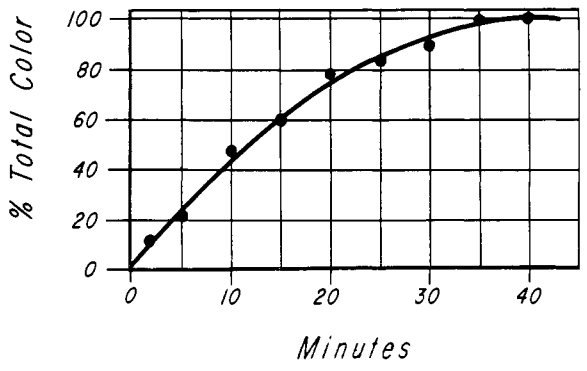

Figure 1. Rate of increase of Duraset color vs. time

m-toluidine. As tomatoes contain a high percentage of water, solid sodium hydroxide is used to keep the concentration at this high level. Lima beans, on the other hand, contain little water so aqueous sodium hydroxide solution can be used. Zinc apparently acts as a reducing agent to prevent oxidation of the amine during digestion and distillation. The wax is added to lessen foaming of the sample.

Treatment of Distillate before Color Formation. Hydrochloric acid is used to react with *m*-toluidine to form the water-soluble hydrochloride. Much of the plant material which is present in the distillate can be removed by an ether extraction. Many of these materials appear to have some reaction with the color-forming reagents. After color formation, their chemical properties follow closely those of the purple dye formed by the *m*-toluidine. Removal is most easily effected before the color reaction.

Color Formation. Sodium nitrite is added to diazotize the *m*-toluidine and the reaction is complete in 5 minutes. The ammonium sulfamate destroys any excess nitrous acid which must be removed because it also forms a purple dye with *N*-naphthylethylenediamine. Ten minutes are sufficient for this step. The *N*-1-naphthylethylenediamine dihydrochloride is then added. Figure 1 shows the time curve of color formation. The spectrophotometric curve has a maximum at 560 mμ (*1*). Although the color of the dye is stable for several days, the color of the interference increases on long standing. The absorbance measurements must be made within 3 hours after color formation is complete.

Extraction after Color Formation. Spectrophotometric curves were run of the purple dye obtained in various pH solutions. Above pH 6, all the dye was present in the basic form as a yellow color. It was hoped that, at a pH close to this transition pH for the desired constituent, the best separation could be obtained between the desired dye and other colored materials still present from the vegetable. By using disodium phosphate it was possible to obtain a pH at which the desired dye was extracted quantitatively into ether, leaving much of the undesired interfering color in the aqueous phase. Then an acidic extraction of the ether layer gave quantitative recovery of the desired constituent.

With any series of treated samples, two untreated samples are also analyzed, one of which contains a known amount of added Duraset. As levels of Duraset residues are usually very low or nonexistent, 0.1 p.p.m. of Duraset was added to the untreated samples. Table I shows typical recovery data obtained from tomatoes, cherries, and strawberries.

The reaction, although sensitive, is relatively nonspecific, and background interferences, unless removed, are large—i.e., the equivalent of several parts per million—compared to the small amount of Duraset being sought. Consequently, a series of separation steps is necessary to keep the background color sufficiently low to determine the Duraset residue accurately. Using the described method, background interferences in untreated samples have absorbance values equivalent to 0.05 to 0.08 p.p.m. of Duraset.

Table I. Recoveries of Knowns

(0.1 p.p.m. of Duraset added in all cases)

Duraset Recovered, P.P.M.[a]	Recovery, %	Duraset Recovery, P.P.M.[a]	Recovery, %
Tomatoes		*Cherries*	
0.078	78	0.098	98
0.092	92	0.079	79
0.107	107	0.090	90
0.088	88	0.070	70
0.080	80	0.080	80
0.096	96	0.073	73
0.099	99	0.062	62
0.087	87	Av.	79 ± 12
0.090	90		
0.073	73	*Strawberries*	
0.099	99	0.065	65
0.086	86	0.088	88
0.084	84	0.085	85
Av.	89 ± 9	0.101	101
		0.085	85
		Av.	85 ± 13

[a] After correction for background interference from untreated sample.

Acknowledgment

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FOOD DISCOLORATION

Reddening of White Onion Bulb Purees

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Purees prepared from succulent inner scales of onion bulbs, when acidified with acetic acid, turn pink to red on storage at room temperature. Joslyn (*31*) observed that this reddening occurred more rapidly and the color was more intense at higher concentrations of acetic acid (5%) than at lower levels (2%), and that addition of salt, even in amounts up to 25%, did not prevent reddening. This reddening was more intense in macerates of white onions than in those prepared from red onion bulbs. In onions susceptible to reddening, pigment formation occurred in mechanically bruised or finely cut tissue and did not occur in carefully cut sliced onions. Sliced onions steamed before crushing yielded purees which did not redden. This pigmentation was confirmed by Cruess (*14*) and more recently investi-

A water-soluble red pigment is formed in acidified macerates of white onion bulb tissue and in bruised, sliced onion tissue during dehydration. The resulting reddening in onion extracts preserved by addition of acetic acid or vinegar is objectionable and the pinking of dehydrated sliced white onions requires manual sorting. Pigment formation in purees is affected by variety, storage conditions, heat treatment, and acidity. Storage at 50° C., after maceration and before acidification, increases rate and intensity of reddening. Heating sliced onions in steam before maceration inhibits reddening. Reddening usually is accelerated in rate and increased in intensity by acidification with acetic acid to pH 3 to 3.5 and does not occur below 2.5 or above 5.5. The isolation and purification by solvent extraction, chromatographic separation on column and paper, its characterization by absorption spectroscopy, and other properties are reported. The pigment was a new nitrogenous water-soluble pigment which differed significantly from all previously reported red plant pigments. Knowledge of its structure and the factors influencing reddening will be useful in explaining the differences in susceptibility to reddening with variety and growing conditions, and in avoiding this by better selection of onion bulbs and control of the factors influencing reddening.

gated by Peterson (*47*). A similar pigment also forms in some varieties of white onions during the initial dehydration (*40*). During commercial dehydration it occurs only in some of the slices and rarely throughout the slice. Pinking during drying is a minor problem in commercial dehydration but necessitates sorting before grinding and packing. Commercially dehydrated onion products (chips, flakes, or powder) after adjustment to a moisture content equivalent to that of fresh tissue usually do not redden at room temperature. The factors influencing reddening of onion purees, and data on the isolation and preliminary characterization of the pigment formed are presented in this paper.

Experimental

Marked differences were observed in reddening between different varieties of onions and between different lots of the same variety. The purees prepared from bulbs of the same lot also varied in rate and extent of reddening. The Southport White Globe, widely used in commercial dehydration, was more susceptible to reddening than other white onions and the yellow and red onions had little, if any, tendency to redden. Reddening was most intense in Southport White Globe onions grown in certain localities. Initially, reddening was more intense in Nevada-grown Southport White Globe onion bulbs than with similar varieties grown in California. Subsequently, Southport White Globe onion bulbs with equal tendency to redden were obtained in California.

Southport White Globe onions grown in Nevada and harvested during the fall of 1954, and the same variety grown in California in 1955, 1956, and 1957 were used in most of the tests. The Nevada-grown onions were obtained through a local commission merchant in February 1955 and apparently were not subjected to slow freezing and subsequent defrosting as occasionally occurs. The California onions were grown in the Stockton or Salinas regions and were obtained from a commercial dehydrater. Smaller quantities of white, yellow, and red onions were obtained locally.

The onion bulbs were peeled free of outer scales, the root and stem tissue cut off, and then sliced. Purees were prepared by blending smaller lots in a Waring Blendor or grinding larger lots in a food chopper. Acids, alkalies, and other chemicals were added to the prepared purees, and these were stored in covered beakers or stoppered flasks at room temperature or in incubators. The chemicals used were C.P. grade but were not purified free of trace contaminants. The enzyme preparations used were obtained from Nutritional Biochemicals Corp. Preliminary qualitative data on the effect of inhibitors and activators tested were obtained by visual observation.

Quantitative data on rate and extent of reddening were obtained by filtering the juice free of suspended particles and measuring the color density of the clear filtrate in a Klett-Summerson filter photometer with green No. 54 filter. The freshly prepared purees were difficult to filter even when bentonite was added as a fining agent. Clear juice was obtained by addition of lead acetate and filtering through a layer of diatomaceous filter aid (Hyflo Super Cel). During storage of the purees, because of activity of the naturally occurring pectic enzymes, separation of pulp and clarification of suspended colloids occurred. This facilitated filtration. Occasionally commercial fungal pectic enzymes were added, but the clarification obtained was variable and incomplete. Absorption and reflectance spectra were measured in a Beckman Model DU spectrophotometer at intervals of 10 mμ and in a Cary recording spectrophotometer Model 11. The pH of the purees and juice was measured with a line-operated, Model H Beckman glass electrode pH meter.

The pigment described was obtained from the Nevada-grown Southport White Globe onion bulbs. Observations were limited to pigment formed in macerates obtained from these bulbs shortly after purchase and in bulbs stored for 1 month at 0° C. The outer dry scales, root, and dry stem were removed, and the inner succulent scales were cut with a stainless steel knife and crushed by blending in a Waring Blendor for 3 minutes at high speed at room temperature in the presence of air. The finely macerated tissue was acidified with glacial acetic acid to pH 3 to 4 and stored either for 2 to 3 days at 20° C. or 2 to 3 hours at 45° C. for pigment formation.

The pigment formed was separated from other water-soluble constituents by solvent extraction, and it was purified by column and paper chromatographic procedures. Identification of the partially purified pigment was made by determination of R_f values by paper chromatography in solvent systems developed by Bate-Smith (*3–5, 7*) and others (*11, 21*), by determination of the characteristic absorption spectra in water and alcoholic solvents, and by qualitative color reactions of Robinson and Robinson (*54*). Absorbance was measured in a Beckman Model DU spectrophotometer at intervals of 10 mμ. Light absorption both in the visible and near ultraviolet was measured also in the Cary recording spectrophotometer, Model No. 11, and absorption in the infrared region was measured in a Beckman Model IR-2 spectrophotometer.

Factors Affecting Reddening of Onion Bulb Purees

General Observations. In onions susceptible to reddening, pigment forma-

tion occurred only in mechanically bruised or finely cut tissue. In sliced onions, carefully cut with a sharp stainless steel knife, pigment formation did not occur. Under commercial conditions it occurred only in obviously bruised portions of cut onions. It is of interest that Morgan (*44*) reported formation of appreciable quantities of pyruvic acid (0.01 gram per 100 ml.) in onion juice from unknown precursor(s) when onion tissues were minced. In crushed or pureed tissue, pigmentation usually occurred first in the surface layers and then gradually extended into the lower layers of the puree. Occasionally, reddening was observed throughout the mass of puree. The pigment formed in the liquid phase, and, on storage, the pulp particles settled out, and the supernatant liquid became clear and red. Initially the sedimented insoluble matter was white and later gradually became yellow to slightly brown in color. Onion tissue macerates, filtered immediately after blending, yielded a clear colorless solution which often reddened as rapidly as the unfiltered puree.

The pigment in solution, both aqueous and alcohol, was unstable, particularly at lower pH values and decomposed to yield yellow- to brown-colored solutions. At room temperatures, from 2 to 3 days were required for maximum reddening and then the concentration of red pigment decreased while yellow-to-brown compounds accumulated. This decomposition was more rapid when the purees were stored at temperatures above 20° C. In fresh purees, stored at temperatures above 60° C., the red pigment did not form and only orange or brown pigments occurred. The general spectral characteristics of the pigment as formed in purees are shown in Figure 1, in which the reflectance spectrum of reddened puree is plotted in comparison with white onion puree. The color changes, during reddening, calculated as changes in C.I.E. tristimulus values and trichromatic coefficients, are shown in Table I. The chief changes observed were increases in purity and brightness and a slight shift toward the red in dominant wave length. The absorption spectrum of the red juice in comparison with that of original colorless juice is shown in Figure 2. This indicates a characteristic maximum at 528 mμ and a minimum at 440 to 450 mμ. The absorption spectrum of purified pigment in water solution is similar, with a maximum at 528 mμ and a minimum at 440 mμ. In comparison, spectral reflectance of the pigment adsorbed on Magnesol is shown in Figure 3. As suggested by Naughton, Frodyma, and Zeitlin (*45*), the latter procedure was tested to determine its suitability to quantitative studies of pigment formation to avoid undesirable changes due to extractive procedures. The preliminary results obtained were promising.

Table I. Color Changes during Reddening Expressed as C.I.E. Tristimulus Values and Trichromatic Coefficients[a]

Color Value	Fresh Puree	Reddened Puree
X	32.969	14.972
Y	35.51	12.89
Z	28.98	11.363
x	0.3349	0.3807
y	0.3607	0.3286
Dominant wave length	570 A.	600 A.
Purity	18.5%	22.5%

[a] Calculated from reflectance data by methods described by Hardy (*24*) and by Judd (*33*).

Varietal Differences. During the investigations, marked differences were observed in susceptibility to reddening between different varieties of onions and even between different onion bulbs of the same variety. In general, white onion bulbs formed pigment more rapidly and pigment formation was more intense than in yellow or red varieties. The Southport White Globe onions were more susceptible to reddening than other white onions. The early spring green onion bulbs did not redden. White onions purchased from local retail markets had widely varying tendencies to redden. The yellow onions, free of outer dry, colored scales, gave a white puree; the chief flavonoid pigment present was isolated and found to be quercetin. The puree prepared from yellow onion bulbs did not redden. The puree prepared from red onion bulbs of several varieties formed a red pigment which was similar in solubility and spectral absorbance and which gave the same R_f value on paper chromatograms developed with butyl alcohol–2*N* hydrochloric acid as did that from White Globe. However, the red pigment formed more slowly and in smaller amounts than from white onions. It did not resemble the naturally occurring anthocyanin pigments present in the outer scale. These were found to be glycosides of cyanidin by the usual anthocyanin color reactions and by paper chromatography. Individual bulbs of a given lot varied in intensity of reddening, indicating genetic variability.

Storage. Onion bulbs usually are harvested when the tops are completely dry, field cured and graded before sacking, and then stored either at room temperature or under refrigeration. The changes that occur in carbohydrate constituents during storage were investigated by Bennett (*9*) in Ebenezer onions, by Karmarkar and Joshi (*34*) in Indian varieties, and by Yamaguchi, Pratt, and Morris (*68*) for Nevada-grown Southport White Globe onions. The optimum storage temperature is considered to be 0° C. (*9, 66*). Yamaguchi, Pratt, and Morris (*68*) reported storage at 0°, 2.5°, and 30° C. resulted in highest yield of sound onions, but that dehydrated onions prepared from bulbs stored at higher temperatures were of better keeping quality—less susceptible to browning than those stored at lower temperatures. During storage, the reducing sugar content at 0° and 2.5° C. increased but decreased at higher temperatures in the range of 5° to 40° C.; the nonreducing sugars in general increased with increase in storage temperatures; the free amino acid content varied from 0.182 meq. per 100 grams at 0° C. to 0.189 at 30° C. and 0.205 at 40° C.; the tendency to brown, however, was highest in onions

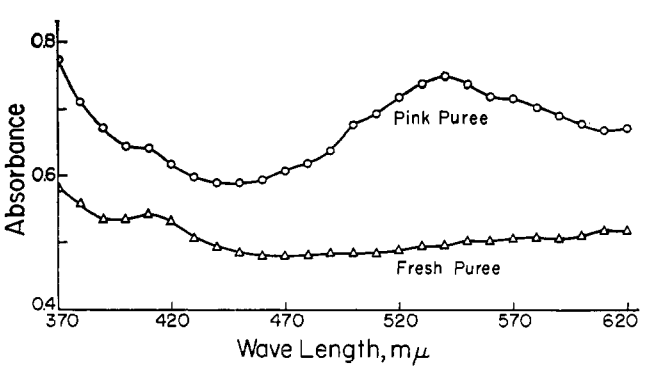

Figure 1. Reflectance spectrum of reddened White Globe onion puree in comparison with white puree

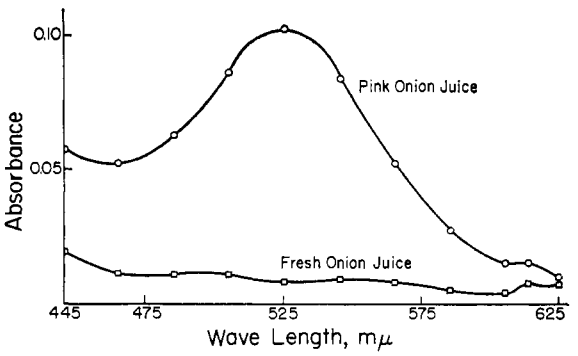

Figure 2. Absorption spectrum of red onion juice in comparison with white juice obtained from purees shown in Figure 1

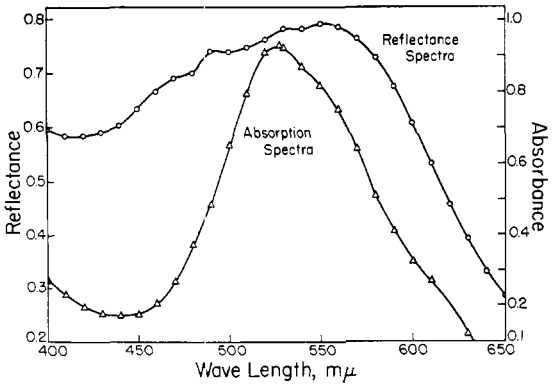

Figure 3. Reflectance spectrum of red pigment adsorbed on Magnesol in comparison with absorption spectrum of purified pigment in water

Table II. Effect of Acidification with Acetic Acid on Red Pigment Formation

Initial pH	Final pH	Colorimeter Reading
3.0	3.26	138
3.2	3.48	122
3.4	3.62	107
3.6	3.79	100
3.8	3.94	107
4.0	4.14	102
4.5	4.30	102
5.2	4.71	101

stored at 0° C. and least in those stored at 30° to 35° C. Changes in carbohydrates in onion bulbs during growth and storage have been investigated (*51*, *62*) and it is significant that Reĭmers (*51*) reported that the higher the relative concentration of disaccharides to monosaccharides, the better the storage quality of onion bulbs. In the onion bulbs used in our investigations, the only soluble sugars found were glucose, fructose, and sucrose.

Marked differences were observed to occur in susceptibility to reddening of purees prepared from onions stored at room temperature and at 0° C. In the original lot of Nevada-grown Southport White Globe onions, storage at room temperature (20° to 25° C.) for several weeks had little effect on rate and intensity of red pigment formation in purees. When stored for 1 month or longer at 0° C., these onions exhibited a greatly increased tendency to redden both in rate and intensity. Puree prepared from cold-stored onions did not exhibit an optimal pH for reddening. Purees prepared from onions as purchased reddened most in the pH range between 3.0 and 4.0 and did not redden below pH 2.5 or above 6.0. The puree or filtered juice obtained from cold-stored onions became equally red at all pH levels from 2.5 to 5.2. After storage for about 1 year at 0° C., purees prepared from these bulbs did not redden. The complete loss in tendency to redden was accompanied by general deterioration in quality, softening of both outer and inner scale tissue.

Effect of pH. The onion purees prepared usually were at pH 5.0 to 5.2. The puree and juice were strongly buffered in the acid region as shown in Figure 4. Reddening increased in rate and extent on acidification, but this varied with type of acid added and the pH to which the purees were adjusted. Of the acids listed, acetic acid was most effective in promoting reddening. Hydrochloric acid, sulfuric acid, *o*- and *m*-phosphoric acids, citric, lactic, or tartaric acid reduced the rate and extent of reddening at the same pH levels. With all acids tested, however, the pH range at which reddening occurred and the optimal pH for reddening was similar to that for acetic acid. During reddening at room temperature, the pH of the purees, acidified with acetic acid to pH 4.0 and below increased, while that of the puree at 4.5 and above decreased. Typical data are shown in Table II for Nevada-grown onions stored at 0° C. for 5 weeks. Hydrochloric acid, in addition to inhibiting rate and extent of pigmentation, also markedly increased the rate of decomposition of the red pigment. Typical of the data obtained on rate and extent of reddening in purees acidified with acetic acid and hydrochloric acid to pH 3.5 is that shown in Figure 5.

When purees from onions held at room temperature were adjusted to various pH levels with acetic acid shortly after preparation, reddening did not occur at room temperature at pH levels below 2.5 or above 5.5. Reddening usually was at a maximum after storage for 48 hours and was most intense at pH 3.0 to 3.5. Typical of the data obtained, Table III shows optimal reddening at pH 3.0. Occasionally reddening was optimal at pH 3.5; in one run, purees adjusted to pH 3.3, 3.4, and 3.6, respectively, reached colorimeter readings of 159, 176, and 216, respectively. The effect of storage is indicated in comparing the data of Table III with that in Table II in which reddening increased as the pH was decreased from 5.2 to 3.0.

The time of addition of acid, however, had more effect than pH on rate and extent of reddening. As the data in

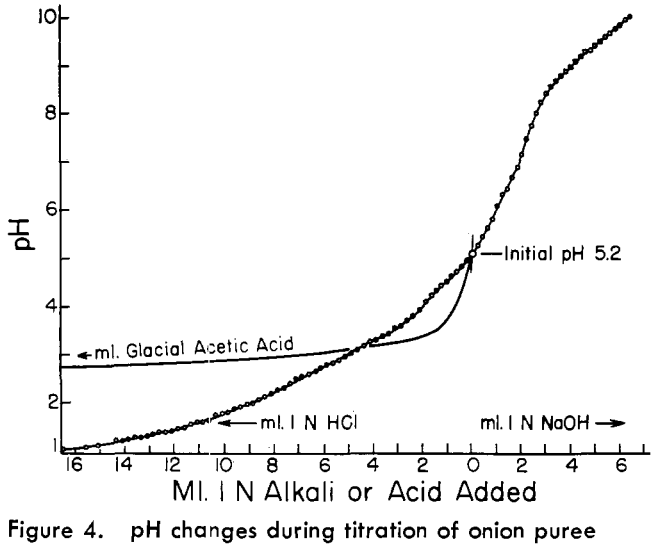

Figure 4. pH changes during titration of onion puree

One-hundred grams of puree titrated with 1*N* hydrochloric acid or 1*N* sodium hydroxide. The data for onion juice were similar, but its pH value was slightly higher at all points. The curve for glacial acetic acid is for onion juice

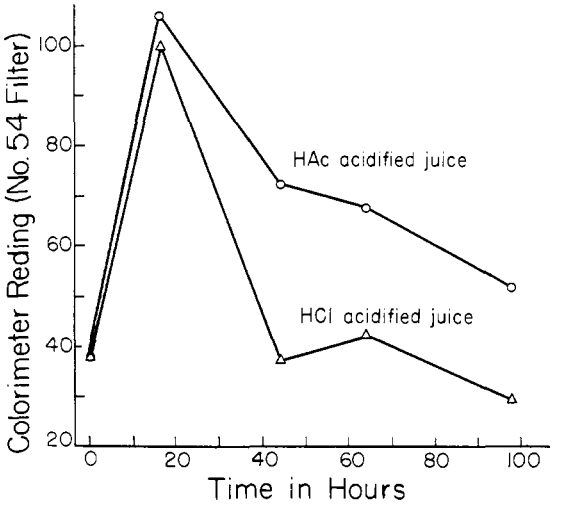

Figure 5. Rate of reddening of puree acidified with acetic in comparison with that acidified with hydrochloric acid to pH 3.5 at room temperature

Table IV indicate, the maximum reddening observed increased when acidification was delayed up to about 7 hours after pureeing. In all cases, the onions were cut and mixed, and aliquots were blended for 5 minutes. This indicates that rapid acidification to pH 3.5 with acetic acid inhibits conversion of some constituent into an active precursor.

From the same lot of onions, purees were prepared, held for 1 hour at 20°, 30°, 40°, 50°, and 60° C., respectively, and cooled to room temperature. Two aliquots of each were adjusted to pH 2.5 and 3.3 by addition of acetic acid, while a third was kept at its original pH of 5.2 and the three lots were stored at room temperature. The data obtained are summarized in Table V, which gives the final colorimeter reading after storage for 2 days. In unheated purees, optimal reddening occurred at pH 3.3, whereas, in purees held at 30° C., reddening increased rapidly on acidification to pH 3.3 and less so up to pH 2.5. In purees held at 40° C. and above before acidification, reddening increased with decrease in pH, particularly in the range of pH 3.3 to 2.5. Similar data were obtained with purees adjusted to a wider range of pH values in the region of 5.2 to 2.3 by addition of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 ml. of acetic acid per 100 grams of puree.

Effect of Temperature. The temperature of storage of the purees before acidification as well as the temperature of storage after acidification affects the rate and extent of reddening. The data obtained on the former effect is presented above. Data on the over-all effect of temperature, after acidification, which influences both the possible formation of a precursor and its conversion, is presented here. Onion purees, both acidified and not, were prepared and stored at various temperatures in the range of 20° to 65° C. As the data in Table VI indicate, the rate of pigment formation increased with increase in temperature. Changes in pigment formed, however, began to occur at 45° C. and were quite marked at higher temperatures. In the temperature range of 20° to 45° C., the purees became pink to red; at 45° C., they were appreciably bluer in tint; at 55° C. and above, they were dark orange to brown in color. At 45° C. appreciable reddening occurred after 0.5 hour and reached a maximum in 2 hours. The respective colorimeter readings after 0.5, 1, 1.5, and 2 hours were 78, 90, 103, and 121. The pH range in which reddening occurred at 45° C. also was 2.5 to 6.0 with a pH optimum between 3 and 4. The pH did not change during reddening at temperatures of 40° C. and above. Storage at temperatures below 20° C. reduced the rate and extent of reddening but even freezing storage at −17° C. did not prevent it.

Table III. Effect of pH on Red Pigment Formation

pH	Colorimeter Reading
4.9	132
4.5	142
4.0	148
3.5	152
3.0	170
2.7	141
2.4	124

Table IV. Effect of Delay in Acidification on Reddening at Room Temperature

Time of Addition of Acetic Acid	Colorimetric Reading of Filtered Juice after 2 to 3 Days' Storage
Before pureeing (in Blendor)	110
After pureeing, min.	
0.5	95
1	118
2	112
4	111
8	135
16	153
32	168
64	161
117	210
Before pureeing, hr.	
0	65
0.5	136
1	151
1.5	158
3	196
7	218
22	161

Purees stored at −17° C. in plastic bags slowly reddened during freezing storage and pigment formation eventually became quite intense, particularly in the concentrated unfrozen juice surrounding the pulp particles.

Effect of Heat and Drying. Heating the sliced onions before maceration had a marked effect on the tendency of the puree to redden. Onion slices steamed for various periods of time, then cooled in ice water and blended, gave purees which, depending upon the time of blanching, at first increased in tendency to form pigment and then decreased. Steaming for 10 seconds or less had no effect on reddening, steaming for 20 seconds gave purees which formed twice as much pigment as unheated onions, steaming for 30 seconds gave purees which formed about four times as much pigment, steaming for 40 seconds gave purees which formed as much pigment as those steamed for 20 seconds, but steaming for 50 seconds or longer completely inhibited pigment formation. The sliced onions steamed for 30 seconds not only gave the most intensely reddened purees but also purees in which the red pigment was formed sooner. They reddened in

Table V. Effect of Temperature and pH on Reddening

Temperature of Initial Storage, ° C.	pH of Puree	Colorimeter Reading
20	5.2	103
	3.3	119
	2.5	95
30	5.2	82
	3.3	158
	2.5	172
40	5.2	144
	3.3	203
	2.5	270
50	5.2	101
	3.3	210
	2.5	460
60	5.2	125
	3.3	172
	2.5	520

Table VI. Effect of Storage Temperature on Color Formation

Temperature, ° C.	Colorimeter Reading, Hr. 2	3
20	89	107
28	106	131
45	153	186
55	191	221
65	192	219

24 hours at 20° C. and remained a vivid reddish-pink even after 7 days' storage. Qualitative tests for peroxidase activity with guaiacol indicated that steaming for 30 seconds resulted in increased peroxidase activity but peroxidase activity was still present in onion slices steamed for 1 minute and then decreased during the period of 1 to 3 minutes and was absent in slices steamed for 4 minutes.

Effect of Salt and Cations. The addition of sodium chloride at levels of 5 to 25% to the onion puree or to the freshly expressed juice inhibited reddening, approximately in proportion to concentration of added salt, but did not prevent it. The addition of sodium chloride to acidified puree or juice had less effect. Thus, in the presence of 10% added sodium chloride, unacidified puree was only very slightly pink after 3 days, but with 1% of added acetic acid it was light pink and with 5% acetic acid it was quite pink.

Acetate ion as such had little effect on rate or intensity of reddening because addition of calcium or magnesium, as acetates or chlorides, had the same effect. In some cases, the addition of calcium, magnesium, or sodium acetate doubled the concentration of pigment formed, in others it had no effect. In one instance, potassium acetate apparently inhibited reddening but this was not confirmed on repeated trials. At pH 3.5, the addition of sodium acetate or bromide, calcium acetate or sulfate,

ammonium acetate, nitrate, or oxalate, magnesium acetate or sulfate, zinc acetate, barium acetate, or lead acetate, even in large excess, had no effect on rate or intensity of pigment formation.

Of the cations tested, only mercuric ion, added as acetate, inhibited reddening; and this only at relatively high concentrations. Lead and zinc, and particularly the former, had a peculiar stabilizing effect on the red pigment. The addition of lead or zinc acetate to onion purees or onion juice, even in concentrations up to saturation, did not inhibit reddening when the salts were added after blending. In purees saturated with lead or zinc acetate, reddening occurred after the suspended solid matter settled out to leave a clear supernatant solution. This finally became as red at the same pH value as purees to which lead or zinc salts were not added. Addition of lead acetate to cut onions, during blending or immediately after, inhibited reddening. This inhibition occasionally extended up to 24 hours, but the final extent of reddening was not affected. Unlike purees containing only acetic acid or acetic acid and other salts, the lead or zinc samples did not lose their red color or become brown. Ordinarily, the red pigment began to decompose and brown after 3 or 4 days' storage at room temperature, but in the presence of excess lead or zinc acetate, the red pigment remained unchanged even after storage for 7 to 14 days.

Effect of Oxidants and Reductants. To determine whether oxidation or reduction was involved in reddening, the purees were deaerated and held in vacuo or under oxygen-depleted nitrogen, and various oxidants and reductants were added. Onion purees and onion juice deaerated and held under vacuum reddened as rapidly as did those exposed to air. Onion purees after evacuation and treatment with nitrogen, previously passed through alkaline pyrogallol, also reddened as rapidly and as intensely as those exposed to air.

No detectable decrease in ascorbic acid content occurred during reddening of fresh onion purees and increase in ascorbic acid levels from the initial level of 10 to 12 mg. per 100 to 50 mg. did not inhibit reddening. The ascorbic acid content of commercially dehydrated white onion slices, however, was appreciably higher than that of the pinked slices (39.9 mg. per 100 grams of dried onions, in comparison with 22.7 mg.)

Acidified onion purees, evacuated with a Nelson vacuum pump or water aspirator and held under vacuum, reddened almost as intensely and only slightly slower than the control. Cut onions, held under vacuum before blending and then blended and stored under vaccum, reddened almost as intensely as aerated controls.

The addition of hydrogen peroxide, at levels of 0.1%, inhibited and, at higher levels, completely prevented reddening. When hydrogen peroxide was added at 0.02% levels, reddening was promoted in rate and extent. The addition of reducing agents—ascorbic acid, cysteine, bisulfite, and hydroxylamine—and of cyanide, at 50 mg. %, inhibited reddening in both unacidified purees (pH 5.2) and acidified purees (pH 3.5), but did not completely prevent it even at levels twice as high.

If oxidation is a factor in reddening only a slight oxidation is necessary. During reddening, the ascorbic acid content of onion purees did not decrease appreciably and significant differences in tannin content were not observed. This would indicate that reddening occurred without appreciable oxidation of these constituents and would preclude accumulation of quinones as intermediates.

Effect of Added Enzymes and Enzyme Inhibitors. Cytochrome bands a, b, and c and a succino-oxidase system were reported to be present in particulate preparations from onion bulbs, but catechol oxidase was not found (*10*). Catalase (*57*), and peroxidase (*17, 57*) activity have been reported; ascorbic acid oxidase has been reported to be absent (*15, 26–29*) or present in very slight amounts (*27*). Substances inhibiting the copper catalyzed oxidation of ascorbic acid are present in onion tissue (*26*), and these apparently also retard purpurogallin formation during the peroxidase catalyzed oxidation of pyrogallol (*17*). All the onion bulbs used in our investigations were very positive in catalase and peroxidase, but negative in polyphenol oxidase activity. In white onion tissue, the peroxidase activity was stronger with catechol than guaiacol in those lots that reddened and weaker with catechol in those lots that did not redden.

Several peroxidase inhibitors were tested to determine their effect on reddening. The general metallo-enzyme inhibitors—sodium fluoride, lead salts, metaphosphoric acid, and trichloracetic acid—were without effect even at high concentrations. Of the heavy metals tested—mercury, lead, and barium—only the mercury salts inhibited reddening. The halides tested –sodium chloride, sodium bromide, and sodium fluoride —were without effect even at high concentrations. Sodium cyanide, however, did inhibit reddening in both plain and acidified purees. The addition of purified horseradish peroxidase, of liver catalase, or of tyrosinase had no effect. Actually the reddening occurred at pH levels at which these enzymes are normally relatively inactive.

The possibility that glycosides occur as precursors and that these are hydrolyzed by glycosidases, either of the usual type or those that are specific for flavonol glycosides (*18, 25*) or of the esterase type (*16*) was investigated. The addition of β-glycosidase or emulsin to fresh or steam-blanched onion puree 1 hour before acidifying had no effect on pigment formation.

Polyphenols have not been found in white onion tissue, and they are present in yellow or red onions only to a small extent in the outer scales. Link and Walker (*38*), for example, reported only an estimated 0.2 to 0.4 mg. of catechol per 100 grams of dry scales. The dry, outer scales make up only a small fraction of the total weight. The permanganate reducing matter expressed as tannin in the Southport White Globe tissue is small, amounting to only 0.33 to 0.36 gram per kg. of onion tissue. The addition of catechol and chlorogenic acid to onion purees had no effect on reddening. The addition of D-catechin was very slightly inhibitory to reddening. The naturally occurring phenolic constituents could not be detected by the usual solvent extraction or lead precipitation procedures used. Acetone extracts of onion tissue did not have a sufficiently high concentration of phenolic constituents to be detected readily by paper chromatography.

The addition of glycine increased reddening slightly both in absence and presence of lead acetate. The effect of glycine was observable at low levels (50 mg. %), noticeable at 0.1%, but increase in concentration of glycine up to 1.0% did not result in an increase in reddening over that obtained at 0.1%. Betaine initially accelerated reddening but had no effect on final extent. Leucine only slightly affected either the rate or extent of reddening.

The filtered onion juice after passage over a Zeo Karb H resin in the hydrogen form did not redden, indicating that cation functions were required. These could not be replaced by any of the amino acids tested, although a chromatographically detectable decrease in amino acids occurred during reddening.

Leucoanthocyanins as Precursors. As the solubility and spectral absorption of the red pigment resembled that of anthocyanins and as it was formed so readily in strongly acidified purees, the possibility that it was formed from a leucoanthocyanin was investigated in some detail.

The vanillin test for leucoanthocyanins (*5*), was used to detect the presence of the colorless precursor of the red pigment. In general, the vanillin test was inconclusive on onion puree and extracts, primarily because all the common reagent grade solvents also gave the test. Ordinary reagent grade butyl alcohol itself, like most of the common solvents, is vanillin positive.

The test also suffers from its lack of objectivity—any change in color from the original colorless to a pink or red is regarded as a positive test. Most of the positive tests obtained with onion tissue were a light pink-orange.

Fresh onion tissue did not give a positive vanillin test, but after approximately a month's storage at 0° C., a weak positive test was obtained. Freshly thawed onion juice (frozen and stored at −10° C. for 3 months) was strongly vanillin positive—especially when thawed rapidly by steam or boiling water. This apparently was similar to steam blanching the onion slices for about 30 seconds before pureeing—as a comparison of vanillin tests of the blanched slices showed an optimum vanillin test at 40 seconds with a negative test at 0 and at 60 seconds. If this juice were boiled with strong hydrochloric acid for a few minutes, it first became yellow and then brown and, at the same time, formed a brown precipitate. This is what would be expected if a leucoanthocyanin were present which yielded a heat labile anthocyanin—the brown precipitate being a leucoanthocyanin polymerization product.

If instead, *n*-butyl alcohol were added to the freshly thawed juice, the vanillin positive substance was extracted by the butyl alcohol, leaving the extracted juice vanillin negative. This butyl alcohol did not redden when incubated with acetic acid. However, when heated with strong hydrochloric acid, it changed from yellow to orange to brown and also formed a brown precipitate. On overnight storage in the presence of 5% hydrochloric acid at room temperature, the butyl alcohol solution became pink. The extracted juice, vanillin negative, still became red upon storage with added acetic acid, just as if nothing had changed. After this red pigment was removed by adsorption onto Magnesol, however, the colorless, slightly brown juice again was vanillin positive.

Discussion

On the basis of the observations reported, several possible mechanisms for pigmentation may exist. First, the conversion of a colorless precursor (leucoanthocyanin) into a red pigment (anthocyanidin) catalyzed at some stage in the reaction sequence by a thermolabile system. Second, the formation of red pigments similar to those formed in the tyrosinase catalyzed oxidation of tyrosine. Third, the polyphenol oxidase–catalyzed, or peroxidase-catalyzed, oxidation of naturally occurring phenols to *o*-quinone and their subsequent reaction with amino acids. Red pigments are formed from catechol and amino acids in the presence of phenolase according to Platt and Wormall (*49*) and Mason (*42*, *43*). There is also the possibility that the phenols present may react directly with amines similar to the well known reaction of gossypol with aniline (*12*).

The formation of the red pigment from macerated onion tissue, however, is not due to the simple phenolase oxidation of tyrosine. Tyrosinase is not present. The red and purple intermediates formed during the oxidation of tyrosine have an absorption maximum in the region of 275 to 305 mμ and a second maximum at 300 to 540 mμ (*42*, *43*). Of these, only dopachrome is red (maxima at 305 and 475). Their spectral absorbtion and other properties are quite different from those observed for the red onion pigment. The pH range in which the onion tissue reddens and the fact that this reddening occurs in the presence of substances known to inhibit tyrosinase (metaphosphoric acid, trichloracetic acid, and lead acetate) mitigate against oxidation of tyrosine or related amino acids as the reaction involved.

The oxidation of a phenol like catechol to the *o*-quinone in the presence of amino acids to form pigments also is unlikely. The concentration of phenolic substances is low, and if these substances are oxidized to *o*-quinones this must occur rapidly and at very low oxygen tensions. Catecholase or similar polyphenoloxidases are absent, and, while peroxidase could catalyze the oxidation of catechols to quinones, the hydrogen peroxide necessary for this must be made available by some terminal oxidase. The relatively high ascorbic acid concentration at which the pigmentation occurs would prevent appreciable accumulation of hydrogen peroxide, and the fact that addition of catalase was without effect and that hydrogen peroxide actually inhibited reddening would rule this out. If either phenolase- or peroxidase-catalyzed oxidation is involved, the result will be the production of a colorless oxidation product, stable under reducing conditions and capable of reacting with amino acids in strongly acid solutions in presence of excess of lead salts.

The only evidence that enzyme activity was actually involved lay in the possibility of preventing reddening by heat treatment before maceration. Acidification or addition of lead acetate during blending or immediately after blending inhibited reddening, which also indicated possible enzyme action. Onion tissues apparently contain a constituent which is converted into a precursor which reddens in acidified onion juice. This substance either requires an enzyme for its conversion into the active precursor or is particularly thermolabile. Its conversion into the active precursor occurs faster at temperatures of about 50° to 60° C. and at a pH of about 5.0. The changes in tendency to redden on cold storage also implicate enzymatic systems. In freshly harvested onions stored at room temperature, the conversion of a precursor into a colorless intermediate must occur quite rapidly because once the tissue is macerated reddening cannot be prevented by heat treatment or enzyme precipitants. If oxidation is involved in this stage it must occur through a system that is rapidly saturated with oxygen at very low oxygen tensions.

The presence of flavonoid compounds and related phenolic constituents in *Allium cepa* tissues has been investigated in relation to genetics and resistance to invasion by pathogenic fungi (*38*, *52*, *65*). Perkin and Hummel (*46*) very early reported the presence of quercetin in the outer scales of yellow onion bulbs and an anthocyanin in red bulbs. Subsequently, the latter was shown to contain a glycoside of cyanidin and the former, glycosides of quercetin (*13*, *47*, *65*). Protocatechuic acid was shown to accompany pigment and to function as inhibitor for pathogenic fungi by Walker and Link (*65*) and others. Free catechol was isolated from pigmented onion scales by Link and Walker (*38*). The bactericidal and fungicidal effect of onion vapors and onion juice have been investigated by Walker, Lindegren, and Bachmann (*64*), Lovell (*39*), Fuller and Higgins (*20*) and others. The naturally occurring anthocyanins and the factors affecting their biogenesis in onion tissue, however, have not been investigated in detail. Chromatographically pure flavonoids have not been isolated from onion tissue, and the identity of anthocyanins previously reported has not been established unequivocally. The outer dry scales and the succulent scales of white onion bulbs have been reported to be free of anthocyanin pigments. Leucoanthocyanins, shown to be widely distributed in plant tissues by Bate-Smith (*2*), have not been reported to occur in onion tissue.

From the results obtained and on the basis of investigations on the isolation and characterization of the pigment formed (*32*, *47*), it is likely that this is similar to the nitrogenous pigment, betanin, of red beets. The actual structures of the nitrogenous anthocyanin–like pigments of beets are still not known (*1*, *2*, *53*), although crystalline preparations of betanin are reported (*59*, *67*). Although it was reported betanin could be further purified by ion exchange chromatography yielding a nitrogen-free pigment (*59*), subsequently crystallized betanin was not nitrogen free (*59*, *67*).

Betanin has been termed a nitrogenous anthocyanin on the basis of solubility, color, and similar properties. From the infrared and ultraviolet absorption spectra data, this is definitely not the case. Betanin cannot be aromatic, and as it probably contains ring

nitrogen, the pigment probably contains at least one pyrrole nucleus (*48*). As the onion pigment is similar to betanin, except for its strong ultraviolet absorption, the term nitrogenous anthocyanin is probably more closely applicable to the onion pigment than to betanin. If the onion pigment were a true nitrogenous anthocyanin, as it appears to be, then it might be expected to arise from a nitrogenous leucoanthocyanin in the same manner as is possible for true anthocyanin pigments.

The anthocyanins may occur in two possible colorless forms, the pseudo base or leucoanthocyanin. The pseudo base and its tautomeric ketone and chalcone can be reconverted easily to the color base on acidification. The conversion of the leucoanthocyanin to the color base is slower, and, in slightly acid solutions, it requires heating but will occur at room temperature in strongly acid solution. During the conversion of leucoanthocyanins into anthocyanidines, amorphous reddish-brown gums form (*6*, *18*, *19*, *35*, *36*). In some cases, the conversion requires an external hydrogen acceptor (O_2) as well as intramolecular oxidation and reduction (*8*, *53*, *55*); but in most cases, the conversion will occur readily in the absence of air. The colorless precursor of betanin is not known but Kozlowski (*37*) isolated white and yellow substances from sugar beets and red beets, which on oxidation by manganese dioxide in 65% sulfuric acid solution yielded violet pigments with absorption spectra similar to those of betanin.

Leucoanthocyanins are widely distributed in plants but usually have been identified by paper chromatographic separation of the anthocyanidins produced from them by treatment with acid. The usual anthocyanidins obtained have been cyanidin, and to a lesser extent, delphinidin. Recently, however, other hydroxylation patterns have been found. King and Bottomley (*35*, *36*) reported the occurrence of flavin-3,4-diol (melacacidin) and Roux (*56*) reported on improved paper chromatographic and absorption spectrophotometric methods for identification of these substances. Simmonds and Stevens (*63*) reported the occurrence of a methylene dioxy bridge in some phenolic constituents of plants.

With the exception of the flavan-3,4-diol isolated by King and Bottomley (*35*, *36*), leucoanthocyanins have not been isolated from plant sources. On the basis of the structure of the flavantriol suspected by Robinson (*55*) to be typical of leucoanthocyanins, its conversion into cyanidin would involve only intramolecular oxidation-reduction resulting in loss of water. If leucoanthocyanins have the structure assigned by King and Bottomley (*35*, *36*) an external hydrogen accepter would be required, and both oxidation and water removal would be involved. This would eliminate pigment formation in the absence of oxidation in onion macerates.

The final alternative is that the onion pigment is formed not by conjugation of *o*-quinone with an amino group to form first amino hydroquinone and then amino quinone as in *o*-diphenol–phenolase systems (*23*, *30*) but by direct conjugation of an acidic phenol with a basic amino acid.

Isolation and Characterization of Pigment Formed

General Observations. The red pigment was soluble in water and formed only in the liquid phase and not in particulate matter present in macerates. It formed as rapidly, or even more rapidly, in clear filtrates obtained from freshly macerated tissue by filtration on a Büchner funnel after addition of bentonite clay or diatomaceous filter aid.

Lead acetate, trichloroacetic acid, and other protein precipitants did not precipitate the pigment or its precursor from onion tissue macerates or filtered onion juice. It also was not precipitated by picrate or other reagents used in separation of anthocyanin pigments—either from aqueous or alcoholic solution over a wide pH range. Lead acetate, however, is commonly used for the separation of the red nitrogenous pigment, betanin, of beets (*50*).

In solution, the pigment was quite unstable, its red color decreased, and yellow to brown pigments formed. In solutions, acidified with hydrochloric acid to below pH 2.5, from 1 to 2 days were required for the conversion into brown products at room temperature. In alkaline solutions, this decomposition was more rapid, usually being complete in several minutes. Regeneration of the original pigment could not be obtained by oxidation with hydrogen peroxide or reduction by zinc in acid solution of the yellow or brown pigments. In its instability in solution, the pigment resembled betanin (*50*).

Solvent Extraction. The pigment was not readily extractable from onion juice or water solutions by the solvents used for separation of anthocyanins and related flavonoids. The pigment was not readily soluble in the usual nonaqueous solvents (ethyl alcohol, acetone, ethyl ether, benzene, petroleum ether, carbon tetrachloride, toluene, or xylene). Of the solvents tested (*n*-amyl or isoamyl alcohol, *n*-butyl alcohol, benzyl alcohol, toluene-cyclohexane, anisole–ethyl isoamyl ether–picric acid), *n*-butyl alcohol and isoamyl alcohol were most efficient. Even with these solvents, repeated extraction removed less than 70% of the pigment from reddened onion juices and then only when hydrochloric acid was added. Saturating the onion juice with salt also increased the proportion of pigment extracted by butyl alcohol. Concentration of the butyl alcohol or amyl alcohol extracts was not possible because of the ease with which the pigment decomposed in solution. It was not possible to concentrate this alcohol extract at temperatures of 20° C. or below, even in high vacuum. At temperatures of 30° C. or above, appreciable decomposition occurred during concentration. Because of these difficulties, preliminary extraction with water-insoluble solvents was discarded.

Adsorption. A wide variety of adsorbents was tested to determine their applicability to separation of the pigment by column chromatography. These included silica gel, titanium and zirconium oxides, magnesia, alumina, cellulose, talc, carbon, and several anion and cation exchange resins. These either did not adsorb the pigment or adsorbed it so strongly that elution with any of several solvent systems was not possible. Magnesol, a commercial anhydrous magnesium silicate, was suitable. This readily and quantitatively adsorbed the red pigment from slightly acid solution and the adsorbed pigment could then be eluted by hot acidified methanol or acetone. It was not necessary to pass the juice through a column of Magnesol as Magnesol could be added directly to the clear juice and mixed, and the juice could be filtered through a sintered-glass funnel; or a layer could be placed on the filter surface, and the onion juice or extract could be filtered through it. While the red pigment was rapidly and completely adsorbed by Magnesol, the yellow or brown pigments passed through the layer without adsorption. The pigment adsorbed on Magnesol was pink to violet in color and could be washed with large amounts of water, methanol, ethyl alcohol, acetone, ethyl acetate, or diethyl ether without elution. The absorbed pigment—after washing first with distilled water, then with alcohol and ether, and drying at room temperature—could be stored indefinitely without detectable change. This procedure was used for separation and storage of pigment for further investigations.

Most of the pigment adsorbed on Magnesol was eluted by hot methanol, acidified by addition of 2% by weight of hydrochloric acid. This was done by filtering boiling, acidified methanol through the Magnesol on a sintered-glass funnel into a suction flask. The methanol extract in the flask was cooled rapidly by evaporation and was rarely above 15° C. Even when large volumes of acidified methanol were used, a small amount of the pigment remained on the Magnesol. This could not be removed by heating the eluent or by any other solvent tested. Ethyl alcohol, ethyl alcohol–phenol, isopropyl, amyl, butyl, and benzyl alcohols, acidified with

hydrochloric acid, were tested as eluents but were not as efficient as methanol. After initial treatment with methanol–2% hydrochloric acid, acetone–2% hydrochloric acid could be used to elute more pigment.

Purification. The pigments eluted from Magnesol by acidified methanol could be concentrated after neutralization of residual acid by sodium bicarbonate. Concentration in the presence of hydrochloric acid caused appreciable decomposition even at low temperatures. The neutralized methanol solution was concentrated in vacuo at room temperatures, and the sodium chloride and excess of sodium bicarbonate which separated were removed by filtration. The pigment present in the concentrate could not be precipitated as a solid by addition of ethyl ether, either with or without addition of hydrochloric acid. It settled out as a dark, blood-red oily layer on addition of two to three volumes of anhydrous ethyl ether. The oily appearance of the ether precipitate was apparently due to the presence of small amounts of water, strongly retained by pigment. When this oil phase was dissolved in one volume of absolute ethyl alcohol, a dark red, extremely hygroscopic solid was precipitable by addition of four volumes of anhydrous ether. This last step often had to be repeated as the pigment would not precipitate as a solid until the residual water content was reduced to a low value. By comparison, the red nitrogenous pigment of beets could be precipitated as a solid from its solutions in methanol by dry ethyl ether only when particular care was taken to exclude water. The presence of even traces of moisture prevent its precipitation. Bentanin is known to be extremely hygroscopic and only recently has it been crystallized (*60, 67*). The ether precipitated pigments could be dried by lyophilization.

The ether-precipitable red pigments or the concentrated methanol extracts separated from Magnesol columns were resolved by chromatography on silica gel columns into one major red pigment, a slower moving minor red pigment, and a minor yellow pigment. Each band of red pigment was accompanied by a colorless fluorescent zone trailing behind, but not completely separated from, the red pigment band. Separation on a dry silica gel column was obtained by premixing the extract or precipitate with freshly dried silica gel, placing this at the top of a silica gel column and developing with butyl alcohol–2*N* hydrochloric acid under positive nitrogen pressure. None of the three pigments so separated could be crystallized, and the colorless impurity could not be completely removed from the major red pigment.

The components of the pigment complex, which appear as fluorescent bands following the red pigments on the silicic acid column, have a high affinity for the red pigments. They could not be separated from the red pigments by any of the chromatographic systems tested. Elution and subsequent concentration of the eluate always resulted in the simultaneous concentration of some of the oil constituents. These were insoluble in acetone but could not be separated completely even by repeated extraction with acetone to remove the acetone-soluble red pigments. The acetone extract still contained some oily matter and the residue still retained some red pigment. The oily constituents were concentrated by precipitation from concentrated methanol solution with acetone. The acetone precipitate so obtained was an amorphous solid which was always pink because of occluded, adsorbed, or coprecipitated red pigment. The acetone precipitate picked up water so rapidly that it settled out as a shiny, oily mass almost immediately after the solvent was drawn off. This became brown at the same time and, once this occurred, none of the red pigment could be recovered.

The pigments present in the two red bands were eluted separately and found to be identical in R_f values in several solvent systems by paper chromatography. They were identical also in absorption spectra. The only difference was in mobility on the silicic acid column. In the study of the properties of the pigment, only the fast-moving red component was used, as it was present in much greater amount than the slow-moving one. The identical R_f values obtained were possibly due more to the "oil" constituents than to the pigments themselves.

The lyophilized, purified red pigment was separable by paper electrophoresis into three components. When spotted on paper in a Spinco paper-strip electrophoretic apparatus and developed with McIlvaine citrate-phosphate buffer at pH 4, two pigment spots were found. After 3 hours at 300 volts, 15 to 20 ma., one red spot moved 3 cm. toward the cathode followed by an incompletely separated fluorescent spot and a more slowly moving minor red spot. At pH 6, with one preparation of purified red pigment, no movement of the pigment was observed. With another preparation, three spots developed. Part of the red pigment was nonmobile, part moved to the cathode and part to the anode. The isoelectric point of the major red pigment was about 6.

Table VII. Comparative R_f Values of Onion Pigment and Anthocyanidins

Pigment	*BuOH-2N HCl*	*HAc-HCl-H_2O*	*m-Cresol-HCl*	*BuOH-HAc-H_2O*
Pelargonidin	0.80	0.68	0.82	
Peonidin	0.72	0.63	0.87	0.38
Cyanidin	0.69	0.50	0.69	0.24
Delphinidin	0.35	0.30	0.52	
Petunidin	0.45	0.45	0.75	
Malvidin	0.53	0.60	0.90	0.54
			m-Cresol-HAc	
Hirsutidin	0.73		1.00	0.51–0.55
Onion pigment	0.77	1.00	1.00	Streak

Paper Chromatography. The major red pigment eluted from the silica gel column was developed by one directional flow in several solvent systems. Butyl alcohol–2*N* hydrochloric acid, water–acetic acid–concentrated hydrochloric acid, *m*-cresol–2*N* hydrochloric acid, and butyl alcohol–acetic acid–water systems were tested. The R_f values obtained at room temperature are shown in Table VII in comparison with those reported for anthocyanidins (*3, 4, 7*). In general, the R_f values were much higher than those for known anthocyanidins.

By comparing the R_f values with the general tendencies of anthocyanidins reported by Bate-Smith (*3*), the red onion pigment (if it is an anthocyanidin type) would probably: be highly methylated, not be a glycoside, have very few free hydroxyl groups, or be acylated, the acyl group being nitrogenous.

The yellow pigment, present only in a very small amount, was investigated to a limited extent. It was not quercetin, the pigment of yellow and brown-skinned onions, and is probably not even a flavonoid. It did not change in color with ferric chloride, did not reduce ammoniacal silver nitrate, did not fluoresce—either in air or ammonia vapor—and did not change color in ammonia vapor. The following R_f values were found when the pigment was chromatographed at 20° C.:

	m-Cresol-HAc-H_2O	BuOH-HAc-H_2O
Yellow pigment	0.08	0.08
Quercetin	0.27	0.74
Rutin	0.26	0.58

Absorption Spectra. The absorption spectrum of the purified red onion pigment in water and in methanol is shown in Figure 6. The absorption spectra shown in Figure 1 include also those of the red pigment after storage at room temperature overnight. The stored solu-

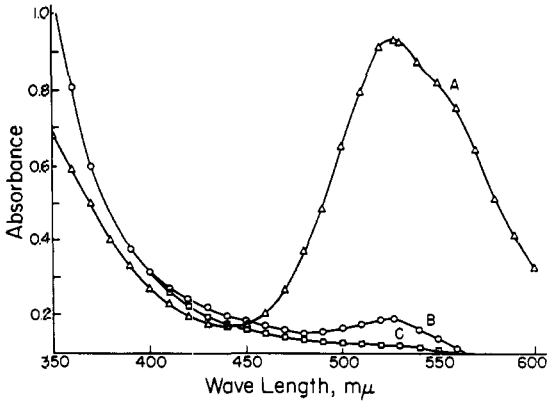

Figure 6. Absorption spectrum of purified pink pigment and brown decomposition products

A. Pink pigment
B. Brown pigment formed during storage
C. Browned pigment solution after percolation through Magnesol

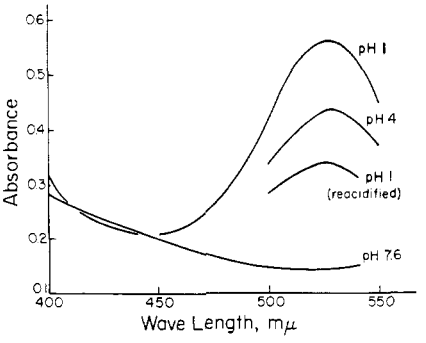

Figure 8. Effect of pH on absorption spectrum of pink pigment

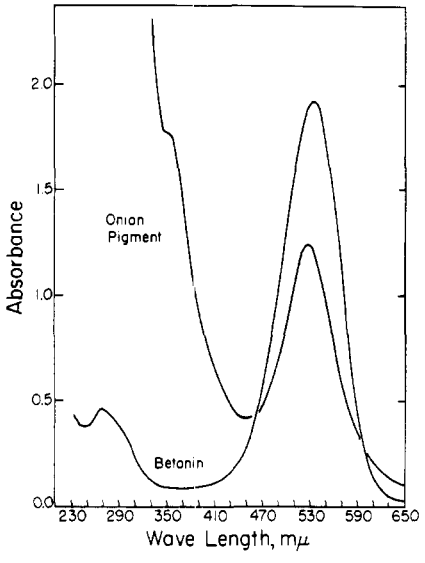

Figure 7. Absorption spectrum of purified onion pigment in comparison with that of purified betanin

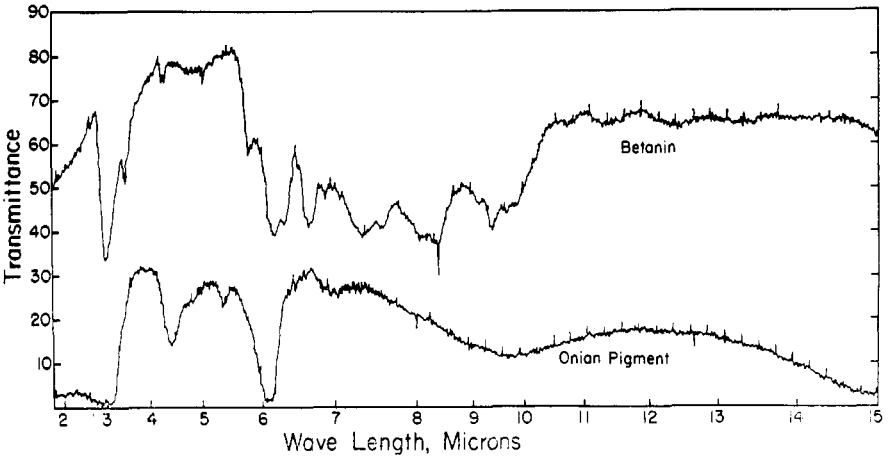

Figure 9. Infrared absorption spectrum of purified onion pigment in comparison with purified betanin

Table VIII. Qualitative Tests for Constituents in Onion Pigment in Comparison with Malvidin and Hirsutidin

	Onion Pigment	*Malvidin*	*Hirsutidin*
Oxidation test	Stable	Stable	Stable
$FeCl_3$ test	Negative	Negative	Negative
Cyclohexanol toluene extraction	Not extracted	Turns organic layer very light blue (slight)	
Aqueous solution	Violet-red	Violet-red	Violet-red
Na_2CO_3 solution	Yellowish orange eventually destroyed	Greenish blue	Greenish blue
Fehling's solution	Very slightly reduced, if at all	Reduces only when boiled	Reduces only when boiled

tion still contained some of the original red pigment, but it was brown in color. When this solution was filtered through Magnesol, the remaining traces of the red pigment were removed. In the visible range, it has a maximum at 528 mμ and resembles betanin more than any of the common anthocyanins (*60*, *61*). In absorption spectra and R_f values, it differs also from other flavonoids reported by Roux (*56*). As shown in Figure 7, the absorption spectrum of the purified onion pigment, both in the ultraviolet and the visible region, differs appreciably from that of purified betanin. Both pigments are red to purple in acid solution and yellow in alkali and both are accompanied in nature by closely related yellow pigments (*1*). Betanin was only recently crystallized and is similar to the onion pigment in nitrogen content (*59*, *60*, *67*).

As the onion pigment in water solution changes from red-violet color in acid to yellow in alkali, the effect of pH on shift in absorption spectrum was investigated. In acid solution, at pH 4 or 1 the absorption spectrum was similar to that in water but the height of the peak at 528 mμ was progressively decreased as the pH was increased from 1 to 7.6 (Figure 8). At pH 7.6, no maximum at 528 mμ was found, and the pigment changed in color abruptly to orange. On reacidification to pH 1, the absorption spectrum was similar to that found at pH 1, although the peak at 528 mμ was lower, as some of the pigment was destroyed by alkali. The pH shift was similar to that reported for malvidin and its glycosides by Sastry and Tischer (*58*). Significant changes in the ultraviolet absorption spectra such as those reported for flavonols investigated by Mansfield, Swain, and Nordström (*41*) between solutions in absolute ethyl alcohol and in sodium ethoxide could not be observed because of the rapid decomposition which occurred in alkaline solutions.

No shift in the absorption maximum in the presence of 0.2% aluminum chloride in an ethyl alcoholic solution of hydrochloric acid (*21*, *22*) was found with the onion pigment. Marked shift in absorption spectra of anthocyanidins and flavonoids hydroxylated in position 3′ and 4′ of the β ring (*21*, *22*, *56*) occur in the presence of aluminum chloride.

Marked differences between the onion pigment and betanin were observed in infrared absorption spectrum. Typical of these are the data shown in Figure 9.

Qualitative Color Tests. On the basis of the qualitative tests of Robinson and Robinson (*54*) for identification (Table VIII), the pigment resembles more closely malvidin or hirsutidin (highly methylated types), or the so-called nitrogenous anthocyanin of beets, betanin.

The ammonium molybdate test of

Blaschko (*11*) indicated that the pigment did not contain free vicinal hydroxyl groups. This is also true of the other three pigments (betanin is not methylated but the others are).

Acid hydrolysis did not yield free reducing sugars and enzyme hydrolysis (*25*), using Aspergillis Anthocyanase B CN 558 (Rohm & Haas Co., Philadelphia, Pa.), failed to occur. This, coupled with the fact that the R_f values are very high, indicates that the pigment is not a glycoside.

Organic Elementary Analysis. A sample of lyophilized red pigment (purified as much as possible, but still known to contain some of the colorless impurity) was analyzed with the following results: C, 53.0%; H, 10.6%; N, 3.6%; S, 0.8%; and OCH_3, 22.8%.

If the molecule contains one nitrogen atom, then the analysis allows only 0.1 sulfur atom. This then is obviously an impurity and the molecule is concluded to be sulfur-free. The sulfur content can be used as one criterion of purity. The value for hydrogen is more than twice as high as it should be for an anthocyanin —or any compound which is unsaturated enough to be so highly colored. This is easily explained by the pigment's great affinity for water. It probably held enough water to render the hydrogen assay unreliable.

Some pigment preparations had a high ash content, while others were very low. It is possible that high ash content was due to mineral constituents picked up from the Magnesol and that the low values are correct. However, the possibility that at least one of the red pigments formed is a metal chelate has definitely not been ruled out.

The nitrogen content varied with each preparation. The most reliable figures appear to be just less than 4%, but some samples contained as much as 8% nitrogen. The purified pigment in aqueous solution does not react with nitrous acid, even when heated. All of the nitrogen present is therefore tertiary.

The nitrogenous portion of the onion pigment may be similar to that present in the tyrosinase- and peroxidase-catalyzed oxidation of tyrosine from which dopachrome and other intermediates have been reported. These, however, differ in spectral characteristics. The red dopachrome exhibits maxima at 305 and 475 mμ, rubreserine at 300 and 480 mμ, and melanochrome at 300 and 540 mμ (*42*). These oxidation products of tyrosine in general are C_8 compounds which contain one atom of nitrogen. Amino hydroquinones and aminoquinones are known to form when *o*-diphenyl compounds are oxidized by phenolase in presence of amines and amino acids (*23, 30, 42, 43*) and amines may react directly with phenolic derivatives. In these cases, however, acid hydrolysis would be expected to liberate the amino compounds. The purified red onion pigment was hydrolyzed in 6*N* hydrochloric acid for a period up to 6 hours at 100° C., and aliquots of the hydrolyzate were chromatographed every half hour for amino acids and sugars. No reducing sugars or sugar derivatives were detected and no ninhydrin reactive constituents were found. A special effort was made to detect proline and hydroxy proline but these also were not found. The pigment itself is not ninhydrin positive, as no fluorescence develops when it is sprayed with ninhydrin. The color of the pigment did not change on heating after spraying with ninhydrin.

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Browning and the Amadori Rearrangement

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The browning of glucose with alanine, glycine, and phenylalanine in solution was compared with the browning of the Amadori rearrangement products of glucose and the corresponding amino acids. The rates of browning and the development of ultraviolet spectra for these systems were recorded. The amount of Amadori rearrangement products formed in a browning system was measured.

THE CHEMISTRY of the Amadori rearrangement and its significance for the nonenzymatic browning of food stuffs has been reviewed (*9*). Reducing sugars are known to condense with amino acids under anhydrous conditions (*1, 7*) and in highly concentrated aqueous solutions (*2-4*). Under these conditions, products from a number of amino acids have been isolated and characterized as Amadori rearrangement products. Richards (*11*) heated glucose and glycine in the dry state at 37° C. and pH 6.7 and obtained a product identified as the enolic form of the Amadori rearrangement product.

In a previous communication, evidence was presented that the Amadori rearrangement took place in bakery products (*10*). In the above references cited, anhydrous or nearly dry conditions prevailed. It was of interest, therefore, to determine whether the rearrangement occurs in dilute aqueous solution, a condition unfavorable to the formation of the nitrogen-substituted aldosylamines, the initial products of condensation. Evidence is presented that the rearrangement occurs in solutions 0.0425*M* in glucose and amino acid and contributes significantly to browning.

Materials and Methods

Amadori Rearrangement Products. 1-Deoxy-1-glycino-D-fructose (DGF), 1-deoxy-1-alanino-D-fructose (DAF), and 1 - deoxy - 1 - phenylalanino - D - fructose (DPhF) were prepared by the procedure of Abrams, Lowy, and Borsook (*1*).

Paper Chromatography. Whatman No. 1 paper, extracted for 24 hours with absolute ethyl alcohol to remove traces of reducing substances, was used. The solvent system of Gottschalk (*7*) (40, 10, and 50; *n*-butyl alcohol, acetic acid, and water) was suitable for these compounds.

Analytical Methods. The absorbance measured at 500 mμ in a Beckman Model DU spectrophotometer was used as a measure of color formation. Development of absorption in the ultraviolet region was also followed with this instrument. Browning observations were made in solutions which were 0.0425*M* in carbohydrate and 0.1*M* in phosphate buffer (pH 6.8), and at a temperature of 93° C.

The method of Abrams, Lowy, and Borsook (*1*) for the determination of Amadori rearrangement products was used with the following modifications. The sample was reacted with 0.1% potassium ferricyanide for 30 minutes at 30 ± 0.05° C. instead of for 5 minutes at room temperature. The changes increased the sensitivity of the method approximately threefold and made it considerably more precise. A straight line was obtained when absorbance was plotted against concentration.

The method as revised is not likely to be specific for Amadori rearrangement products or reliable with a mixture of compounds. However, in this case the compounds were first separated by paper

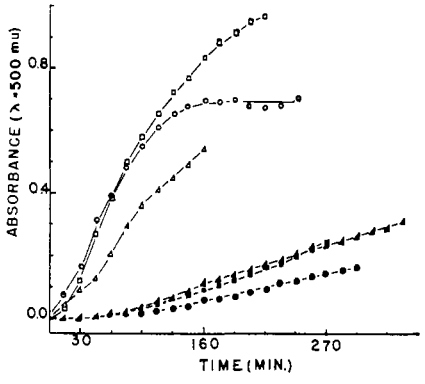

Figure 1. Browning of glucose with three amino acids compared with the corresponding Amadori rearrangement products

○ DAF ● Glucose and alanine
□ DGF ■ Glucose and glycine
△ DPhF ▲ Glucose and phenylalanine

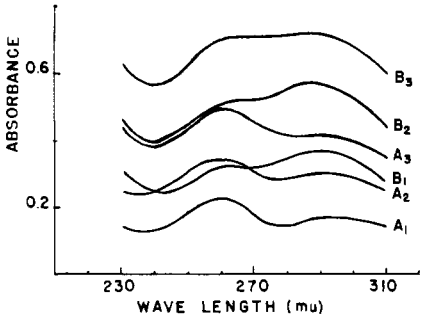

Figure 2. Comparison of ultraviolet spectra of heated DPhF (*B* curves) and of heated solution of glucose plus phenylalanine (*A* curves). Heating times in minutes: A_1-135, A_2-225, A_3-285, B_1-45, B_2-75, and B_3-165

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