step serves only in the stabilization of the o-quinonoid structure and in the preservation of its reactivity through repeated reductions and re-oxidations. The amino acid oxidation is initiated with a condensation between the substituted o-quinone and another amino acid to form a quinone imine which subsequently undergo a rearrangement to the imino acid structure. The quinone imine is then hydrolyzed into keto acid and amino phenol. The latter is oxidized to the original substituted o-quinone with the release of ammonia.

Several workers have obtained evidence consistent with the formation of aldehyde via the amino acid oxidation mechanism of figure 2. Bokuchava and Popov (1954) reported

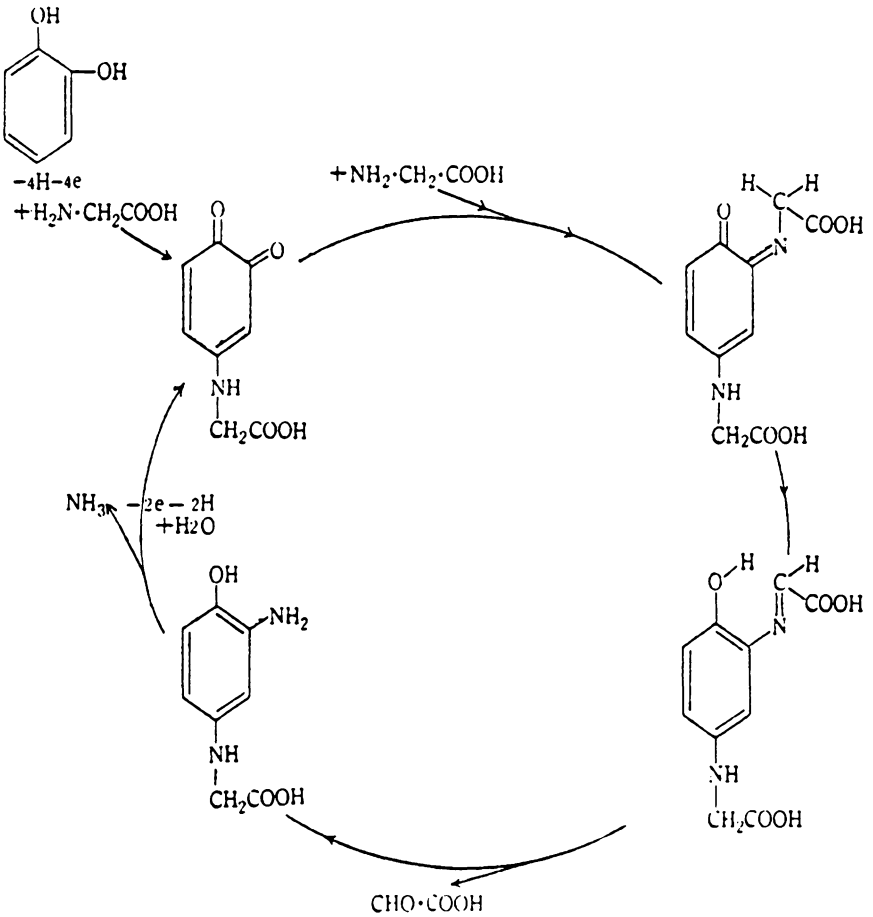

Figure 2 Tentative scheme of the continuous oxidation of amino acid by the mono-substituted o-quinonoid color compounds (Trautner and Roberts, 1950)

that when various amino acids were incubated with hot aqueous solutions of tea tannin or with tea leaves, aroma characteristic for each amino acid was formed. They suggested that the aroma was due to the formation of aldehydes by a mechanism involving the oxidation of amino acids by quinones present in tea tannin. Popov (1956) demonstrated the formation of carbon dioxide, ammonia and aldehyde during the oxidation of amino acid in a reaction system containing tea PPO, catechin and amino acid. He suggested the involvement of PPO in the mechanism and proposed that the reaction involves an oxidative deamination of amino acids by o-quinones formed during PPO catalyzed oxidation of catechin. Bokuchava et al. (1968) reported that addition of alanine to tea products enhanced the formation of acetaldehyde. In tobacco leaves, PPO and phenolic compounds were reported to take part in the oxidative deamination of amino acids during drying (Antonenko et al., 1975). The reaction was accompanied by oxygen absorption, the release of carbon dioxide and ammonia, and the formation of brown pigment.

The second possible mechanism is the Strecker degradation, involving the decarboxylation of the amino acid, followed by a deamination resulting in the release of an aldehyde (Chuyen et al., 1972).

Several experiments reported in the literature suggested that this mechanism exists in tea, cocoa and wine. Using radio tracer techniques with ^{14}C-amino acids, Co and Sanderson (1970) and Saijo and Takeo (1970a) demonstrated that leucine, isoleucine, valine and phenylalanine were converted to isovaleraldehyde, 2-methyl butyraldehyde, isobutyraldehyde and phenylacetaldehyde, respectively, when these amino acids were added to fermenting (oxidizing) fresh tea leaves, and to model systems containing tea enzyme extract and tea polyphenol. Enzymes were required because tea leaves or enzyme extract thereof inactivated by heat gave no aldehyde (Co and Sanderson, 1970; Saijo and Takeo, 1970b). They suggested that the mechanism involved the enzymic oxidation of tea polyphenols to o-quinones by PPO, followed by a non-enzymic reaction between the quinones and the amino acids to form aldehydes via the Strecker degradation.

Bailey et al. (1961), Rohan (1964), and Darsley and Quesnel (1972) proposed that the formation of aldehydes in cocoa beans during fermentation, drying and roasting was also via the Strecker degradation of amino acids. The volatile aldehydes formed by interaction of amino acids with quinones produced by PPO were suggested to be the source of wine bouquet (Singleton, 1969).

Motoda (1979) reported the formation of aldehydes from amino acids in model systems containing d-catechin, amino acids and a microbial PPO purified from Alternaria tenuis strain A-2. When the microbial PPO was added to the extract of tea leaves, cocoa beans and coffee beans, isovaleraldehyde was formed and the formation was enhanced when leucine was also included in the reaction mixture. This indicates that this mechanism does not seem to be restricted to a certain enzyme-substrate system. The enzyme only functions to produce the quinone. Saijo and Takeo (1970b) reported that addition of H_2O_2 to a mixture containing (-)-epicatechin, amino acid and tea enzyme extract led to the formation of greater amounts of aldehyde, suggesting that peroxidase can catalyze the enzymic oxidation of epicatechin to o-quinone which further result in formation of aldehyde.

Both mechanisms outlined above could possibly exist in bananas, because bananas possess a similar quinone-producing system involving PPO and dopamine. Griffith (1959) reported that dopamine is the only major phenolic constituent in bananas that contributes to browning of the fruit. Waalkes et al. (1958) reported that the concentration of dopamine in banana pulp is about 8 µg/gr fresh weight, much smaller than the concentration in the peel. The synthesis and

accumulation of dopamine in the peel of ripening banana were studied by Buckley (1965). The dopamine content was 0.7 to 0.8 mg/gr fresh weight of peel tissue at harvest and increased during ripening to a level of 1.0 to 1.2 mg/gr fresh weight.

Palmer (1963) indicated that PPO from bananas catalyzes the oxidation of dopamine more readily than other phenols tested. Banana PPO has optimum activity at pH 7.0 with dopamine as substrate. The reaction mechanism for the enzymic oxidation of dopamine by banana PPO followed by polymerization of the active quinones to form the black melanin pigment is shown in figure 3 as reported by Palmer (1963). Studies on the oxidation of polyphenols by PPO and on the formation and the structure of melanins are discussed in more detail by Mason (1959), Kertesz and Zito (1962), Corse (1965), Pierpoint (1966), Singleton (1972) and Butt (1980).

Earlier studies done by Hultin and Proctor (1962) indicated that enzymes are required for the development of banana flavor. They demonstrated that the addition of a crude enzyme extract from ripe banana pulp to a heat processed banana puree stimulated the regeneration of banana aroma, particularly when supplemented with valine,oleic acid or pyruvic acid. Crouzet et al. (1980), and N'galani and

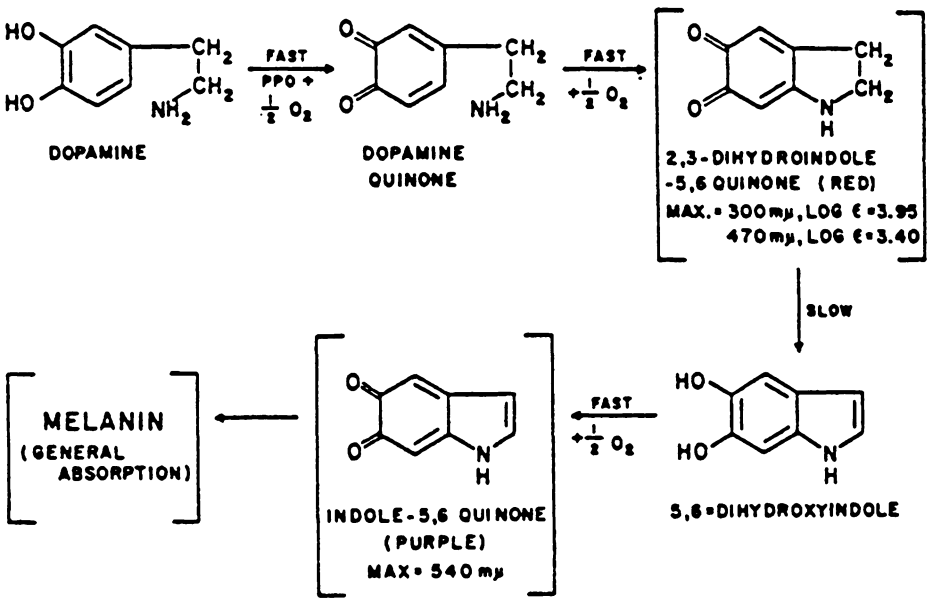

Figure 3 Proposed mechanism for the oxidation of dopamine catalyzed by banana polyphenoloxidase (Palmer, 1963)

Crouzet (1980) reported that the addition of an enzyme extract from banana pulp to banana powders free of volatile components caused regeneration of alcohols, carbonyl compounds and esters reported to be the volatile aroma components of banana. They indicated that the presence of alanine and leucine aminotransferases and alcohol dehydrogenase in the extract suggested that the formation of aldehyde and alcohol was via transamination and decarboxylation of the amino acids. And the formation of esters was explained by the presence of transacylase in the extract. They also indicated that the possibility of the involvement of PPO could not be excluded and suggested that the quinone formed may react with the amino acid via Strecker degradation.

By combining all the possible mechanisms outlined above, an overall reaction scheme could then be postulated for the production of aldehyde, alcohol and ester in bananas (figure 4). The reaction would begin with the enzymic oxidation of dopamine catalyzed by PPO (as in figure 3) and/or POD to form dopamine quinone. Nagle and Haard (1975) reported the presence of POD in bananas, which according to Saijo and Takeo (1970b) could catalyze the oxidation of (-)-epicatechin to o-quinone. Other enzymes such as laccase and cytochrome c oxidase could also catalyze the oxidation

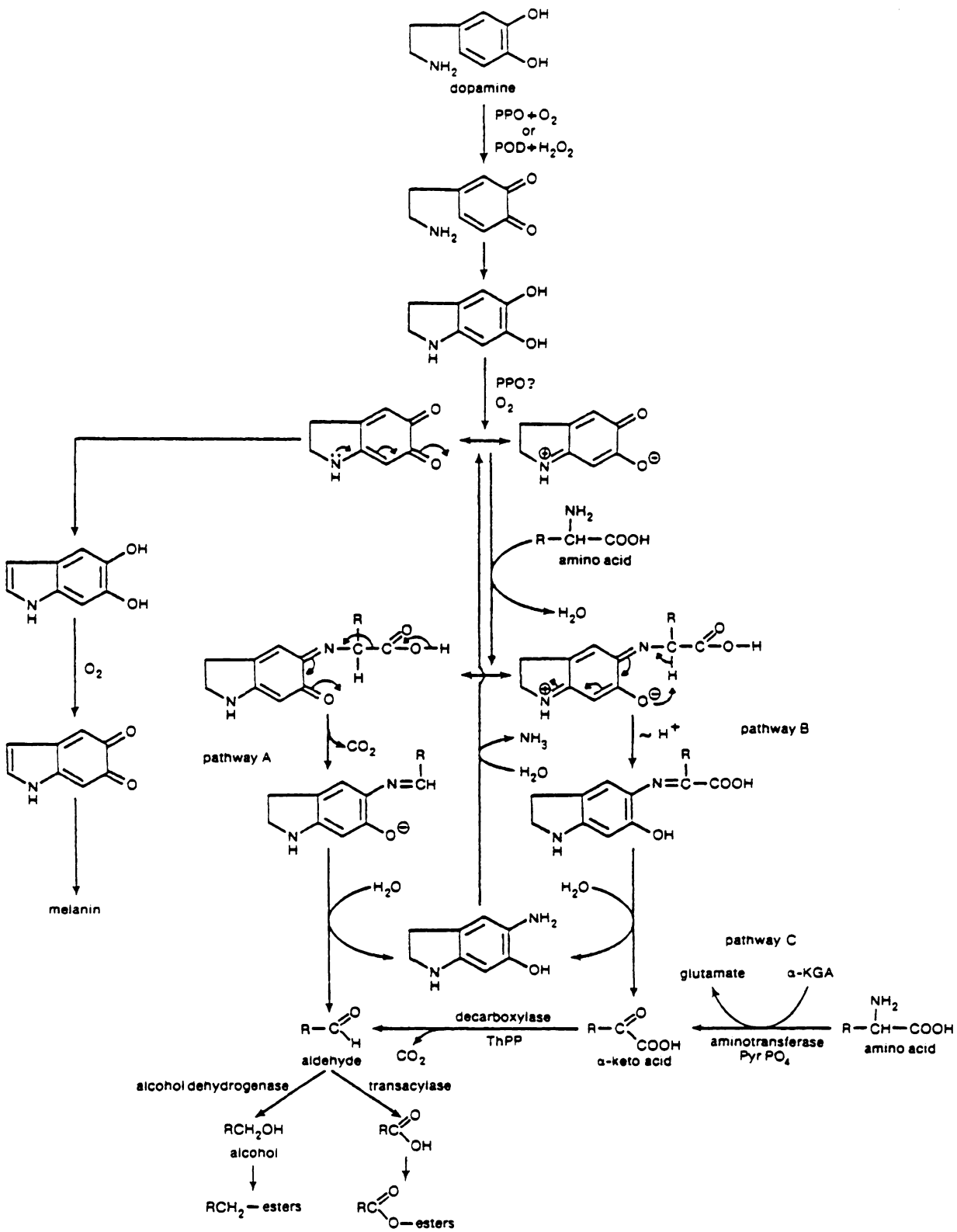

Figure 4 Proposed mechanism for the formation of aldehyde, alcohol and ester from amino acid in bananas

A = Strecker degradation (Pickenhagen and Palmer, 1971)
B = amino acid oxidation (Trautner and Roberts, 1950)
C = Ehrlich mechanism (Tressl, 1970c)
Dopamine oxidation and melanin formation (Palmer, 1963)

$Pyr PO_4$ = pyridoxal phosphate
ThPP = thiamine pyrophosphate
α-KGA = α-ketoglutaric acid

of dopamine directly or indirectly (Palmer, 1963). Laccase is a p-diphenol oxidase which is also capable of oxidizing o-diphenols. Oxidized cytochrome c formed during oxidation catalyzed by cytochrome c oxidase, can oxidize dopamine to the corresponding quinones.

The substitution of nitrogen in the 4 position of the catechol to stabilize the o-quinone (figure 2), as proposed by Trautner and Roberts (1950), is accomplished in the scheme of figure 4 by closing of the ring to form the indole compound. At this point, there are two resonance forms of the dopachrome quinone and then two resonance forms of the quinone imine are produced. One resonance form would be expected to undergo decarboxylation followed by a deamination, releasing an aldehyde (Strecker degradation, figure 4, pathway A). The other would be expected to undergo oxidative deamination and release an α-keto acid (amino acid oxidation proposed by Trautner and Roberts, 1950, figure 4, pathway B). Another mechanism of producing α-keto acid is via enzymic transamination of the amino acid (figure 4, pathway C) as proposed by Tressl (1970c,d). This step requires aminotransferase, α-keto glutarate and pyridoxal phosphate. The α-keto acid formed via both pathways B and C could be decarboxylated enzymically to produce the aldehyde. The aldehyde would then be further

oxidized to form the acyl or reduced to form the alkyl components of the esters.

III. MATERIALS AND METHODS

A. PREPARATION OF ENZYME EXTRACT FROM BANANAS

Banana enzyme extract was prepared as described by Archer and Palmer (1975). The enzymes were extracted from banana pulp with a 0.02 M phosphate buffer solution (pH 7.0) which contained 1% polyethylene glycol (Carbowax 6000, Mann Research Laboratories, New York, NY) to protect the enzymes from precipitation by tannins. Four grams of ripe banana pulp were homogenized in 36 ml of the extraction medium, in a Du All glass tissue grinder (Kontes, Vineland, NJ). The homogenate was centrifuged for 15 min at 20,000 x g in a Sorvall Superspeed Automatic Refrigerated Centrifuge Model RC2-B (Sorvall Inc., Newton, CT) and the supernatant was used for the study. Preparation of enzyme extract was performed between 0 and 4 °C. The enzyme extracts were stored frozen in small polyethylene bottles until used.

B. ENZYME ASSAYS

Polyphenoloxidase (PPO), peroxidase (POD), laccase and cytochrome c oxidase can oxidize dopamine directly or indirectly to dopamine quinone (Palmer, 1963). The enzyme extract possibly contained some or all of these enzymes. Therefore, the extract was assayed for the presence of the above enzymes as described in the following sections.

In general, the enzyme assays were performed at 25 °C in a Spectronic 710 (Bausch & Lomb, Rochester, NY) equipped with a Fisher Recordall Series 5000 recorder (Fisher Scientific Co., Pittsburg, PA). The enzyme activities were determined from the rates of reactions measured as the initial change in absorbancy per minute at the appropriate wavelength, in a cell of 1 cm pathlength. The enzyme reactions were initiated by the addition of enzyme extract and the change in absorbancy followed for 2 to 3 minutes. The substrates were checked for autoxidation by measuring the absorbancy for several minutes prior to adding the enzyme extract. Non-enzymic reactions were measured in reaction mixtures containing boiled enzyme extract.

1. Polyphenoloxidase (PPO)

Activity of PPO was assayed by measuring the initial rate of production of dopachrome quinone from dopamine as described by Palmer (1963), and Archer and Palmer (1975). The reaction mixture, consisted of 1.5 ml of freshly prepared 0.01 M dopamine hydrochloride (Sigma Chemical Co., St Louis, MO), 1.35 ml of 0.1 M phosphate buffer (pH 7.0) and 0.15 ml of enzyme extract. The initial linear increase in absorbancy per minute was measured at 470 nm and the unit activity of PPO calculated. One unit of PPO activity was defined as the amount of enzyme which catalyzes the transformation of 1 µmole subtrate per minute under the conditions of the assay. The initial rates were proportional (R-square 0.99) to the amount of enzyme added.

2. Peroxidase (POD)

POD activity was determined by the method of Nagle and Haard (1975). Hydrogen peroxide (H_2O_2) was used as the hydrogen acceptor and o-dianisidine (Sigma Chemical Co., St Louis, MO) as the hydrogen donor. The H_2O_2 solution was prepared from 0.1 ml of 30% H_2O_2 in 100 ml of 0.01 M phosphate buffer (pH 6.0). The assay medium contained 0.8 ml of a 1% (w/v) solution of o-dianisidine in methyl alcohol, 10 ml of the H_2O_2 solution and 0.01 M phosphate

buffer (pH 6.0) in a final volume of 100 ml. Into a cuvette were placed 2.0 ml of the assay medium, 0.9 ml distilled water and 0.1 ml banana enzyme extract. The rate of oxidized o-dianisidine formation was measured at 460 nm. The presence of H_2O_2 in the enzyme extract was checked in mixtures of which H_2O_2 had been omitted. The POD unit in this present study was expressed in terms of equivalent PPO unit as described in the Results section.

3. Laccase

A modified method of Peisach and Levine (1965) was used to determine laccase activity with p-phenylenediamine as substrate. The total volume of reaction mixture in each cuvette was 3 ml, which consisted of 0.75 ml of 0.1 M p-phenylenediamine dihydrochloride (Sigma Chemical Co., St Louis, MO), 2.15 ml of 0.2 M phosphate buffer (pH 7.0) and 0.1 ml of banana enzyme extract. Rates of the reactions were measured at 490 nm.

4. Cytochrome c Oxidase

Cytochrome c oxidase was assayed according to Smith (1955) except for the preparation of reduced cytochrome c solution. Three ml of a 1.0 mM cytochrome c solution (Sigma Chemical Co., St Louis, MO) in 0.1 M phosphate buffer (pH

7.0) was reduced by adding approximately 0.1 mg sodium dithionite. The cytochrome c was checked for complete reduction by pipetting 0.05 ml of the above solution into a cuvette containing 2.45 ml of 0.1 M phosphate buffer (pH 7.0) and measuring absorbance at 550 nm, the absorbancy maximum for the reduced form of cytochrome c. Additional sodium dithionite was subsequently added to the solution until no further increase in the absorbancy of the diluted mixture was observed. One ml of the reduced cytochrome c solution was passed through a G-10 Sephadex column (Sigma Chemical Co., St Louis, MO) to remove excess sodium dithionite. A 0.1 M phosphate buffer (pH 7.0) solution was used to elute the cytochrome c. The pink cytochrome c band, which eluted ahead of the dithionite, was collected and diluted to 10 ml with the same buffer solution.

The reaction mixture contained 0.5 ml reduced cytochrome c, 2.4 ml of 0.1 M phosphate buffer (pH 7.0) and 0.1 ml enzyme extract. The rate of the reaction was measured as the initial decrease in absorbancy at 550 nm due to oxidation of cytochrome c.

C. FORMATION OF ALDEHYDES FROM AMINO ACIDS IN REACTION MIXTURES CONTAINING ENZYME EXTRACT FROM BANANAS

The formation of aldehydes was measured in reaction systems containing combinations of dopamine, amino acids, banana enzyme extract and hydrogen peroxide as described below.

The effects of the amounts of dopamine, leucine and enzyme extract on the production of isovaleraldehyde were studied on factorial combinations of three levels each of dopamine, leucine and enzyme. The three levels of leucine were 25, 50 and 75 mM, the levels of dopamine were 0.05, 0.5 and 5 mM, and the amounts of enzyme extract added were 0.75 ml of a 1:10 dilution (0.04 units PPO activity/ml final reaction mixture); 0.15 ml (0.07 units/ml) and 0.75 ml (0.36 units/ml) of undiluted enzyme extract. The optimum levels of dopamine, leucine and enzyme extract found in this part of the study were further used in the following studies.

Formation of IVA was measured in reaction systems with or without the addition of H_2O_2 to observe if POD could function in the formation of aldehydes from amino acids as reported by Saijo and Takeo (1970b) in tea experiments. A H_2O_2 solution was prepared from 0.1 ml of 30% H_2O_2 solution in 10 ml of 0.1 M phosphate buffer (pH 7.0), and 0.7 ml of this solution added to the reaction mixture.

Valine was also tested as the amino acid precursor. Reaction mixtures were prepared containing leucine or valine alone, or in combination.

All the mixtures were prepared and incubated in 60 ml separatory funnels which would be used later for extraction. The incubations were carried out at pH 7.0, the optimum pH for banana PPO when oxidizing dopamine (Palmer, 1963). The reactions were initiated by the addition of enzyme extract. All reaction mixtures were incubated at 25 °C for 2 hours. The 2 hour reaction time was chosen based on the data obtained by Saijo and Takeo (1970b), and Luh and Palmer (1973). An experiment performed to check the reaction time indicated that a 15 min reaction time yielded aldehyde concentrations which were just detectable, while easily measurable concentrations were produced after 2 hours. However, extending the time to 10 hours caused the amount of aldehyde to drop down to one third of the 2 hour value. The aldehyde probably reacted further to form alcohols and/or acids, reacted with other compounds present in the reaction mixture and some could have evaporated.

The composition of individual reaction mixtures and other parameters will be given in detail in the Results section.

D. SPECTROPHOTOMETRIC ANALYSES OF THE REACTION MIXTURES

Spectrophotometric analyses were performed on the reaction mixtures in conjunction with the aldehyde formation studies to establish a relationship between aldehyde production and dopachrome quinone formation from dopamine. Reaction mixtures (total 2 ml), identical to the mixtures prepared in separatory funnels for aldehyde formation, were prepared separately in cuvettes. The absorbancy of these mixtures were then measured with a recording spectrophotometer at 470nm (Palmer, 1963).

E. CONVERSION OF ALDEHYDES TO 2,4-DINITROPHENYLHYDRAZONE DERIVATIVES FOR IDENTIFICATION AND QUANTIFICATION

Any aldehydes formed in the 10 ml reaction mixtures prepared in separatory funnels were then converted to the corresponding 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives by a method adapted from Selim (1977). A 2,4-dinitrophenylhydrazine (2,4-DNP) reagent was prepared by dissolving 0.025 g of 2,4-DNP (Eastman Kodak Co., Rochester, NY) in 10 ml of 6 N hydrogen chloride, heating to 100 °C, then diluting to 25 ml with distilled water (Vogel, 1966). One ml of the 2,4-DNP reagent, 10 ml isooctane and 15 ml water were added to the reaction mixtures in the separatory funnels at the completion of the designated reaction time.

The mixtures were shaken on a Burrell Wrist Action Shaker (Burrell Corp., Pittsburg, PA) for 90 min. The 90 min shaking time was determined to be sufficient in preliminary studies in which IVA was added to blank reaction mixtures containing 25 ml water and 1 ml of the 2,4-DNP reagent. The reaction mixtures were shaken for different lengths of time up to 135 min with 10 ml isooctane and carried through the extraction procedure described further below. At 90 min the conversion of IVA to 2,4-DNPH derivative had leveled off at 85%. Increasing the shaking time to 135 min did not improve the conversion. This conversion was lower than that reported by Selim (1977) for propionaldehyde. Repeating the experiment with IBA gave 87% conversion after 90 min shaking time. The difference from Selim's result may be due to the fact that in this experiment the aldehydes were added to the isooctane fraction rather than the water fraction. When authentic IVA-2,4-DNPH was carried through the extraction procedure, 98% of it was recovered after 90 min shaking.

Following the 90 min reaction with the 2,4-DNP reagent, the aqueous layer of the mixture in the separatory funnel was then drawn off into a second separatory funnel and extracted with another 10 ml of isooctane for 5 min. The two isooctane fractions were combined and 10 ml of this was

extracted twice with 5 ml acetonitrile, both times shaken for 5 min. The acetonitrile fractions were collected in a 15 ml centrifuge tube, concentrated to 4.0 ml under nitrogen in a 40 °C water bath and filtered through a 0.45 µm Nylon filter (Rainin Instrument Co. Inc., Woburn, MA) using a Swinnex-25 Millipore Filter Assembly (Millipore Corp., Bedford, MA). The resulting solution was analyzed quantitatively for its aldehyde content by HPLC and the identities of the aldehydes were confirmed in representative samples by TLC and flash exchange GC, as described below.

F. QUANTITATIVE AND QUALITATIVE ANALYSES OF THE 2,4-DNPH DERIVATIVES OF IVA AND IBA

1. Preparation of Authentic 2,4-DNPH Derivatives

The method of Shriner et al. (1980) was used to prepare the standard 2,4-DNPH derivatives from IVA and IBA. A freshly prepared 2,4-DNP solution (0.4 g of 2,4-DNP, 2 ml concentrated sulfuric acid, 3 ml water and 10 ml of 95% ethanol) was added to a solution of 0.5 g of aldehyde in 20 ml of 95% ethanol. The resulting mixture was allowed to crystallize overnight at room temperature. The crystals were recovered and recrystallized from 30 ml of 95% ethanol.

The melting point of the crystals was determined using a Gallenkamp melting point apparatus (Gallenkamp, London, U.K.) kindly lent by Dr. D.G.I. Kingston (Chemistry Dept.,

Va Tech, Blacksburg, VA). The melting point of IVA-2,4-DNPH was 122.5 °C and IBA-2,4-DNPH was 181 °C. The reported melting point of IVA-2,4-DNPH is 123 °C and IBA-2,4-DNPH is 182 °C (Shriner et al., 1980).

The wavelength of maximum absorbancy of 0.01 mg/ml 2,4-DNPH solutions in acetonitrile was determined using a Coleman 124 Double Beam Spectrophotometer equipped with a Model 56 recorder (Perkin-Elmer Corp., Norwalk, CT). The maximum wavelength of both 2,4-DNPH's were 362 nm, while that of 2,4-DNP was 293 nm.

2. High Performance Liquid Chromatography

A model 6000A Chromatography Pump (Waters Assoc., Milford, MA) equipped with a 20 µl Rheodyne Injector Port (Rheodyne Inc., Berkeley, CA) and a Waters Assoc. model 440 Absorbance Detector set at 365 nm and 0.05 aufs was used. The column was a C18 Radial Pak Cartridge placed in a RCM-100 Radial Compression Module (Waters Assoc.) A C18 Corasil Reversed Phase Guard-Column (Waters Assoc.) was installed ahead of the Radial Pak Column. All data were recorded with an OmniScribe Recorder (Houston Instruments, Austin, TX).

Acetonitrile:water (3:2) was used as the solvent at a flow rate of 3.0 ml/min (Selim, 1977), 450 to 600 psig. The

acetonitrile was filtered through type FH filters of 0.5 µm pore size (Millipore Corp., Bedford, MA) while water was filtered through type A/E glass fiber filters of 0.3 µm pore size (Gelman Instrument Co., Ann Arbor, MI). The acetonitrile:water solvent was degassed for 10 sec under water pump vacuum before use.

Approximately 0.1 ml aliquots of sample and standard solutions (0.01 mg/ml) were injected into the 20 µl Rheodyne injector port with a 1 cc plastipak disposable syringe (Becton-Dickinson, Rutherford, NJ). The retention times of the 2,4-DNPH samples were compared to the standards. Quantitation was achieved from peak height measurements relative to the peak heights of the standards. The standard calibration curve of IVA-2,4-DNPH in acetonitrile was found to be linear (R-square 0.99) over the range of concentrations tested .

3. Gas Chromatography of Aldehydes Regenerated from 2,4-DNPH

A flash-exchange method modified from Ralls (1960) was used to confirm the formation of aldehydes in the sample mixtures. Acetonitrile extracts of the samples and standard solutions containing 15 to 100 µg 2,4-DNPH derivatives were combined with three parts by weight of α-ketoglutaric acid in acetonitrile in 1 ml reacti- vials (Pierce Chemical Co.,

Rockford, IL). The mixtures were evaporated to dryness under nitrogen in a 40 °C water bath. The closed reacti-vials were then heated in a 250 °C heating block (Pierce Reacti-Therm heating module, Pierce Chemical Co.) until the mixture melted (approximately 1 min). A 0.2 ml sample of the headspace was withdrawn with a 1 ml gas-tight syringe (Hamilton Co., Reno, NV) and injected into a 12% SP 1000 on 60/80 Chromosorb Column (Supelco Inc., Bellefonte, PA) maintained at 70 °C. A model 600 Varian-Aerograph Gas Chromatography with a flame ionization detector and a model 650 Aerograph Hydrogen Generator (Wilkens Instrument & Research Inc., Walnut Creek, CA) equipped with an OmniScribe recorder (Houston Instruments, Austin, TX) was used. The flow rate of hydrogen was 30 ml/min, nitrogen 20 ml/min and air 300 ml/min. The retention time of the peaks were compared to peaks of the headspace of authentic IBA and IVA.

4. Thin Layer Chromatography

The acetonitrile extracts were further concentrated 30 fold. Eight µl of the concentrated samples and standards (0.2 mg/ml) were spotted with 2 µl Drummond Microcaps disposable micro-pipettes (Bolab Incorporated, Derry, NH) on a type KC18DF reversed phase plate (Whatman Inc., Clifton, NJ) and the plate was developed twice with methanol:water

(4:1). The developed plate was examined for spots under visible and UV light using a model UVSL-58 Mineralight Lamp (Ultra Violet Products Inc., San Gabriel, CA). The IVA and IBA hydrazones and the 2,4-DNP reagent were readily visible as yellow spots. The distances travelled by the sample spots were compared to the standard spots.

IV. RESULTS

A. ANALYSIS OF ISOBUTYRALDEHYDE (IBA) AND ISOVALERALDEHYDE (IVA)

1. Quantitative Analysis of IBA and IVA

A rapid and reproducible method was needed to analyze for low concentrations of IBA and IVA in enzyme incubation mixtures. Two approaches were tried as indicated below.

a. Gas chromatography (GC).

Two possibilities here would be to (1) extract the aldehydes from the incubation mixture and then to analyze the extract or (2) analyze the aldehydes in headspace samples taken from over the incubation mixture.

Attempts to separate aldehydes in standard solutions in ether or carbon disulfide on a 2100 SP Carbowax column (Supelco Inc., Bellefonte, PA) at about 50 °C were unsuccessful, as the aldehyde peaks were poorly separated from the solvent peaks.

Analysis of headspace over aqueous solutions of the aldehydes under the same GC condition yielded two well separated major peaks. However, it proved difficult to prepare a standard solution of the aldehydes in water, in

terms of obtaining equilibrium between the headspace and solution, and thus obtaining a reproducible relationship between the concentration of aldehyde in the water and in the headspace. Earlier studies on methods of determining vapor-liquid equilibria for dilute organics in aqueous solution (Kieckbusch and King, 1979) revealed that quite specialized and involved techniques were required to assure reproducible equilibration.

Problems with interference from other volatile components in the headspace over incubation mixtures could be foreseen. It was known from earlier studies (Luh and Palmer, 1973) that banana enzyme extracts often contain levels of volatiles that can interfere. Also, the extracts would probably contain enzymes which further convert the expected aldehydes to alcohols, acids or esters. Quite sophisticated techniques such as capillary column GC or combined GC-mass spectrometry would be required to assure adequate separation and identification of the expected aldehydes, in the presence of other volatile components derived from the enzyme extract or enzyme action.

Based on the available information and on the preliminary results described above the GC approach was abandoned.

b. High performance liquid chromatography (HPLC).

The aldehydes formed in the reaction mixtures were subsequently quantitatively determined as the 2,4-dinitrophenylhydrazone (2,4-DNPH) derivatives using HPLC as adapted from Selim (1977). Isobutyraldehyde (IBA), isovaleraldehyde (IVA or 3-methyl butyraldehyde) and 2-methyl butyraldehyde (2-MBA) were expected to be formed from valine, leucine and isoleucine, respectively and attempts were made to separate the three authentic aldehyde-2,4-DNPHs on a C18 μBondapak column under conditions utilized by Selim (1977) to separate straight-chain aldehydes. IBA-2,4-DNPH was easily separated from IVA- and 2-MBA-2,4-DNPH, however changing the solvent composition (acetonitrile:water = 60:40, 50:50, 45:55, 40:60) and the flow rate (2-3 ml/min) did not improve the separation of IVA-2,4-DNPH from 2-MBA-2,4-DNPH. Therefore, the μBondapak column was replaced with a C18 Radial Pak cartridge placed in a Radial Compression Module. Changing the solvent composition and the flow rate still did not improve the separation of IVA-2,4-DNPH from 2-MBA-2,4-DNPH. Solvent programming gave worse separation. Methanol:water (3:2) showed the same results as acetonitrile:water (3:2).

Since 2-MBA is not a major flavor compound in banana and isoleucine content of banana remains constant during

ripening (table 1, and Tressl and Drawert, 1973), it was decided to discontinue the attempts to separate IVA-2,4-DNPH from 2-MBA-2,4-DNPH and not to include isoleucine or 2-MBA in this present study. However, it should be recognized that small amounts of 2-MBA-2,4-DNPH may be present in the IVA-2,4-DNPH peaks.

The Radial Pak column was finally selected for separating IVA- and IBA-2,4-DNPH as it gives longer retention times and the peaks are better separated.

Figure 5 shows a typical HPLC chromatogram of IVA- and IBA-2,4-DNPH in an acetonitrile extract of a reaction mixture containing 0.5 mM dopamine, 37.5 mM leucine, 37.5 mM valine and enzyme extract (0.07 units/ml reaction mixture). As expected, IVA-2,4-DNPH had longer retention time (10.2 min) than IBA-2,4-DNPH (7.5 min) since IVA-2,4-DNPH has a lower polarity and was therefore retained longer on the reversed phase column. Control runs established that the extraneous peaks represented impurities in the 2,4-DNP reagent.

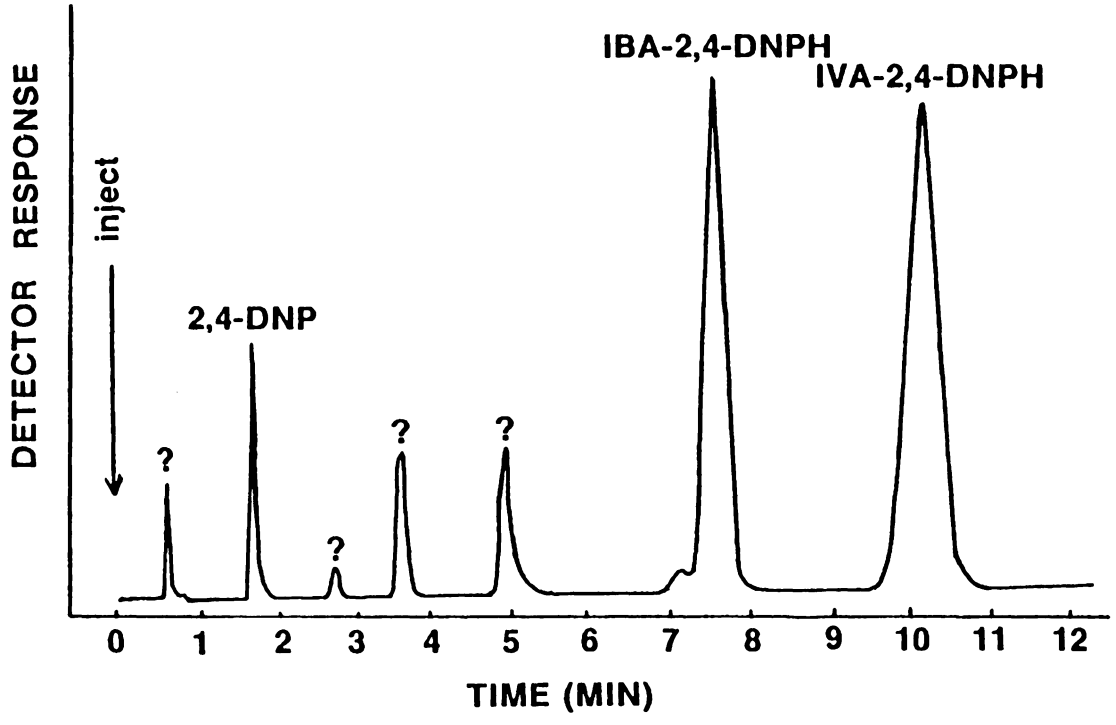

Figure 5 Typical HPLC chromatogram of the 2,4-DNP and 2,4-DNPHs in acetonitrile extract of the incubation mixtures

C18 Radial Pak reversed phase column (10 cm x 7 cm I.D.) at ambient temperature.
Mobile phase acetonitrile:water (3:2) at a flow rate of 3 ml/min (500 psig).
UV detection at 365 nm, 0.05 aufs.
2,4-DNP = 2,4-dinitrophenylhydrazine
IBA-2,4-DNPH = isobutyraldehyde-2,4-dinitrophenylhydrazone
IVA-2,4-DNPH = isovaleraldehyde-2,4-dinitrophenylhydrazone

2. Confirmation of Identity of the IBA- and IVA-2,4-DNPH Derivatives

a. Flash exchange gas chromatography.

Flash exchange GC confirmed the identity of the aldehyde hydrazones and thus confirmed the presence of IVA and IBA in the extracts. A typical chromatogram is shown in figure 6. The composition of the reaction mixture was the same as used for the HPLC chromatogram. The additional peak in the gas chromatogram seemed to be a decomposition product since this peak was also present when authentic IVA-2,4-DNPH was carried through the flash exchange procedure. No peaks were found when α-KGA, with or without 2,4-DNP reagent, was carried through the procedure.

b. Thin layer chromatography.

The production of IVA and IBA in the reaction mixtures were also confirmed by thin layer chromatography (TLC). A typical chromatogram of the well separated IVA- and IBA-2,4-DNPH is shown in figure 7. The composition of the reaction mixture was the same as used for the HPLC chromatogram. The extraneous spots found on the TLC plates again represented impurities from the 2,4-DNP reagent.

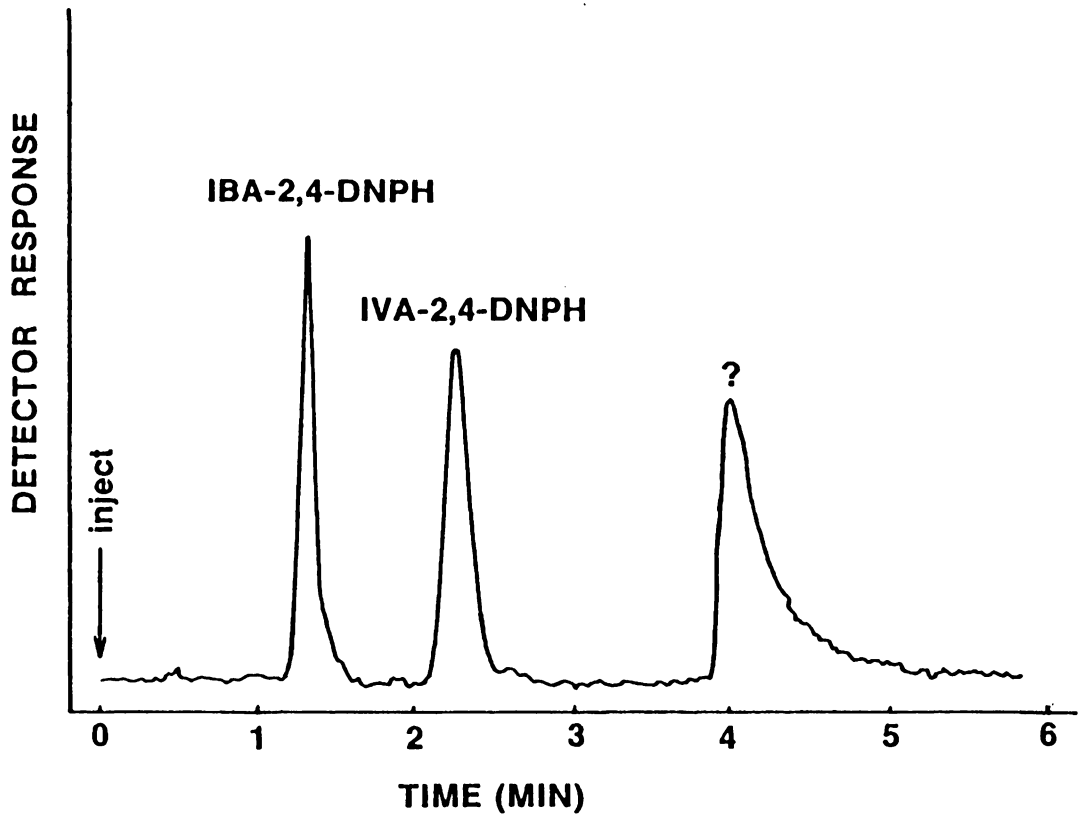

Figure 6 Typical flash exchange GC chromatogram of the 2,4-DNPHs in acetonitrile extract of the incubation mixtures

12% SP 1000 60/80 Cromosorb column (stainless steel, 5 ft x 1/8 in. O.D.) at 70°C. The flow rate of H_2 30 ml/min, N_2 20 ml/min and air 300 ml/min.

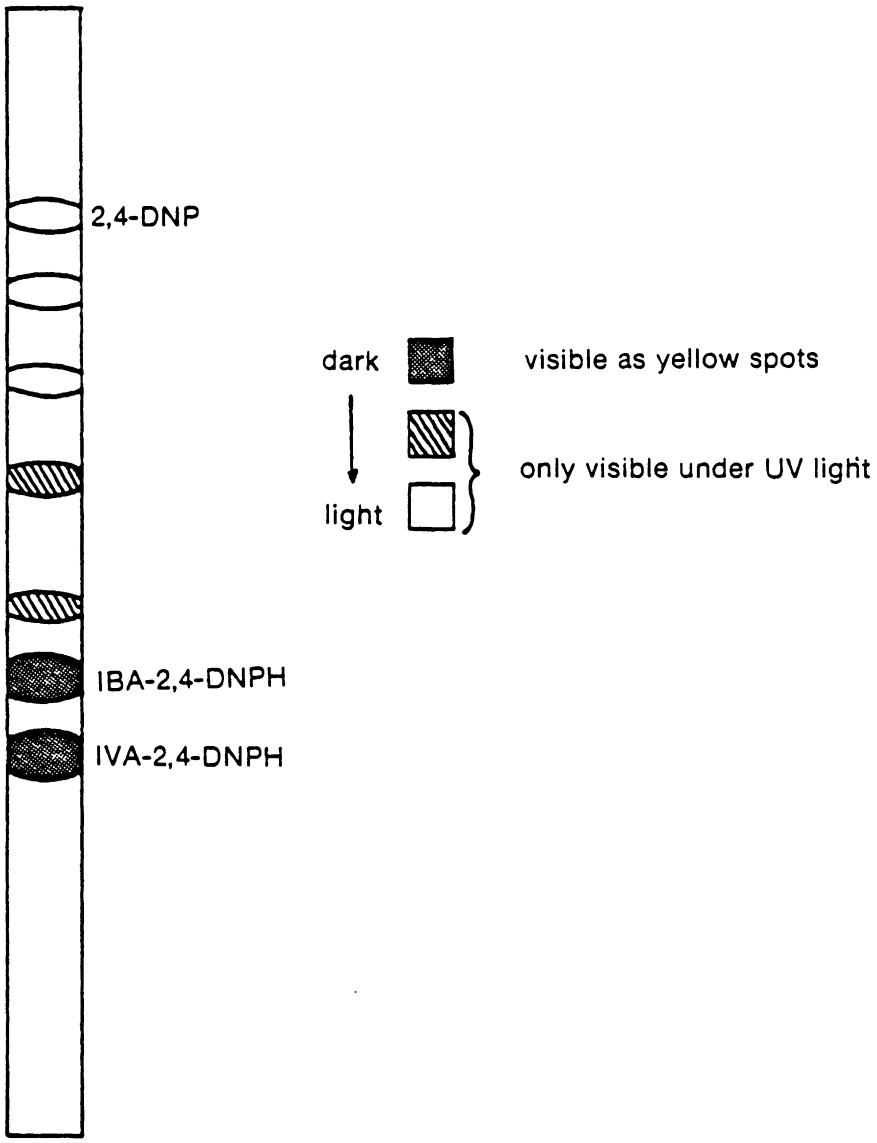

Figure 7 Typical TLC chromatogram of the 2,4 DNP and 2,4 DNPHs in acetonitrile extract of the incubation mixtures

Developed twice with methanol:water (4:1) on a Whatman KC18DF reversed phase plate.

c. Mass spectrometry.

Another possible method to confirm the identity of the hydrazones was mass spectrometry (MS). Attempts were made to perform this technique. The spots were scraped off the TLC plates and the 2,4-DNPHs dissolved in acetonitrile. The resulting acetonitrile extract was then placed into a probe, the acetonitrile evaporated and the 2,4-DNPHs analyzed by a mass spectrometer (Varian MAT 112 MS data system, Varian MAT, Bremen, W. Germany). The mass spectra of the authentic 2,4-DNPHs agreed with those reported by Stanley et al. (1975). However, the spectra of the unknown had many more peaks than the authentic 2,4-DNPHs with some peaks at mass numbers greater than the expected molecular ion peak. Presumably the extra peaks represent impurities in the solvent or foreign material extracted from the TLC plate. Since the extract had been concentrated about 30 fold for TLC analysis and then evaporated to dryness for doing the MS analysis, any impurities in the extracts were greatly concentrated. It was clear that considerable additional work would be required to obtain representative and interpretable mass spectra of the 2,4-DNPH samples.

Since the combination of HPLC, flash exchange GC and TLC data adequately identified the hydrazones, no further studies with MS were attempted.

B. EFFECT OF ENZYME AMOUNTS AND REACTANT CONCENTRATION ON THE OXIDATION OF DOPAMINE AND FORMATION OF IVA

1. Preliminary Experiments

a. Spectrophotometric method for optimizing the PPO catalyzed production of quinone.

It was reasoned that optimum rates of aldehyde production would occur when the production of quinone from dopamine was optimized. A spectrophotometric determination, using the increase in absorbancy (A) at 470 nm resulting from production of the red dopachrome quinone as a measure of quinone production (Palmer, 1963), was used in this study to optimize the production of quinone. Using this method, Palmer (1963) had shown that 3 mM dopamine saturated banana PPO but that concentration above 12 mM actually inhibited PPO, resulting in a decreased rate of production of quinone. The saturation of banana PPO at about 5 mM dopamine and a significant inhibition at higher concentration (20 mM) was confirmed in the present study (figure 8). A 5 mM concentration was therefore selected for further studies, as recommended by Palmer (1963).

The amount of PPO was then increased, holding the substrate at 5 mM. There was a significant increase in the initial rate of production of quinone over the range of 0.06 to 0.44 units/ml (figure 9). However, the rate of production leveled off quickly with time (presumably a

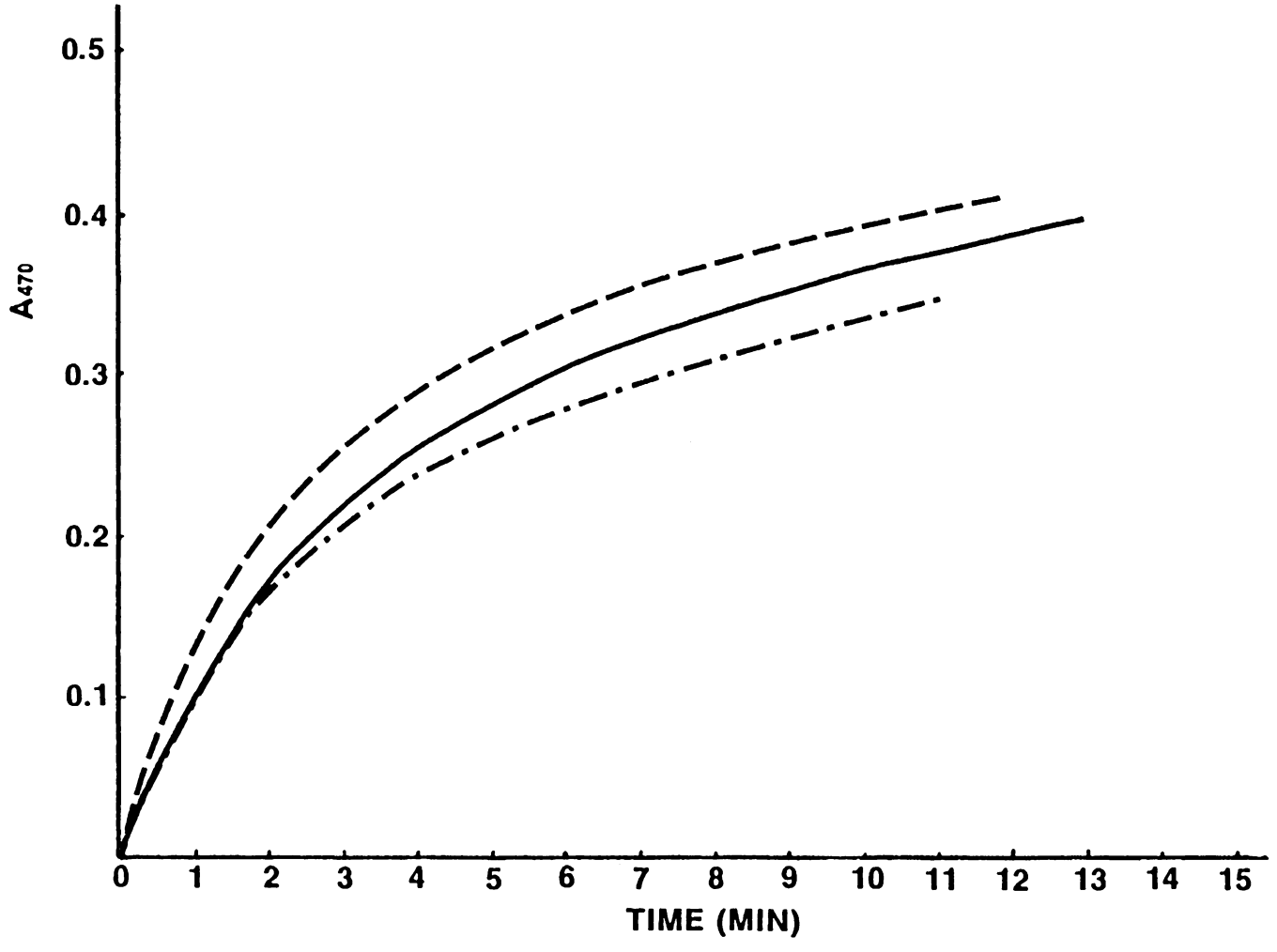

Figure 8 Effect of dopamine concentration on the PPO catalyzed oxidation of dopamine

Enzyme constant at 0.06 units/ml reaction mixture.
Variable dopamine concentration: ——— 2mM; — — — 5mM; — • — • 20mM.

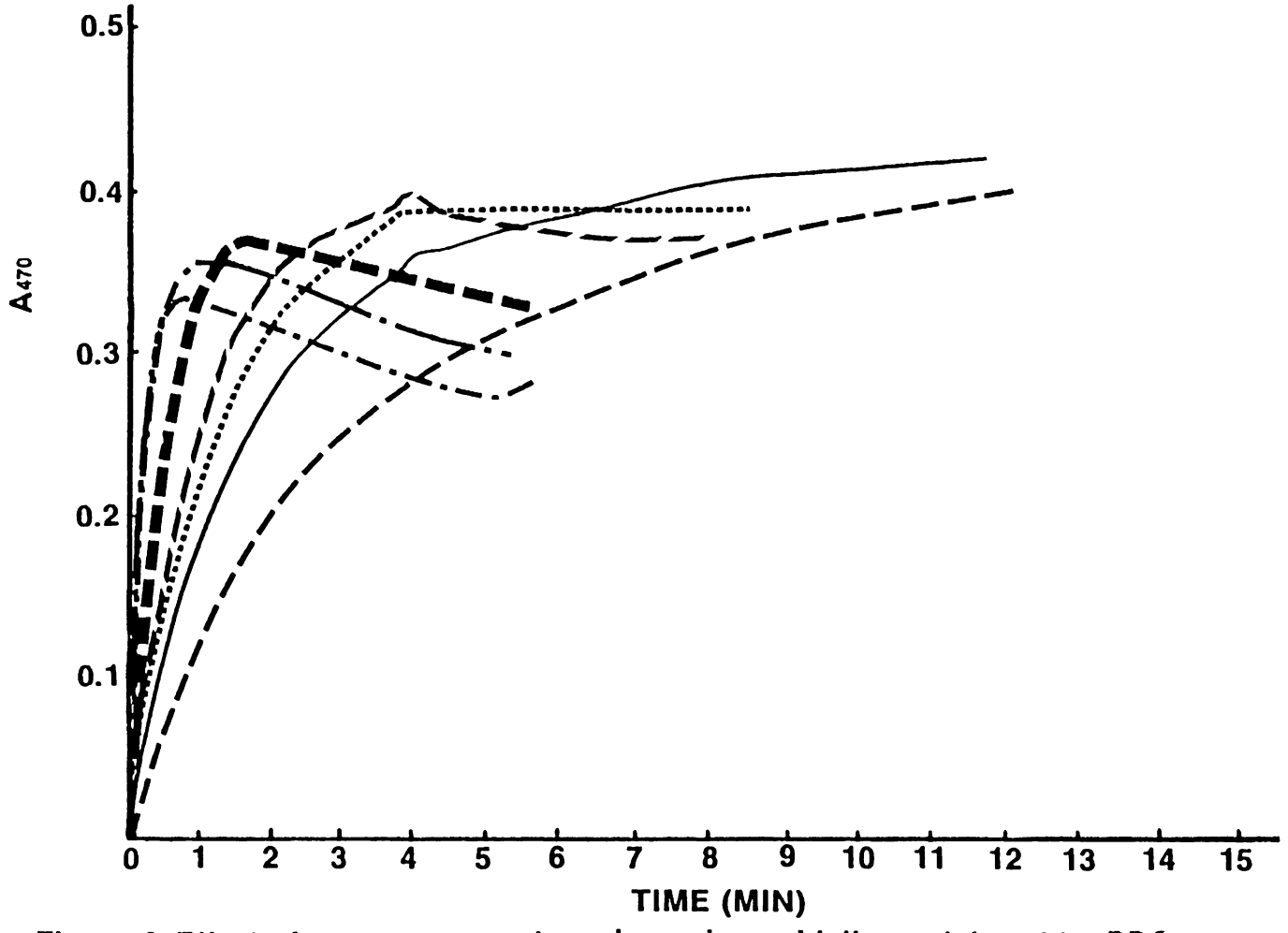

Figure 9 Effect of enzyme amount on dopamine oxidation catalyzed by PPO.

Dopamine constant at 5 mM.
Variable enzyme added: — — — 0.06 units/ml reaction mixture; ——— 0.09 units/ml;
····· 0.12 units/ml; — — — 0.15 units/ml; ■ ■ ■ 0.29 units/ml;
- ——— - 0.44 units/ml; — - — - — 0.58 units/ml.

consequence of the well known product inhibition). At high enzyme levels the quinone concentration peaked out and then dropped significantly presumably resulting from limited oxygen availability since oxygen was also a reactant in the oxidation of dopamine. The drop in quinone concentration might have also resulted from further reactions of the active quinones. The most prominent reaction of the quinones is polymerization to form black melanin pigments. At low levels of enzyme the concentration of quinone was continuing to increase slowly when terminated at 13 min. Considerably more experimentation would be required to explain the results of figure 9, in terms of the rate of formation of initial quinones, and of subsequent products through to the melanins (as outlined in figure 3). However, in terms of choosing conditions for optimizing aldehyde production, it seemed likely that use of enzyme levels which gave a slower, but extended increase in A at 470 nm (e.g. 0.06-0.09 units/ml in figure 9) would yield greater production of quinone and subsequently aldehyde, assuming that aldehyde production resulted from the reaction between quinone and amino acid.

b. Effect of leucine on the spectrophotometric determination of the reaction mixtures.

This experiment was designed to determine the effect of leucine on the reaction mixtures of section a above. Since the quinone subsequently reacted with amino acids to form aldehyde, it seemed likely that the A at 470 nm which represented quinone concentration would be lower with increasing concentrations of leucine. Experiments were run with constant amounts of dopamine (5 mM) and enzyme (0.08 units/ml reaction mixture), but increasing concentrations of leucine (0 to 50 mM), as shown in figure 10. In general, the addition of leucine to the mixtures did not change the shape of the curve significantly. The initial rates of reactions were relatively the same, slightly higher with higher concentrations of leucine and the curves leveled off at slightly higher absorbancies with increasing concentrations of leucine. It was observed during the course of these experiments that the incubation mixtures containing leucine tended to develop a yellowish cast. Presumably the higher absorbancies observed in the presence of leucine resulted from an overlap of the absorption spectra of the dopachrome quinone and the unidentified yellow intermediate at 470 nm. The results of Saijo and Takeo (1970b) indicated a similar overlap of the absorption spectra when phenylalanine was included in tea PPO-catechin reaction mixtures.

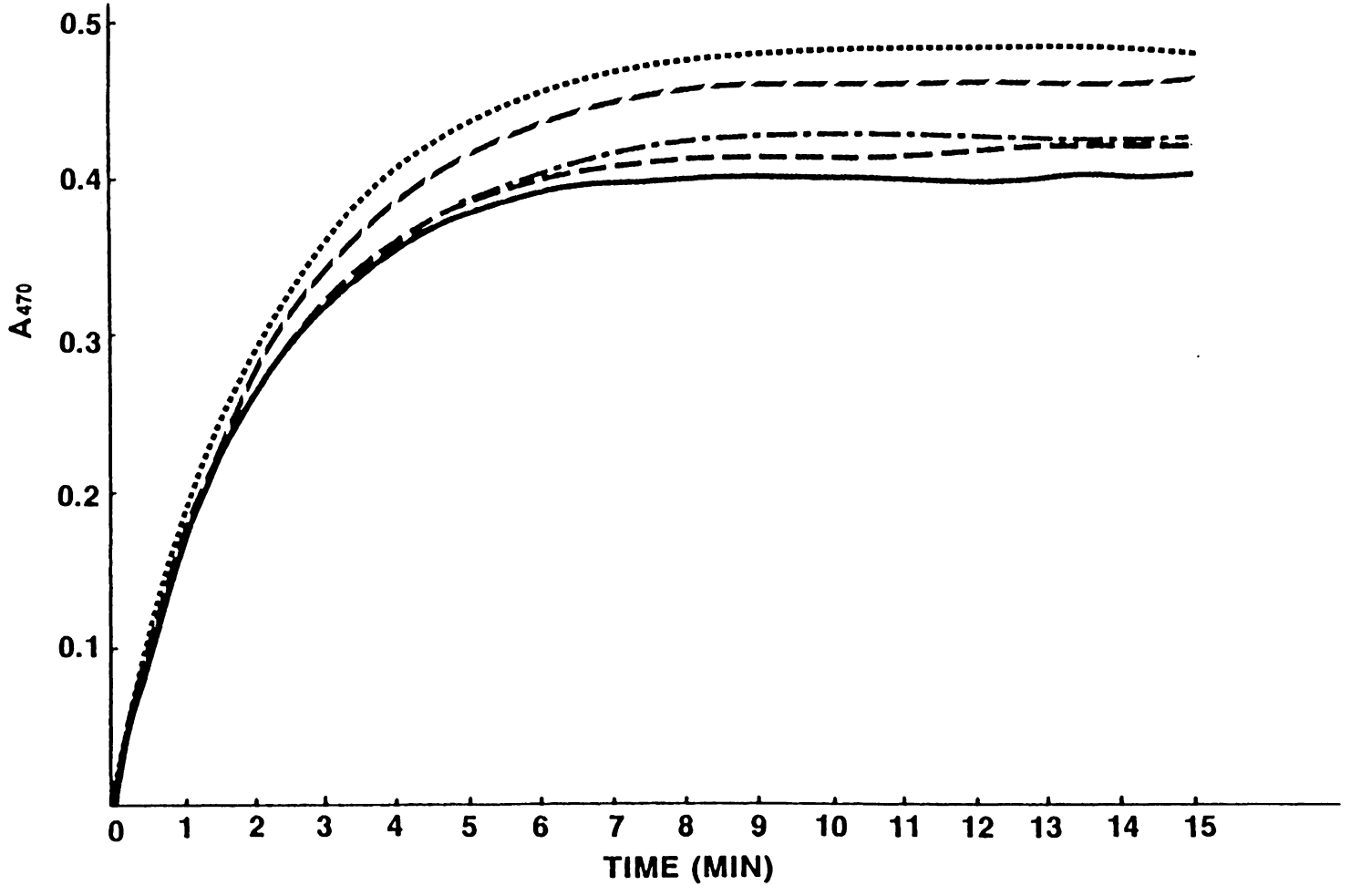

Figure 10 Effect of leucine concentration on the spectrophotometric determination of the incubation mixtures

Dopamine constant at 5 mM.
Enzyme constant at 0.08 units/ml reaction mixture.
Variable leucine concentration: —— 0 mM; — — 1 mM; —·— 5 mM; — — 25 mM; ····· 50 mM

c. Relationship between spectrophotometric determinations and actual aldehyde production.

Further experiments were designed to relate the spectrophotometric determinations developed above (sections a and b) with the actual production of aldehyde so as to predict the formation of aldehyde from the shape of the A curves at 470 nm vs time. Ten ml reaction mixtures were prepared in separatory funnels containing a constant amount of dopamine (5 mM) and leucine (50 mM), but with increasing amounts of enzyme. Aldehyde production was measured. Mixtures of the same compositions (total 2 ml) were also prepared in cuvettes and the A at 470 nm measured as in the preliminary experiments mentioned above (sections a and b). The mixtures were only incubated for 15 min, since the A had become essentially constant at this time, indicating that little additional quinone was being produced (figure 11), possibly because of product inhibition on the enzyme or because there was a balance between the production and the consumption of the quinone.

As indicated in the data for aldehyde production under figure 11, measurable IVA was formed in each of the reaction mixture tested. No simple relationship between IVA formed and the spectrophotometric curves could be seen, probably because at each time after the initial 3 min, the A at 470 nm is a sum of the absorption of the red quinones, the

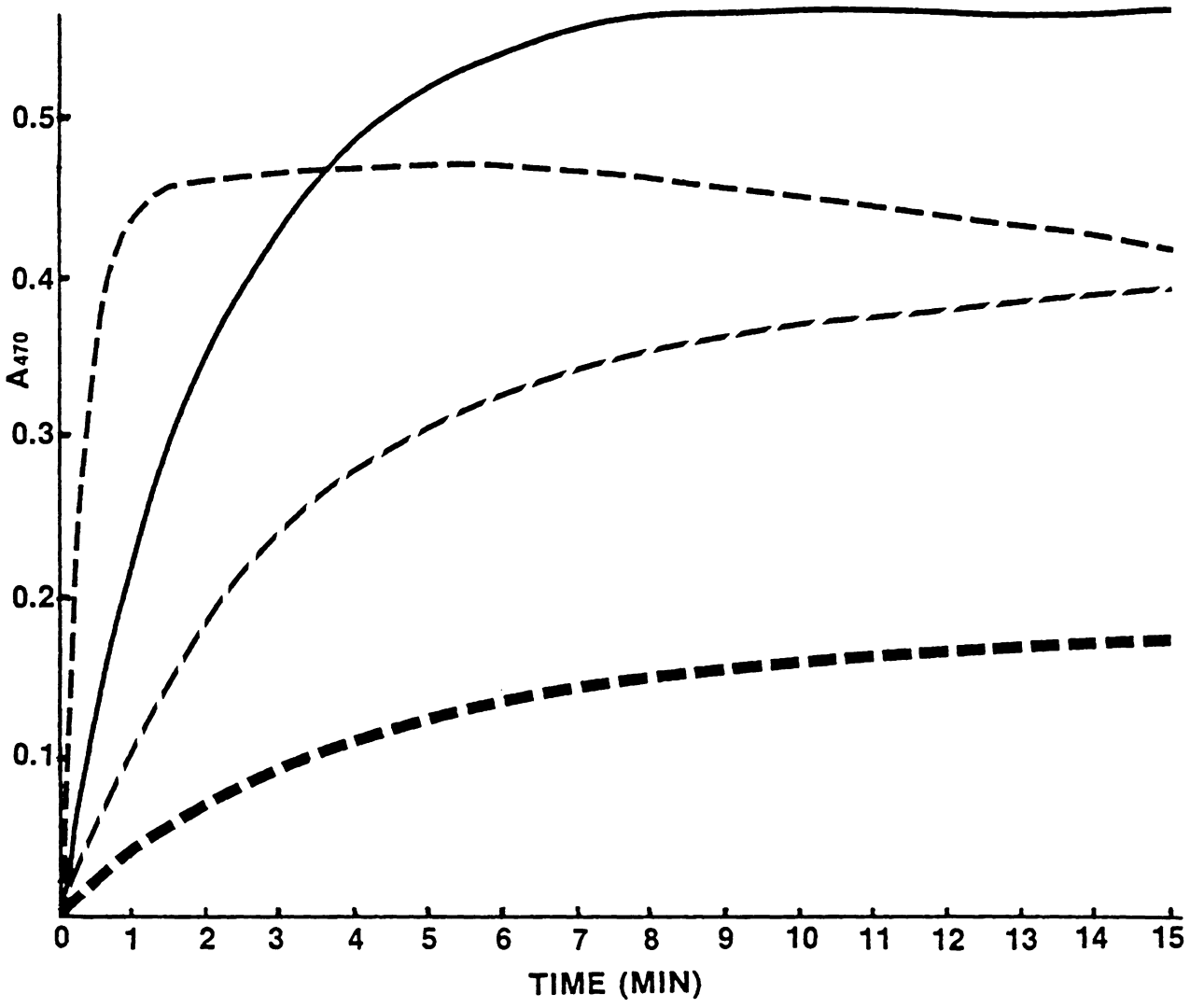

Figure 11 Relationship between absorbancy of reaction mixtures and time

Dopamine constant at 5 mM, leucine at 50 mM.
Enzyme (E) amount added and IVA (A) formed are:

No.	E (units/ml)	A (μmoles)
1	0.003	0.08
2	0.03	0.15
3	0.06	0.22
4	0.3	0.17

yellow intermediate compound and the black melanin pigments. However, in terms of predicting the amount of aldehyde formed, the data indicated that an enzyme level (0.06 units/ml) which gave a relatively slow but extended increase in A at 470 nm (figure 11, curve 3) yielded the largest amount of aldehyde (as hypothesized in section a above). At enzyme levels lower than 0.06 units/ml (figure 11, curve 1-2), aldehyde production increased with increasing initial rate and final A. At high enzyme levels which showed a sharp initial increase in A followed by a dropping off of A (figure 11, curve 4), less aldehyde was formed.

2. Analyses of IVA Production as Influenced by Factorial Combinations of Dopamine, Leucine and Enzyme

Based on the preliminary data of section B.1., more detailed analyses of IVA production were conducted on factorial combinations of three levels each of dopamine, leucine and enzyme. The levels of dopamine tested were 0.05, 0.5 and 5 mM. The highest concentration was the saturating substrate level for PPO (Palmer, 1963 and preliminary data, figure 8) and 0.5 mM was reported to be optimum for production of phenylacetaldehyde from phenylalanine by tea enzymes (Saijo and Takeo, 1970b). Leucine was tested at 25, 50 and 75 mM based on preliminary experiments which indicated that aldehyde production

increased with leucine concentration up to 50 mM (figure 10) and that readily measurable amounts of aldehyde were produced at 50 mM. Enzyme levels of 0.03, 0.07 and 0.36 units/ml were selected on the basis of the preliminary data. The two lower levels represented the region which showed the slow but extended increase in A; the highest represented an enzyme level which yielded a rapid initial increase in A at 470 nm, followed by a drop in A (figure 9).

Table 2 shows the µmoles of IVA produced with each combination of dopamine, leucine and enzyme. Under the chosen experimental conditions, analysis of variance (ANOVA) (table 3) indicated significant differences ($P \leq 0.001$) in IVA production among dopamine and leucine concentrations. Duncan's Multiple Range Test at alpha level of .05 (table 4) shows that the largest amount of aldehyde was formed at the intermediate dopamine concentration (0.5 mM), but that production of aldehyde increased as leucine concentration was increased.

The ANOVA indicated no significant difference in IVA production among enzyme levels. However, significant interaction ($P \leq 0.01$) between dopamine and leucine was also shown via ANOVA (table 3). Therefore, comparison between the enzyme levels should be performed by combining the 3 levels of leucine at each level of dopamine and the 3 levels

Table 2 IVA formed in reaction mixtures (μmoles)

	E_1 0.03 units/ml			E_2 0.07 units/ml			E_3 0.36 units/ml		
	L_1 25mM	L_2 50mM	L_3 75mM	L_1 25mM	L_2 50mM	L_3 75mM	L_1 25mM	L_2 50mM	L_3 75mM
D_1 0.05mM	0.02	0.09	0.27	0.03	0.12	0.30	0.01	0.08	0.18
D_2 0.5mM	0.06	0.22	0.67	0.10	0.38	0.91	0.09	0.32	0.56
D_3 5mM	0.04	0.12	0.37	0.03	0.14	0.48	0.05	0.19	0.55

E = Enzyme
L = Leucine
D = Dopamine

Table 3 Analysis of variance of the effect of dopamine, leucine and enzyme levels and of their interactions on the production of IVA

Source of variation	Degrees of freedom	Mean Square
Dopamine	2	0.1363 **
Leucine	2	0.4296 **
Enzyme	2	0.0113 NS
Dopamine x Leucine	4	0.0314 *
Dopamine x Enzyme	4	0.0085 NS
Leucine x Enzyme	4	0.0043 NS
Error	8	0.0037 NS

*$P \leq 0.01$

**$P \leq 0.001$

NS $P > 0.05$

Table 4 Duncan's Multiple Range Test of the IVA means

Grouping	Mean(μmoles)	Dopamine
A	0.37	D_2
B	0.22	D_3
C	0.12	D_1

Grouping	Mean(μmoles)	Leucine
D	0.48	L_3
E	0.19	L_2
F	0.05	L_1

Means with the same letter are not significantly different at alpha level = .05

of dopamine at each level of leucine. Based on this analysis (table 5) intermediate enzyme levels produce significantly higher IVA ($P \leq 0.05$) at intermediate dopamine (0.5 mM) or at highest leucine (75 mM) levels.

Development of a quick assay to predict aldehyde production was again attempted by relating the A of the reaction mixtures and the aldehyde formed. The A vs time curves of 7 of the factorial combinations from table 2 are shown in figure 12. Assay conditions and IVA production for each combination is listed below figure 12. The A vs time curves are similar to the curves in figure 11, except that the reaction time was extended to 2 hours. A complex relationship was again observed between the spectrophotometric readings of the reaction mixtures and the aldehyde produced in the mixtures. Increasing enzyme levels are shown in figure 12 as curves 2, 3 and 7. Similar results as in figure 11 were found here. The largest amount of aldehyde was produced at intermediate enzyme level (0.07 units/ml); at this level the curve showed a slow but extended increase in A. Increasing levels of dopamine are shown in figure 12 as curves 1, 3 and 6. A greater amount of aldehyde was produced at the intermediate 0.5mM dopamine level, a level lower than the saturating dopamine concentration for banana PPO of 5 mM (Palmer, 1963). This

Table 5 Means of IVA formed (μmoles) and grouping of enzyme levels according to Duncan's Multiple Range Test

	Means of IVA formed (μmoles)					
	D_1	D_2	D_3	L_1	L_2	L_3
E_1	0.13^a	0.32^b	0.18^d	0.04^e	0.15^f	0.44^g
E_2	0.15^a	0.46^c	0.22^d	0.06^e	0.21^f	0.56^h
E_3	0.09^a	0.32^b	0.26^d	0.05^e	0.20^f	0.43^g

Means with the same letter (among dopamine or among leucine levels) are not significantly different.

Alpha level = .05

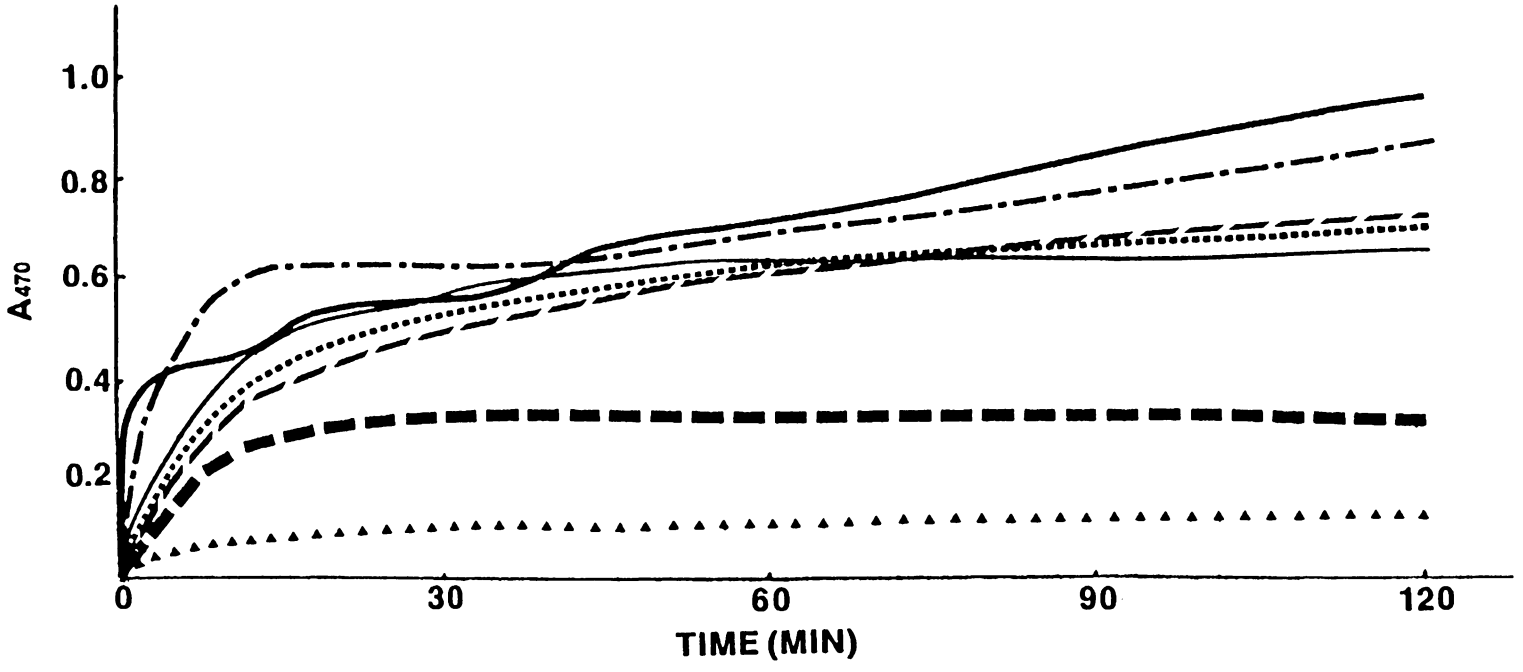

Figure 12 Relationship between absorbancy and time in selected reaction mixtures of the factorial study

Dopamine (D), leucine (L) concentrations and enzyme (E) amount added, and IVA (A) formed are:

No.	D (mM)	L (mM)	E (units/ml)	A (μmoles)
1	0.05	75	0.07	0.30
2	0.5	75	0.03	0.67
3	0.5	75	0.07	0.91
4	0.5	50	0.07	0.38
5	0.5	25	0.07	0.10
6	5.0	75	0.07	0.48
7	0.5	75	0.36	0.56

seemed to be in agreement with the hypothesis that a slow but extended increase in A will produce greater amounts of aldehyde. At highest dopamine and enzyme levels the rapid formation of quinones supported the production of black melanins, as reflected by the blackening of the reaction mixtures and consequently by the high absorbancies of curves 6 and 7 (figure 12). Curves 5, 4 and 3 in figure 12 shows increasing levels of leucine. Although there was only a slight increase in the initial rate and the final A with increasing leucine levels, its repeatability within the factorial experiment assured that there was a direct correlation between the curves and the aldehyde production at increasing levels of leucine. Further experimentation will be nescessary, however, to explain this complex relationship. A more sophisticated spectrophotometric analysis using a computerized spectrophotometer could be useful for revealing the actual mechanism and kinetics of the reaction by simultaneously measuring the rate of production and accumulation of the various colored intermediates.

C. FORMATION OF IBA AND IVA IN REACTION SYSTEMS CONTAINING BANANA ENZYMES

The optimum conditions for aldehyde production found in the factorial experiment described above were used in this part of the study, i.e. the intermediate dopamine concentration (0.5 mM), highest leucine concentration (75 mM), intermediate enzyme level (0.07 units PPO activity/ml final reaction mixture) and 2 hour reaction time.

In reaction systems containing dopamine, enzyme extract and amino acid, IVA was formed when ℓ-leucine was added and IBA when ℓ-valine was added (table 6, No. 1-3). Incubation without the enzyme extract (table 6, No. 4) or with enzymes inactivated by heat (table 6, No. 5) produced much smaller amounts of IVA, suggesting that the reaction was enzymatically catalyzed and that the small amounts of IVA can be produced by the non-enzymatic autoxidation of dopamine. No aldehyde was found in a dopamine free incubation mixture (table 6, No. 6), indicating that dopamine was required for the reaction to take place and that no significant amounts of aldehyde or dopamine was present in the enzyme extract. The requirement for dopamine and enzyme suggested that PPO was the enzyme involved in the reaction, since banana PPO has a high affinity for this o-diphenol (Palmer, 1963) which is the only substrate for enzymatic browning found in bananas (Griffith, 1959). When

Table 6 The production of isobutyraldehyde (IBA) and isovaleraldehyde (IVA) in different incubation mixtures

No.	Incubation mixture composition	Aldehyde found	Amount of aldehyde formed (μmoles)
1	Leucine + dopamine + enzyme extract	IVA	0.66
2	Valine + dopamine + enzyme extract	IBA	0.49
3	Leucine + valine + dopamine + enzyme extract	IVA	0.26
		IBA	0.29
4	Leucine + dopamine	IVA	0.01
5	Leucine + dopamine + enzyme extract (inactivated)	IVA	0.02
6	Leucine + enzyme extract	None	0
7	Dopamine + enzyme extract	None	0
8	Leucine + dopamine + enzyme extract + H_2O_2	IVA	1.15
9	Leucine + dopamine + H_2O_2	IVA	0.01
10	Leucine + enzyme extract + H_2O_2	None	0
11	Dopamine + enzyme extract + H_2O_2	None	0

Reaction systems 1-2, 4-11: 75 mM amino acid, 0.5 mM dopamine HCl, 0.6 mM H_2O_2 and 0.07 units/ml enzyme, 0.2 M phosphate buffer (pH 7.0) in a 10 ml total volume. In systems 4, 6-11, components were omitted as noted.

Reaction system 3: 37.5 mM of each amino acid, 0.5mM dopamine HCl and 0.07 units/ml enzyme, 0.2M phosphate buffer (pH 7.0) in a 10 ml total volume.

Reaction system 5: Same as 1, except that the enzyme extract had been heated in boiling water for 5 minutes.

All incubations for 2 hr at 25°C.

no amino acids were added to the reaction mixture (table 6, No. 7), no aldehydes were formed. This indicated that the aldehydes were being formed from the amino acids as hypothesized and that the enzyme extract did not contain significant amounts of amino acids that could interfere with the results.

Independent assays confirmed the presence of POD in the enzyme extract. More IVA was produced from leucine when H_2O_2 was included in the incubation mixture (table 6, No. 8). No POD activity was observed in an enzyme assay mixture unless H_2O_2 was added, indicating that the enzyme extract did not contain significant amounts of H_2O_2. However, the addition of H_2O_2 had no effect on mixtures which were free of dopamine, enzyme extract or amino acid (table 6, No. 9-11), suggesting that POD catalyzes formation of aldehyde via the same mechanism as PPO. The POD unit is thus expressed in terms of equivalent PPO units calculated from the μmoles of dopamine oxidized per minute. Addition of H_2O_2 to a PPO assay mixture increased the PPO units by 1.1 fold.

Laccase and cytochrome c oxidase could also catalyze oxidation of dopamine directly or indirectly (Palmer, 1963). In a preliminary experiment, oxidized cytochrome c was found to be able to oxidize dopamine. However, according to the

appropriate assays these enzymes were not found in significant amounts in the extract.

In the present study it is thus confirmed that aldehyde could be formed from amino acid in a reaction with quinone produced from PPO and POD catalyzed oxidation of dopamine. However, Tressl (1970c,d) proposed that the formation of aldehyde from amino acid was via enzymic transamination and decarboxylation. Attempts were therefore made to produce aldehyde via Tressl's mechanism. In preliminary experiments using headspace analysis, Pickenhagen and Palmer, 1971, and Luh and Palmer, 1973 were able to produce aldehyde in model systems containing amino acid, enzyme extract, α-keto glutaric acid (α-KGA) and pyridoxal phosphate. Using the experimental method outlined in this present study, attempts were made to produce aldehyde in the α-KGA model system. However, based on earlier studies (Ichihara and Koyama, 1966) relatively large amounts of α-KGA (about 50 μmoles) would be required in this reaction, while the expected yield of aldehyde would only be 0.5 to 1 μmole. In control runs containing authentic IVA, α-KGA, 2,4-DNP and water, it proved difficult to recover the IVA (as its 2,4-DNPH) in the presence of this 50 to 100 fold excess of α-KGA which also reacted to form 2,4-DNPH. Since considerably more work needs to be done to obtain reliable results in the α-KGA model system, no further studies were attempted.

V. DISCUSSION

From the spectrophotometric analysis and aldehyde production of the factorial study, it seemed that rapid production of quinone in large amounts either by adding high levels of dopamine or enzyme, supported formation of melanins and depressed formation of aldehyde. Similar results were obtained by Saijo and Takeo (1970b) in model systems containing tea enzymes, (-)-epicatechin and phenylalanine. They suggested that the two reactions, polymerization of quinones to form melanins and reaction between quinone and amino acid to produce aldehyde (figure 4), are competitive. In systems containing higher concentrations of o-quinone derived from epicatechin, most o-quinone polymerized and the production of aldehyde was depressed. Hence, they suggested that the polymerization rate of o-quinone is higher than the rate of the reaction between o-quinone and amino acid.

In figure 4 three possible pathways to produce aldehyde are proposed: the Strecker degradation (Pickenhagen and Palmer, 1971), amino acid oxidation (Trautner and Roberts, 1950) and transamination followed by decarboxylation (Tressl

et al., 1970c). In the present study, it was demonstrated that leucine and valine presumably react with the quinone produced from the PPO and POD catalyzed oxidation of dopamine, followed by either a Strecker degradation (figure 4, pathway A) or an amino acid oxidation mechanism (figure 4, pathway B) to produce IVA and IBA, respectively. Which quinone actually reacts with the amino acid is not known. Any quinone produced after 2,3-dihydroindole-5,6-quinone in the mechanism of figure 3 as proposed by Palmer (1963) or even small polymers thereof could possibly react with the amino acid. This finding, however, does not necessarily rule out the possibility that α-keto acid and aldehyde could be produced via transamination-decarboxylation (figure 4, pathway C) as proposed by Tressl et al. (1970c). They demonstrated the incorporation of ^{14}C-label from labeled amino acids into the corresponding α-keto acids and aldehydes in banana tissue slices. Since banana tissue slices were used, the ^{14}C-label could have been incorporated into the corresponding α-keto acids and aldehydes via pathway B and/or C of figure 4.

From the evidence to date, it is clear that aldehydes could be formed in bananas from the corresponding amino acids via any or all of the pathways of figure 4. Additional experimentation with appropriately labeled amino

acids and possibly with specific inhibitors of the particular pathway will be required to establish which pathway(s) actually exists or predominates in bananas. The use of ripening banana slices (Palmer and McGlasson, 1969) would permit introduction of suspected precursors and/or inhibitors at the appropriate ripening stage.

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THE FORMATION OF ISOVALERALDEHYDE FROM ℓ-LEUCINE AND ISOBUTYRALDEHYDE FROM ℓ-VALINE CATALYZED BY BANANA POLYPHENOLOXIDASE AND PEROXIDASE

by

Imas Artati Setiabasa

(ABSTRACT)

Possible mechanisms for the formation of isovaleraldehyde from ℓ-leucine and isobutyraldehyde from ℓ-valine in bananas are described. These aldehydes are key intermediates for the development of the branched-chain alcohols and esters, which are prominent components of banana flavor volatiles. When amino acids were incubated with dopamine and banana enzyme extract, these aldehydes were formed. Greater amounts of aldehydes were produced when hydrogen peroxide was included in the reaction mixture. It is proposed that the reaction mechanism involves oxidation of dopamine to o-quinones catalyzed by polyphenoloxidase and peroxidase, followed by the non-enzymic reaction of the quinone with the amino acid to form the corresponding aldehyde. At levels of leucine,

polyphenoloxidase (PPO) and dopamine tested, aldehyde production increased with leucine concentration (up to 75 mM) and tended to peak at relatively low levels of PPO (0.1 units/ml) and dopamine (0.5 mM).