Reactions Involved in Formation of a Pink Pigment in Onion Purees

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The reactions involving and the conditions affecting the formation of pink pigments from precursors isolated from purees of white onion were investigated. A colorless ether-soluble precursor reacts with certain amino acids in onions to form a second colorless compound insoluble in ether. The latter compound then reacts with formaldehyde or naturally occurring carbonyls to form the pigment. The final pigment-forming reaction proceeds at about seven times the rate of the first reaction and has an optimum near pH 4.8. Pigment formation is inhibited by the sulfhydryl group of cysteine. Studies with isotopes indicate that amino acids and formaldehyde are incorporated in the pigment molecule. The rate and extent of pigment formation and the color of pigment formed were affected by the kinds of amino acids and carbonyls.

During the commercial dehydration of white onion flakes, occasionally pink pigments develop which lower the quality of the dried product (Lukes, 1959). Some of the horticultural and chemical factors affecting the formation of these pigments in onion purees were reported by Yamaguchi *et al.* in 1965. These pigments also form in onion purees and have been the object of studies by Joslyn and Peterson (1958, 1960). The purified pigment was studied in some detail, and the coloration was enhanced by addition of small quantities of formaldehyde. They concluded that probably there were no enzymes involved, but that there were two reactions leading to the formation of the pigment. A natural carbonyl was considered to be one of the final reactants. However, Lukes (1959) demonstrated that a preliminary enzymic reaction was necessary to form an ether-soluble compound, which he named color developer. He found that the pigment formed when the color developer was incubated with certain amino acids. From these reports, the precursors and the reactions are not entirely understood. This paper reports on studies of the colorless precursors and the reactions involved in the pigment formation.

Materials and Methods

All extracts were prepared from the juice of mature Southport White Globe onions, *Allium cepa.* L., obtained with a juicing attachment on a Hobart food chopper.

The time course of pigment formation in mixtures of various extracts and solutions was followed by measuring changes in absorbance of the solutions at 520 mμ, the wavelength of maximum absorption by the pigment. Absorbance was measured with a Bausch and Lomb Spectronic 20 spectrophotometer. Except during actual measurements, the reaction mixtures were kept in a constant temperature water bath maintained within 1° C. of the desired temperature. The absorbance of highly colored solutions was calculated from measurements of diluted samples. Absorption spectra of purified pigment preparations were determined in a Beckman Model DU spectrophotometer.

Radioactivity of ^{14}C-labeled compounds and reaction mixtures was determined with a Nuclear Chicago gas flow counter. Liquid samples containing nonvolatile ^{14}C constituents were evaporated in planchettes prior to measurement. Samples containing ^{14}C-labeled formaldehyde were treated with $KMnO_4$ and NaOH to prevent loss by volatilization during evaporation in the planchettes. The $^{14}CO_2$ released from glycine-1-^{14}C was flushed from the reaction tube with a stream of humidified air which had been passed through a bubble tube containing dilute H_2SO_4 and thence through a bubble tube containing NaOH. The trapped $^{14}CO_2$ was precipitated as the barium salt and collected on a small filter paper. The precipitate on the paper was then transferred to a planchette, dried, and the activity counted.

Experimental and Discussion

Colorless Precursors and Pigment-Forming Reactions. Color developer was obtained by extracting onion juice with ether as described by Lukes in 1959. The ether was evaporated in an air stream and the residue was extracted with water. The presence of the color developer in this extract was confirmed by incubating a small amount in a 1.0% aqueous solution of glycine containing 10 p.p.m. of formaldehyde. After 24 hours at 40° C., the mixture was a deep red color. Both color developer extract and glycine were necessary for pigment formation. Slight pinking occurred in the absence of formaldehyde, indicating the presence of what might be a naturally occurring carbonyl in low concentration.

Since the work of Joslyn and Peterson in 1960 indicated that most of the colorless precursor had been formed in the first 2 hours of incubation at 50° C., the following experiment was performed to determine if this was true also for the color developer.

Twenty-milliliter portions of fresh onion juice were subjected to different temperatures and then extracted with several 20-ml. portions of ether. The ether extracts were evaporated, and 9 ml. of a 1% glycine solu-

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tion and 1 ml. of a 100-p.p.m. formaldehyde solution were added to each residue. Then the mixtures were incubated at 40° C. for 24 hours for color development. The amounts of pigment formed are shown in Table I. The largest amount of color developer was extracted with the ether from the fresh cold juice. Less than half as much was extracted from juice heated for only 10 minutes at 50° C. Color developer was almost totally absent from juice heated to 100° C. for 10 minutes. Evidently, the color developer either was destroyed or was converted to another colorless precursor insoluble in ether by standing or by heating. The latter was shown to occur in the following experiments.

A sample of fresh onion juice was filtered until it was sufficiently clear so that the time course of pigment formation could be followed by changes in absorbance. Formaldehyde was added (10-p.p.m. final concentration) to one portion of the juice and the mixture incubated at 40° C. Pigment formation in this sample began slowly and the rate of formation increased gradually during the 6-hour period the reaction was followed (Figure 1). Another portion of the juice was incubated without formaldehyde at 50° C. for 3 hours, during which time very little pigment formed. This sample then was cooled to 40° C. and formaldehyde was added as in the first sample. The rate of pigmentation after addition of formaldehyde was very rapid, followed by a decline in rate. To explain this observation, it can be hypothesized that without added formaldehyde, another colorless precursor (pigment precursor) was formed during incubation of the juice; and upon addition of formaldehyde, the mixture reacted very rapidly with the pigment precursor to form the pigment.

This evidence together with Lukes' findings made it plausible to propose the following reactions as leading to pigment formation:

$$\text{Unknown precursor} \xrightarrow[\text{(very fast)}]{\text{enzyme reaction}} \text{color developer (colorless, ether-soluble)} \quad (1)$$

$$\text{Color developer} + \text{amino acid} \xrightarrow[\text{(slow)}]{} \text{pigment precursor (colorless, ether-insoluble)} \quad (2)$$

$$\text{Pigment precursor} + \text{formaldehyde} \xrightarrow[\text{(fast)}]{} \text{pink pigment} \quad (3)$$

Studies of Reactions 2 and 3 were made with color developer extracts, pure amino acids, and formaldehyde. The optimum concentrations of the three components and the best conditions for pigment formation were found through trial and error. It was necessary to determine the optimum amount of color developer extract for each new preparation. Below the optimum concentration, the amount of pigment formed was proportional to the quantity of color developer used. Pigment formation was inhibited with concentrations of $3 \times 10^{-4}M$ (9 p.p.m.) formaldehyde. Glycine was the standard amino acid used, with an optimum concentration near $0.1M$.

The formation of what the authors call pigment precursor (Reaction 2) was confirmed by following the time course of pigment formation at 50° C. in mixtures of color developer and glycine subjected to different incubation temperatures and times before addition of formaldehyde. When the three components were incubated together at 50° C., there was a delay of about 30 minutes before much pigment was formed (Figure 2). After this the rate of pigmentation was constant. When the color developer–glycine mixture was heated to 100° C. for 2 minutes before formaldehyde at 50° C. was added, the initial rate was very rapid. However, after 30 minutes the rate of pigment formation approached that of the sample which had not been given a pretreatment. Pretreatments up to 30 minutes at 100° C. resulted in increasing initial rates of pigment formation, but periods longer than 10 minutes did not give appreciable increases. The initial rapid rate of pigment formation followed zero-order kinetics, but the reaction became more complex as the concentrations of pigment precursor and formaldehyde became limiting.

Color Developer, Properties, and Methods of Extraction. To minimize the conversion of color developer to pigment precursor, the onion bulbs were cooled to

Table I. Effect of Temperature and Duration of Incubation on Ether-Soluble Color Developer

Treatment before Ether Extraction		Absorbance at 520 mμ[a]
Temp., ° C.	Time, min.	
0	0	4.10
0	720	3.70
50	10	1.70
100	4	0.26
100	10	0.14

[a] Pigment formed following treatment by incubating mixture at 40° C. for 24 hours. Mixture contained 10 ml. ether extract of onion juice, 9 ml. glycine, and 1 ml. of 100-p.p.m. formaldehyde.

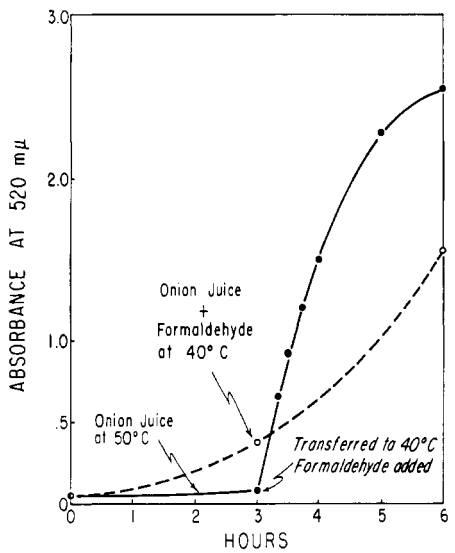

Figure 1. Effect of incubation on pigment formation in onion juice at 50° C. prior to addition of formaldehyde

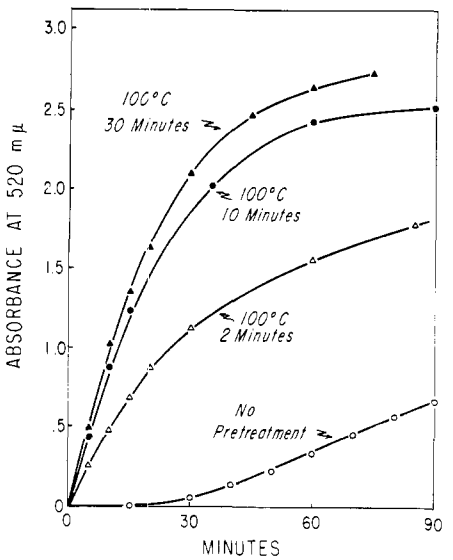

Figure 2. Effect of duration of pretreatment of color developer and glycine mixture at 100° C. on subsequent color formation with formaldehyde at 50° C.

0° C. The juice was expressed rapidly and then filtered through cheesecloth to remove most of the pulp. The juice (about 1 liter per batch) was extracted twice with equal volumes of cold ethyl ether in a 3-liter separatory funnel. It was necessary to centrifuge the emulsion to separate the ether phase. The combined extracts were evaporated by directing a stream of air into the container at room temperature. The residue was extracted with 100 ml. of distilled water, which then was filtered to remove the gummy residue. The color developer solution had a distinct onion odor and tended to become cloudy. However, the color developer was fairly stable, and solutions stored at room temperature for several weeks maintained their capacity to form pigment.

Color developer also was prepared by salting out a 1 to 1 mixture of cold fresh onion juice and acetone. When the concentration of NaCl reached a critical level, a yellow, acetone-rich phase separated from a saline aqueous phase. The acetone layer was transferred to another separatory funnel, diluted 1 to 1 with water, and again salted out to concentrate further and purify the color developer extracts.

Attempts were made to purify the color developer by paper chromatography, using the following solvents: acetone–water (8 to 2, v./v.), 1-butanol–acetic acid–water (4:1:5, v./v., upper phase), methanol–1-butanol–benzene–water (2:1:1:1, v./v.), water saturated with *tert*-amyl alcohol, 2-propanol–ammonia–water (10:1:1, v./v.), and petroleum ether saturated with methanol.

The color developer was located at about an R_f of 0.90 by formation of the pigment in the presence of glycine and formaldehyde in all solvent systems except petroleum ether saturated with methanol. In the latter solvent the tests were positive at the origin. A compound giving a red-purple color with Ehrlich's reagent coincided with the color developer. Ehrlich's reagent, 5% *p*-dimethylamino benzaldehyde in 18*N* H_2SO_4, is used to detect pyrroles. The color developer also gave a weak positive test for a pyrrole nucleus with 0.005*M* $FeCl_3$ in 35% perchloric acid (Salkowski's reagent). Thiosulfinates (Fujiwara *et al.*, 1955) also were detected at the same R_f, in addition to a compound which absorbed ultraviolet radiation (257 mμ). The presence of thiosulfinates with the color developer was believed to be coincidental, since synthetic dipropylthiosulfinate did not react with Ehrlich's reagent, absorb ultraviolet radiation, or form pigment with glycine and formaldehyde. Dipropylthiosulfinate was prepared by the method described by Fujiwara *et al.* in 1955. The compound in color developer extracts which gave a positive test with Ehrlich's reagent always was associated with color developer activity, so this test was used as a semiquantitative assay of the color developer.

The absorption spectra of the color developer extract before and after being heated in water solution are shown in Figure 3. The absorption maximum at 250 mμ was enhanced by heating and a shoulder was formed at about 340 mμ. When crystalline glycine was added to the cooled color developer, there was no change in the absorption spectrum. When the color developer–glycine solution was heated, the absorption in the ultraviolet region was enhanced, with another shoulder evident at 290 mμ. Absorption in the ultraviolet region changed very little after incubation of the mixture with formaldehyde, but there was a new absorption maximum at 520 mμ, due to the presence of the pigment.

Pigment formed in onion purees had an intense absorption in the ultraviolet region (Joslyn and Peterson, 1958; Yamaguchi *et al.*, 1965). The color developer–glycine-formaldehyde pigment had much less ultraviolet absorption, and the ratio of absorption at 250 mμ to absorption at 520 mμ decreased when the pigment was purified by absorption techniques (Joslyn and Peterson, 1958). Part of the ultraviolet absorption probably was due to unreacted color developer or to compounds not related to the pigment. The high ultraviolet absorption by pigment formed in onion puree

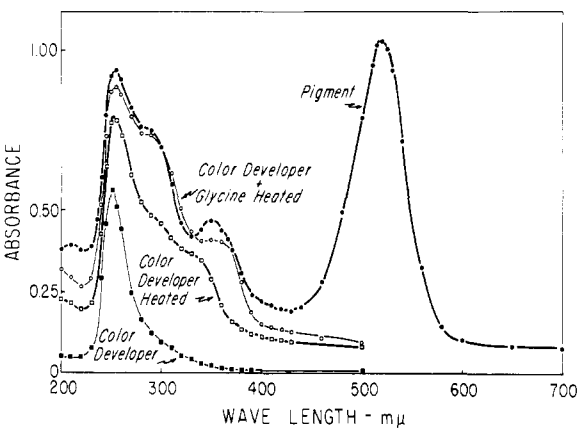

Figure 3. Absorption spectra of (■) color developer, (□) color developer heated, (○) color developer with glycine heated (pigment precursor), and (●) pigment formed from mixture of color developer, glycine (0.1*M*) and formaldehyde ($3 \times 10^{-4}M$)

probably resulted from incorporation of amino acids, which absorb ultraviolet radiation.

Factors Affecting Pigment Formation. TEMPERATURE. The temperature coefficients (Q_{10}) of Reactions 2 and 3 were calculated from the slopes of the straight-line portions of the time course curves in Figure 4. The Q_{10} of each of the reactions was close to 3.0. The time course curves of the mixtures given a pretreatment of 10 minutes at 100° C. are those of Reaction 3 discussed above. Reaction 2 is illustrated by the rate of pigment formation without the pretreatment. Here, the rate of pigmentation is limited by the rate of reaction between color developer and amino acid. Reaction 3 is about 7 times faster than Reaction 2.

pH. The two reactions involved in pigment formation were studied separately. The effect of pH was first determined for the final reaction (Reaction 3) by adjusting the pH after the formation of pigment precursor, just before addition of formaldehyde. There was a sharp optimum at pH 4.6 to 4.8, as indicated by the total amount of pigment formed after 90 minutes at 50° C. (Figure 5).

The effect of pH on Reaction 2 was studied by measuring the pigment which formed in mixtures in which the pH was varied before the high temperature treatment of the color developer–glycine mixture. This reaction was affected very little by pH in the range of pH 3 to 8. During the formation of pigment precursor, sufficient hydrogen ions were released to change the initial pH from the range of 5.0–6.5 to 4.8–5.0. Since the initial pH of most of the mixtures subsequently studied fell within this range, it was seldom necessary to adjust the pH to 4.8, the optimum for pigment formation.

OXIDIZING AND REDUCING AGENTS. Joslyn and Peterson (1958) observed that pigment formation in onion purees was inhibited by H_2O_2 at a concentration of 0.1% but was enhanced at a concentration of 0.02%. Also, they found that reducing agents such as ascorbic acid and cysteine inhibited pigment formation when added at the rate of 50 mg. per 100 grams of puree. The authors found that only the final pigment-forming reaction was affected when oxidizing or reducing reagents were added to the color developer–glycine-formaldehyde mixtures. Low concentrations of hydrogen peroxide increased the initial rate of pigmentation, with the maximum increase at $1.8 \times 10^{-3}M$ H_2O_2 (Table II). However, the total amount of pigment formed after 60 minutes at 50° C. declined with increasing concentrations of H_2O_2. Both the initial rate and the total amount of pigment formed were inhibited by low concentrations of cysteine. Inhibition was complete at $5 \times 10^{-3}M$ cysteine.

CARBONYL COMPOUNDS. Pink pigments were formed in onion puree in the presence of diacetyl, acetoin, methyl ethyl ketone, and glycolonitrile. The unsaturated ketone, 5-hexene-2-one, caused a blue-green pig-

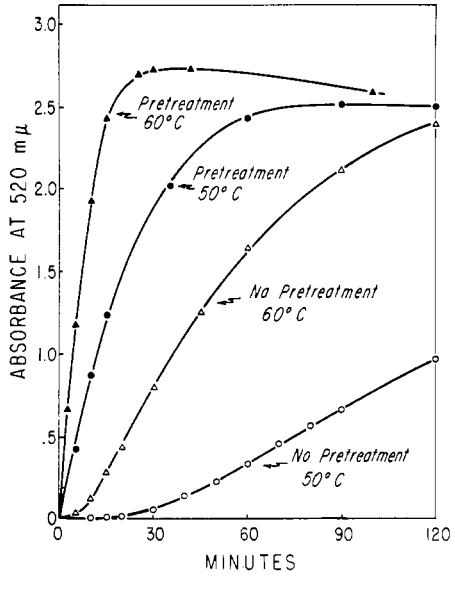

Figure 4. Effect of 10-minute pretreatment of color developer and glycine solution at 100° C. on subsequent pigment formation with added formaldehyde at 50° C. and 60° C.

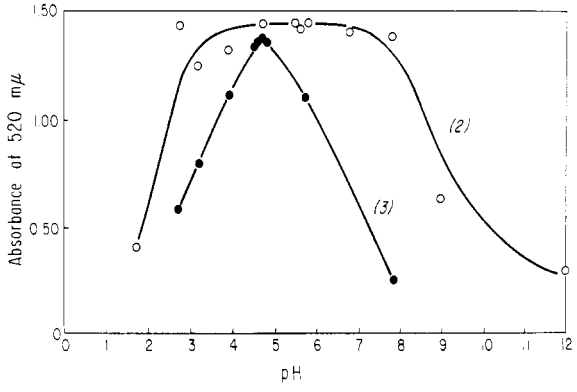

Figure 5. Effect of pH on formation of pigment precursor (Reaction 2) and formation of pigment (Reaction 3). See text

Table II. Effect of Hydrogen Peroxide and Cysteine on the Initial Rate and Extent of Pigment Formation[a]

Reagent	Molar Concn.	Initial Rate[b]	Total Pigment[c]
Water	...	100	100
Hydrogen peroxide	2.2×10^{-4}	157	95
	9.0×10^{-4}	203	71
	1.8×10^{-3}	236	64
	3.6×10^{-3}	220	63
	9.0×10^{-3}	200	57
Cysteine	5.0×10^{-4}	13	34
	1.0×10^{-3}	7	19
	5.0×10^{-3}	0	0

[a] Reaction mixture contained 0.5 ml. of color developer and 3.75 ml. of 1% glycine, heated 10 minutes at 100° C., followed by 4.75 ml. of treatment solutions and 1.0 ml. $3 \times 10^{-3}M$ at 50° C.
[b] Relative to water with 0.030 absorbance unit at 520 mμ per minute.
[c] Relative to water with 1.98 absorbance units at 520 mμ after 1 hour.

ment, while no color was observed in acetaldehyde, acetone, acetoacetic ester, propionaldehyde, or mesityl-oxide (Joslyn and Peterson, 1960). Of these carbonyls, methyl ethyl ketone, acetone, acetaldehyde, and propionaldehyde have been found in onions (Carson and Wong, 1961; Spare and Virtanen, 1961; Wilkins, 1964). The authors did not evaluate the pigment-forming qualities of many carbonyls with the purified system, but did observe that acrolein, another unsaturated carbonyl at a concentration of $2.5 \times 10^{-4}M$ caused the formation of a blue pigment. The formation of a blue pigment when garlic puree was mixed with onion puree was reported earlier (Yamaguchi *et al.*, 1965). Blue pigment formed rapidly when an ether extract of fresh garlic juice was incubated with pigment precursor. This indicates that a carbonyl, possibly unsaturated, was formed in garlic puree and was responsible for formation of the blue pigment in the garlic-onion mixtures.

Amino Acids. Lukes (1959) found that several amino acids were able to form pigment when incubated with his color developer extracts, but no color was formed with asparagine, histidine, tryptophan, or threonine. However, when the authors' standard procedure was followed, using $0.1M$ solutions of these amino acids and adding formaldehyde to the preformed pigment precursors, pigments did form. A more complete examination of the pigment-forming ability of several amino acids was undertaken, using $0.1M$ solutions where possible. The solutions were adjusted to pH 5 to 6 before formation of pigment precursor, and pigment intensity was determined at 50° C. with formaldehyde at $3 \times 10^{-4}M$. The initial rate and total amount of pigment formed with each amino acid are given in Table III. The absorption spectra of most of the pigments formed were similar, with an absorption maximum near 520 mμ. However, the hydroxy amino acids serine, threonine, and δ-hydroxylysine formed orange-pink pigments having a second maximum at 450 mμ (Figure 6).

Pigment did not form with proline, glycine methyl ester, sarcosine, betaine, ethanolamine, or ethylamine. Both a free primary amino group and a free carboxyl group were required. Cysteine did not form pigment, owing to its action as a reducing agent.

The data in Table III indicate the following structures influence pigment formation:

Chain Length. A normal four-carbon chain was optimal.

Amino Position. The alpha-amino acid was more reactive than the beta-amino acid in straight-chain compounds, but with branched chains, the beta-amino acid was more reactive than the alpha-amino acid.

Ring Structures. The benzene ring in phenylalanine enhanced pigment formation, but the indole nucleus of tryptophan inhibited its formation.

Hydroxyl Groups. OH on aliphatic amino acids decreased rate and extent of pigmentation.

Dicarboxylic Acids. Two carboxyl groups had induced higher initial rates of formation, but a lesser total amount of pigment formed.

Table III. Relative Rate and Extent of Pigment Formation as a Function of Kind of Amino Acid

Amino Acid	Molar Concn.	Initial Rate[a]	Total Pigment[b]
Glycine	0.10	100	100
DL-α-Alanine	0.10	129	109
β-Alanine	0.10	56	107
DL-Serine	0.10	42	28
DL-α-Amino-*n*-butyric	0.10	152	130
DL-Threonine	0.10	22	26
γ-Amino-*n*-butyric	0.10	54	79
α-Amino-isobutyric	0.10	2	13
β-Amino-isobutyric	0.05	114	96
DL-Valine	0.10	120	107
DL-Morleucine	0.10	112	98
L-Leucine	0.10	124	96
DL-Isoleucine	0.10	85	83
DL-Phenylalanine	0.10	172	172
L-Tyrosine	(saturated)	(fast)[c]	40
DL-Tryptophane	(saturated)	(slow)[c]	(low)[c]
DL-Methionine	0.10	116	103
L-Cysteine	0.10	0	0
L-Cystine	(saturated)	8	17
L-Cysteic acid	0.10	77	23
s-Methyl-L-cysteine	0.10	192	126
s-Methyl-L-cysteine sulfoxide	0.10	33	61
s-Ethyl-L-cysteine	0.10	188	119
L-Asparagine	0.10	93	95
DL-Aspartic	0.10	140	44
L-Glutamic	0.10	132	53
DL-Lysine	0.10	31	59
DL-Lysine	0.05	28	63
DL-δ-Hydroxylysine	0.05	47	31
L-Arginine	0.10	92	116
L-Histidine	0.10	29	27

[a] Relative to glycine, which was 0.107 absorbance unit per minute at 520 mμ.
[b] Relative to glycine, which was 2.53 absorbance units.
[c] Solution turbid, unable to clarify for absorbance measurement.

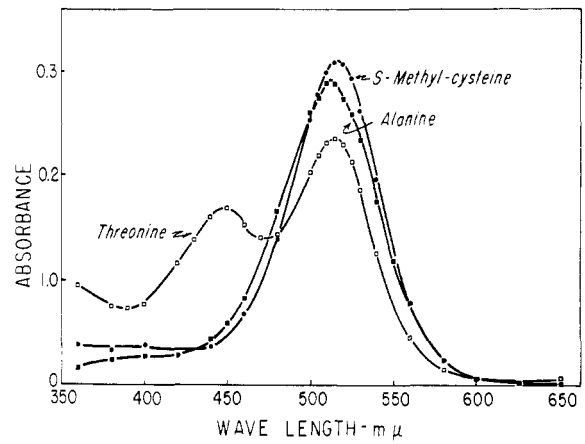

Figure 6. Absorption spectra of pigments formed with onion color developer and threonine, alanine, or 5-methylcysteine

Formaldehyde added for pigment formation

Basic Amino Acids. Additional amino groups had variable influences.

The S-alkyl derivatives of cysteine were highly reactive. This is in contrast to the inhibition by the free sulfhydryl of cysteine.

Incorporation of Labeled Precursors. Incorporation of amino acid and carbonyl compound into the pigment molecule was confirmed by the use of ^{14}C-labeled glycine and formaldehyde. Both glycine-1-^{14}C and glycine-2-^{14}C were used to study the incorporation of the amino acid. Almost twice as much ^{14}C was incorporated from the glycine-2-^{14}C as from the glycine-1-^{14}C (Table IV). This indicates that one of every two molecules of glycine incorporated was decarboxylated. That a decarboxylation did occur was confirmed by trapping $^{14}CO_2$ which formed in a reaction mixture of glycine-1-^{14}C and color developer. As shown in Table V, some $^{14}CO_2$ was released from glycine-1-^{14}C before the addition of color developer, but about four times that quantity was formed in the presence of color developer. Pigment formed in the presence of ^{14}C-labeled formaldehyde and purified by absorption on Magnesol contained 20% of the ^{14}C initially present in the reaction mixture (Table VI). An additional 7% of the ^{14}C not recovered was assumed to be present in pigment which could not be eluted from the Magnesol.

Conclusions

The color developer compound was not identified, but its reaction with Ehrlich's reagent indicates it may contain a pyrrole nucleus. The reaction of color developer with amino acids to form the final colorless compound, pigment precursor, must be complex. Differential incorporation of 1-^{14}C- and 2-^{14}C-labeled glycine and the decarboxylation of glycine-1-^{14}C indicate that two molecules of amino acid are involved in the formation of pigment precursor. One of the amino acid molecules apparently is incorporated intact, while the second is decarboxylated during incorporation. The kind of amino acid can affect the color of pigment formed, but the majority of amino acids tested gave the typical pink to red color observed when the pigment formed in onion bulb macerates.

The final pigment-forming reaction involves the condensation of the colorless pigment precursor with formaldehyde or another reactive carbonyl. The color of pigment formed is a function of the carbonyl involved. Some carbonyls have been reported to inhibit pigment formation in onion purees (Joslyn and Peterson, 1960), while others enhance the formation of the normal pink color. The unsaturated carbonyls 5-hexen-2-one (Joslyn and Peterson, 1960) and acrolein (Yamaguchi *et al.*, 1965) caused the formation of blue-green pigments in onion purees.

The concentration of the natural pigment-forming carbonyls present in onion purees or in onion slices during dehydration probably is the most important factor determining the rate and extent of pigmentation.

Table IV. Incorporation of Glycine-1-^{14}C and Glycine-2-^{14}C in the Pigment

Glycine in Reaction Mixture		Purified Pigment		
Position labeled	Total c.p.m.	Total activity, c.p.m.	Total units[a]	Specific activity,[b] c.p.m.
1-^{14}C	10.5×10^6	11.4×10^3	3.84	2.98×10^3
2-^{14}C	11.4×10^6	20.3×10^3	3.48	5.83×10^3

[a] Volume times absorbance at 520 mμ. [b] C.p.m. per unit of purified pigment adjusted to equal activities of starting solutions.

Table V. Incorporation of Glycine-1-^{14}C in the Pigment and the Release of $^{14}CO_2$

Sample	C.P.M. $\times 10^{-3}$	% of Total
Total glycine-1-^{14}C added	5250	100
$^{14}CO_2$ evolved in absence of color developer	3.6	0.07
$^{14}CO_2$ evolved in presence of color developer[a]	15.9	0.30
Unreacted glycine-1-^{14}C	5180	98.70
Purified pigment	19.4	0.40

[a] Heated to 100° C. for 15 minutes.

Table VI. Incorporation of Formaldehyde ^{14}C in the Pigment

Sample	C.P.M. $\times 10^{-3}$	%
H^{14}CHO initially present in reaction mixture	36.5	100
^{14}C not adsorbed by Magnesol (unreacted H^{14}CHO)	26.5	73
Pigment eluted from Magnesol	7.5	20
Pigment remaining on Magnesol	...	7[a]

[a] Calculated by difference.

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