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FOOD DISCOLORATION

Reddening of White Onion Tissue

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Factors affecting the formation of the water-soluble, red nitrogenous pigment in acidified white onion purée were investigated. The presence and absence in onions of several compounds are reported. Added acetic acid was not necessary for pigment formation, and its great enhancing effect was traced to the 1 to 3 p.p.m. of formaldehyde which occurs in reagent glacial acetic acid as an impurity. Other similar compounds such as diacetyl, acetoin, and glycolonitrile can replace formaldehyde in the reddening reaction, and studies were made in an effort to identify the naturally occurring carbonyl compound. A type-reaction mechanism for the pigment formation is postulated and discussed.

A WATER-SOLUBLE RED pigment is formed in macerates of white onion bulb tissue when the purée is allowed to stand after acidification with acetic acid. In an earlier paper (*5*), it was shown that the pigment formation in purées was affected by variety, storage conditions, and heat and acid treatments.

New evidence affecting pigmentation has been found, and it has been possible to develop the problem considerably. The acetic acid specificity had been fully explained and, along with this, a general type sequence of steps is postulated. These are presented in this paper.

Experimental

Southport White Globe onions obtained from the same sources as previously reported (*5*) were used, as well as some onions from the same lots used in past work. In addition, one lot grown in Washington State was obtained. No significant difference was found between those from Washington and those from the Stockton region of California.

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Methods of preparing the samples, adding reagents, extracting, and measuring the pigment formed were identical with those previously reported.

Properties of the Pigment

The mpure pigment, prepared in the usual manner, could be purified further by adding sodium hydroxide until the aqueous-alcoholic solution reached pH 9.5. The pigment remained in solution, but a colorless, amorphous nitrogen-free precipitate settled out. After filtering rapidly, the clear solution was reacidified to avoid excessive destruction of pigment at the high pH. Acetone was added and the precipitated salt filtered off. Then the pigment was precipitated by ether addition. The solid was redissolved in absolute ethyl alcohol, filtered, and reprecipitated with ether to remove the remaining traces of inorganic salts. The solid preparation was then lyophilized for 24 hours and used immediately.

The infrared absorption spectrum of the pigment, prepared as above, was determined by using the potassium bromide pellet technique. The data obtained are plotted in Figure 1.

The same pigment preparation was used for organic elementary analysis, with the following results:

Element	Per Cent
C	27.96
H	6.70
N	7.88
O	44.06
Ash	13.4

A comparison of the infrared data and the elementary analysis with the previously published data (*5*) shows the relative purity of the two preparations.

The pigment coupled readily with diazotized sulfanilic acid, forming a compound which was orange in acid and red in base. The diazonium salt was prepared in the classical manner by dissolving 10.5 grams of sulfanilic acid in 100 ml. of 2.5% sodium carbonate by boiling. After cooling the solution, 3.7 grams of sodium nitrite were added, stirred until dissolved, and poured into a beaker containing 50 grams of ice and 10 ml. of concentrated hydrochloric acid. For coupling, sodium hydroxide was added to the aqueous solution of the onion pigment, then the diazotized sulfanilic acid suspension was added, stirred, and allowed to stand. The red solid was filtered off, redissolved in 95% ethyl alcohol, giving an orange solution, and precipitated as a yellow oil by ether addition. After redissolving in absolute methanol, the material was precipitated as an orange solid by addition of ether.

Factors Affecting Pigment Formation

During the first stages of this research, a difference was observed in the rate of pigment formation which depended upon the time elapsed between puréeing and acidifying the tissue. Active precursors of the pigment appeared to form as soon as the tissue was puréed, and the addition of acetic acid caused the precursor already formed to react, forming the red pigment. At the same time, formation of more precursor completely stopped.

Assuming, for simplicity, that there are only two steps involved in pigment formation—the change which occurs immediately after puréeing to form the precursor, and the coupling of the precursor with another compound in the presence of acetic acid—an attempt was made to study the first step.

Sixteen hundred grams of purée were separated into four 400-gram lots. Each was held at 22°, 40°, 50°, or 60° C. for 1¼ hours, cooled to room temperature, and divided into eight 50-gram portions, and varying amounts of acetic acid were added to each. The visual color, pH, and Klett reading were measured after 48 hours at room temperature. In every case the color was increased with increased acetic acid concentration and there was a definite temperature optimum for the first step near 50° C.

Samples of fresh purée were heated rapidly to 50° C. in a hot water bath and stored at 50° C. for varying lengths of time. They were then cooled, acidified to pH 2.7 to 2.8, and allowed to redden at room temperature for 24 hours (Table I).

The initial step was apparently unaffected by calcium ion, but was inhibited by acetic acid and lead. Blending with calcium chloride or addition of calcium salt immediately after puréeing had no effect on color formation, but addition of lead acetate or acetic acid before blending or immediately after puréeing inhibited color formation.

Effect of Enzyme Treatment

The first step might seem to be enzymatic, but none of the usual enzyme inhibitors, except sodium cyanide, had any appreciable effect when added to fresh purée. A number of different enzymes from other sources were added to fresh purées. Whether these were added to fresh purées or to freshly steam-blanched purées, the results were identical. None of the following enzymes added to the purée, at a level of 0.1 gram per 100 grams of purée, had any effect upon pigment formation, when the mixture was incubated for 3 hours at 35° C., followed by acidification and storage at room temperature.

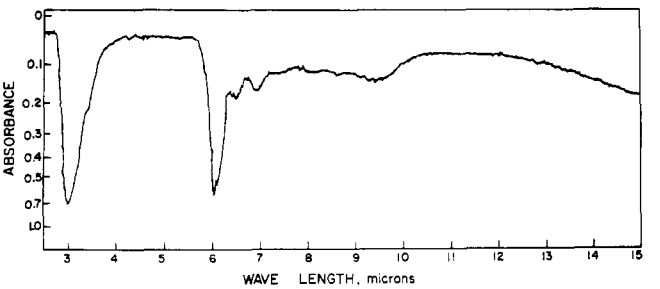


Figure 1. Infrared spectrum of the purified onion pigment

Pectinase	Anthocyanase	Hydralase	Peroxidase
Pectinol	Bromelain	Mylase P	Tyrosinase
Emulsin	Diastase of malt	Rapidase	Acid phosphatase
Clarase	Papain	Taka-diastase	β-Glucosidase
Mylase	Multizyme	Catalase	Pepsin
Trypsin			

The above enzymes were also added to dehydrated onion preparations obtained commercially; none had an appreciable effect on pigment formation.

An attempt was then made to isolate a possible enzyme occurring naturally in the onion tissue which was catalyzing the initial reaction. As reported previously (*5*), the whole pinking system is soluble and when onion juice was dialyzed for 48 hours, then acidified with glacial acetic acid, no pigment formed.

Freshly prepared purée was frozen at −15° C., thawed, and filtered cold. Acetone at 0° C. was mixed with the cold juice to a final concentration of 50%. The precipitate was washed with cold 50% acetone. More material was precipitated at 75% acetone and washed as before. A third solid preparation was obtained by thawing frozen purée directly in acetone solution, to give a final acetone concentration of 50%, and filtering. The various soluble fractions were used in the following manner.

Onions were peeled and sliced, and the slices were steam-blanched for 5 minutes to inactivate all natural enzymes. The material, after being puréed, was divided into samples for mixing with the acetone-precipitated preparations described above. The purées were mixed and stored for 1 hour at 50° C., cooled, acidified to pH 3, and stored at 20° C. for possible reddening. None of the solid preparations added caused reddening. When the 50% acetone solution was evaporated to less than half its volume in vacuo, to remove most of the acetone, it began to redden. With added acetic acid, it became a vivid pink in 2 hours at 20° C.

Acetic Acid Specificity

Figure 2 shows the amount of pigment formed in a typical experiment in relation to the amount of acetic acid added and to the pH of the final solution. The points are corrected for volume changes due to the large amounts of acetic acid required to lower the pH in the lower ranges. Obviously, the acetic acid concentration is the important factor and not the pH. Because previous experiments had shown conclusively that neither acetate concentration nor hydrogen ion concentration per se was limiting, it was suspected that the reddening was caused by an impurity in the acetic acid used.

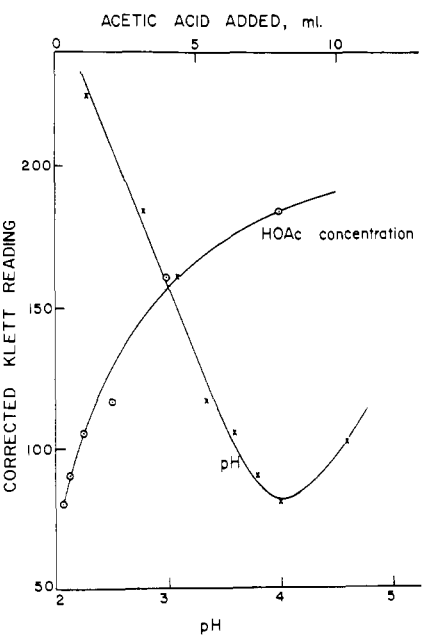


Figure 2. Onion tissue reddening as a function of acetic acid concentration and pH

Table I. Rate of Formation of Precursor

Storage at 50° C., Min.	Klett Reading	Visual Color[a]
0	50	White
10	214	(Pink) ++
20	193	++
40	276	+++
80	284	+++
160	355	++++
320	187	+

[a] Number of plus signs indicates degree of redness.

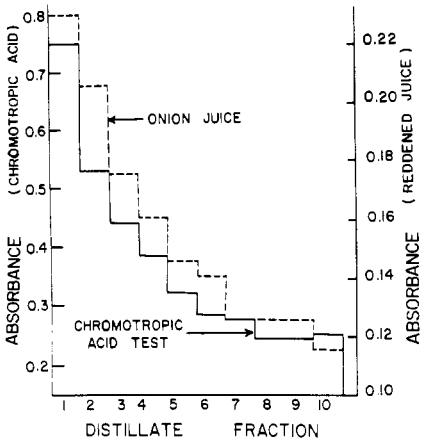


Figure 3. Comparison of onion tissue reddening in the presence of various distillate fractions of acetic acid with the absorbance of chromotropic acid tests of the same acetic acid fractions

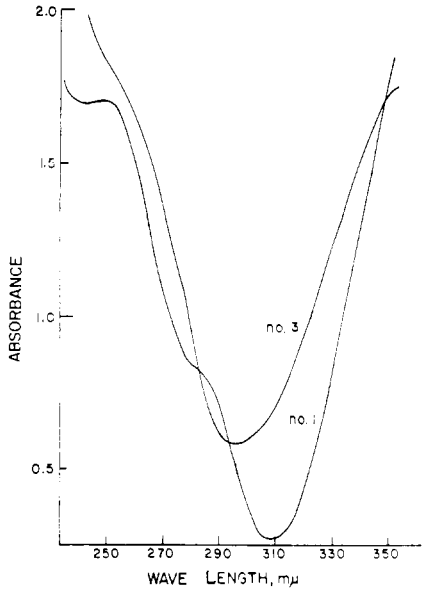


Figure 4. Ultraviolet spectra of DNP derivatives of naturally occurring carbonyl compounds

A "pure" glacial acetic acid was prepared from sodium acetate and sulfuric acid. This was used as a control by adding equal amounts of it and "commercial" reagent glacial acetic acid to identical purée samples. The pure sample did not redden, while the commercial sample did. In addition, the pure acetic acid was found to be chromotropic acid negative—the commercial was positive.

A fractional steam distillation of commercial acetic acid was carried out. One liter was steam-distilled at a rate of about 1 ml. per minute. Ten 25-ml. fractions were collected and 1 ml. of each was mixed with 0.5 ml. of 10% chromotropic acid and 5 ml. of sulfuric acid. After heating at 100° C. for 30 minutes, they were cooled and the absorbance read in 1-cm. tubes at 570 mμ.

Table II. Absorbances of Chromotropic Acid Tests (570 Mμ)

Distillate Fraction	Fresh Purée	Steam-Blanched	Held at 50° C. for 1 Hour	Reddened Purée
1	0.85	0.24	0.58	0.58
2	0.46	0.20	0.44	0.38
3	0.34	0.175	0.36	0.27
4	0.315	0.17	0.27	0.25
5	0.25	0.15	0.22	0.23
6	0.235	0.14	0.17	0.20
7	0.22	0.13	0.16	0.17

Two hundred and fifty milliliters of fresh onion juice were prepared, heated at 50° C. for 1 hour, cooled, brought to pH 3.5 by the addition of sulfuric acid, and divided into ten 25-ml. fractions. Five milliliters of each of the acetic acid distillate fractions were added to the corresponding samples of juice and after reddening, they were filtered and the absorbance was read at 550 mμ. The results are plotted along with the distillate chromotropic acid tests (Figure 3). A complete study of the acetic acid impurity was undertaken and it was shown to be formaldehyde (*12*). An optimum formaldehyde concentration for reddening near 3 p.p.m. was found.

Several other carbonyl compounds, including both diacetyl and acetoin, methyl ethyl ketone (but not acetone), and glycolonitrile also gave red pigments in onion tissue under similar conditions. The compound 5-hexene-2-one yielded a blue-green pigment; other carbonyl compounds (acetaldehyde, acetoacetic ester, and dihydroxyacetone) were inhibitory. Hydrogen peroxide also increased pigment formation during the second step. Hydrogen peroxide was added to the purée after acidification (formaldehyde addition), showing an optimum for pigment formation at 0.002 *M* hydrogen peroxide. Hydrogen peroxide, like acetic acid, inhibited the first step.

Carbonyl Constituents

Onion purée, stored without added formaldehyde for nearly 1 week, formed a very small amount of red pigment. This formaldehyde-free, red onion pigment was isolated in the same manner as the previous pigment. The absorption spectrum differs slightly from that of the formaldehyde pigment in the visible (*14*), and is quite different in the ultraviolet (*11*). Thus, formaldehyde does not occur naturally, even in traces, in these onions. However, the red pigments formed from the addition of diacetyl, etc., to the purées were not studied further and it is assumed that one of these carbonyl compounds occurs naturally and reacts with the precursor formed in the first step to give the red pigment. Lukes (*8*) showed that microbial contamination was not involved in onion reddening. The naturally occurring carbonyl compound was studied by the following procedure.

Two hundred grams of fresh onion purée were diluted with 300 ml. of water and steam-distilled at a rate of 5 ml. per minute. Ten 25-ml. fractions were collected and tested with chromotropic acid as before. Fraction 1 was turbid and had a strong onion odor. Fraction 2 was less turbid, etc., until fraction 7 was clear. The chromotropic acid results were similar; number 1 was a brownish purple, number 2 light brown (no purple), and the others were successively less positive. All of the substances giving a purple chromotropic acid color were in the first fraction. Each fraction was added to 50 ml. of a saturated solution of dinitrophenylhydrazine (DNP) in 2*N* hydrochloric acid and allowed to stand. After 24 hours, the orange precipitates in the first four fractions (no precipitate formed in the others) were filtered off, washed, and dried. The combined solids were then dissolved in a very small amount of benzene–petroleum ether (40 to 60) and the solution was passed over a dry silicic acid–Hyflo (1 to 1) column. Elution with the same solvent mixture gave three bands: number 1, buff colored; 2, yellow; and 3 (at the origin), dark orange.

The column was extruded, the bands were cut off, and the hydrazones present were eluted with acetone, precipitated with water, and recrystallized from hot 95% ethyl alcohol.

Hydrazone 1 formed red-orange needles, melting point 158–9° C. The ultraviolet spectrum is given in Figure 4, although no further identification was made.

Hydrazone 2 formed orange needles, melting point 154–5° C. Mixed melting point with the pure derivative of propionaldehyde showed no depression. The reported existance of propionaldehyde in onion tissue (*7*) is therefore confirmed.

Hydrazone 3 was present in such small amounts that a definite melting point could not be obtained. The ultraviolet absorption spectrum of its dilute solution in 95% ethyl alcohol is given, however.

It is not known whether one of these volatile carbonyl compounds is responsible for the positive chromotropic acid test in onion purée. To determine whether the chromotropic acid positive fraction changed during the process, four stages of reddening were investigated: fresh onions, purée held at 50° C.

for 1 hour, purée held at 50° C. for 1 hour and then allowed to redden, and purée from steam-blanched fresh onions. Two hundred grams of each of these purées were steam-distilled, as before, and after comparing their chromotropic acid tests (Table II), the DNP derivatives of each distillate sample were prepared.

A visual comparison of the curves for the first few distillate fractions indicated that there might be a volatile inhibitor and a nonvolatile reactant present in onion tissue. The inhibitor, being more volatile than the other compounds present, was lost on storage at 50° C. allowing the added formaldehyde or a similarly acting compound to combine with the reactant to give the pink pigment. It is clearly seen that no steam-volatile compound was used up during the reddening reaction, although volatile compounds are present.

Nitrogenous Constituents

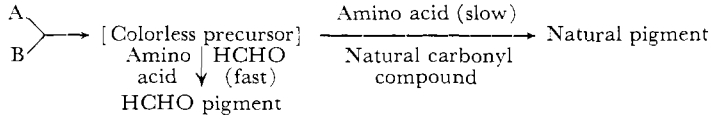
Which amino acids enter into the pinking reaction is not certain. Lukes believes that glycine is the most likely, although other amino acids are able to form similar pigments (*8*). Earlier (*5*, *10*) it was shown that at least three pigments were formed under the present conditions. All the experimental work has dealt with the major pigment.

One kilogram of fresh onion purée was clarified by pectinase treatment, then filtered until a clear juice was obtained. This was divided into three parts; one was passed over a 1 × 10 inch anion exchange column (Duolite A-2), one over a cation exchange resin (Duolite C-3), and the third was given no ion exchange treatment. All three were then acidified with glacial acetic acid and stored at 37° C. for reddening. The cation-free onion juice was colorless, while the anion-free and control samples reddened after 12 hours.

Following the general procedures used by Hulme (*4*), onion juice was passed over a Zeokarb H cation exchange resin. After thorough washing, the column was eluted with 0.5*N* ammonium hydroxide. The ammonia eluate was evaporated to less than 10 ml. on a hot plate, at which point crystals of tyrosine began to form in the neutral solution. The solution was cooled, filtered, and passed over a Dowex 50 × 4 cation exchange column (1 cm. in diameter × 70 cm. long). Elution was carried out by using a 0.2*M* borate buffer at pH 8 and containing 0.1*M* sodium chloride. One-milliliter fractions were collected; Figure 5 shows the ninhydrin reaction plotted *vs.* the fraction number (*15*). In Figure 5, 1 kg. of onion juice was used, the ammonia eluate was reduced to exactly 10 ml. (after removal of excess tyrosine), and 0.5 ml. was used on the final Dowex column.

None of the compounds in the bands was found to react with formaldehyde in acetic acid solution to give a red pigment. Band *A* was shown by usual paper chromatographic methods to contain tyrosine; band *B*, histidine. The actual concentration of these amino acids present was determined by the α-nitroso-2-naphthol method of Ottaway (*9*) for tyrosine and the diazotized sulfanilic acid method of Fraenkel-Conrat and Singer (*3*) for histidine. It was found that 35 mg. of tyrosine and 21 mg. of histidine occur in 1 kg. of the Southport White Globe onions used in this study. The complete absence of arginine was shown by the α-naphthol–hypobromite reaction of Acher and Crocker (*1*). (The original Zeokarb column was eluted with 0.1*N* sodium hydroxide, in this case, to ensure the removal of any arginine present.) Proline and hydroxyproline were also absent. In model systems, no definite effect was detected in any case, although methylhistidine, sarcosine, canavanine, ornithine, citrulline, histamine, tyramine, prolylglycine, anserine, and carnosine were added to the purée in addition to all the "common" amino acids. None gave a red color with formaldehyde in acetic acid solution and none resulted in an increase in pigment formation when added to onion purée, either before or after a 50° C. holding period. None of the proteolytic enzymes (papain, pepsin, trypsin, bromelain) added to onion purées exerted any effect upon pigment formation. Obviously, amino acid concentration is not a limiting factor.

The simplest total system possible is shown below:



The evidence presented above makes it questionable whether any step is enzymatic. If not, compound A or B must be heat-labile. Both the colorless precursor and the naturally occurring carbonyl compound are extracted by ether and neither A nor B is extracted (*8*). Step 1 is inhibited by acetic acid, hydrogen peroxide, and lead salts. The maximum colorless precursor is formed in 2 hours at its optimum temperature, 50° C. The last step must be compound, because Lukes finds the amino acid to be the only component missing from the ether extract. The present authors find formaldehyde to be the only component missing from the incubated purée. Acetic acid and hydrogen peroxide increase the speed of step 2.

Varietal Differences

Differences in reddening tendencies of different varieties of onions have been reported (*5*). Other plants closely related to the onion also undergo spontaneous coloring reactions. It has been known for some time that garlic (*Allium sativum*) bulbs, when puréed and acidified with acetic acid, become green (*2*, *13*). Joslyn and Sano (*6*) showed that the green pigment formed did not behave like chlorophyll and was not formed by the action of a naturally occurring phenolase or peroxidase. In the light of the onion work described here, it became desirable to determine whether formaldehyde entered into the garlic greening system.

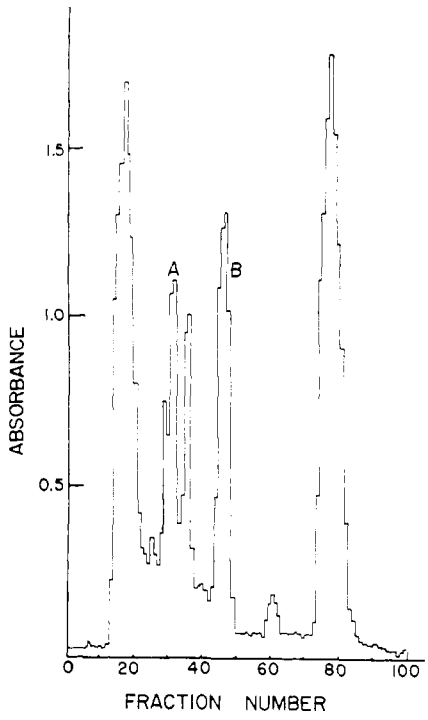


Figure 5. Amino acids in Southport White Globe onions

Two hundred and twenty grams of peeled garlic cloves were puréed and held at 50° C. for 5 minutes. After cooling, 20 ml. of formaldehyde-free acetic acid were added and it was divided into two lots; 0.1 ml. of 0.1*M* formaldehyde was added to one. The dark green pigment formed in both in about 2 hours at 20° C. at the same rate and to the same extent. Fifty grams of fresh garlic juice were treated as above, except that no acetic acid was added to either sample. Twenty-four hours were required for an appreciable amount of green pigment to form in the samples, and again, the formaldehyde sample contained the same amount of pigment as did the blank. The acidified samples were both dark green, while the unacidified samples were both very light green. Formaldehyde alone, or in the presence of acetic acid, had no effect upon garlic greening.

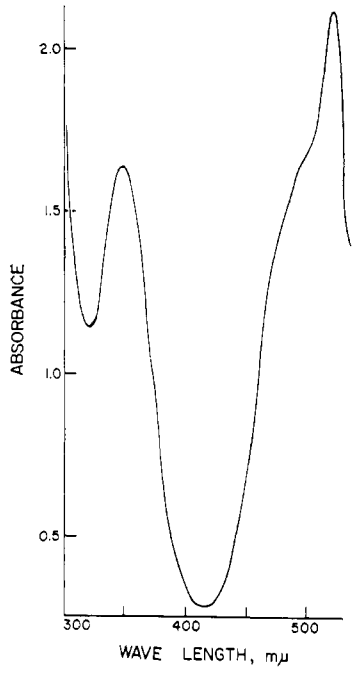


Figure 6. Absorption spectrum of pink solution obtained from *Allium giganteum* juice

Leaf and stem samples of *Allium giganteum* and bulb, stem, and flower samples of *Allium christophii* (both central Asian species) were crushed with and without acetic acid and with and without formaldehyde. The *christophii* samples remained white and no water-soluble pigment formed in the leaf tissue of *giganteum*. A mercaptan odor was given off immediately after cutting the *christophii* bulbs. The flowers are similar but larger than typical onion flowers and contain a small amount of preformed red pigment. This pigment was not identified, and no additional pigment formed after crushing. *Giganteum* leaves yielded a strong manure-like odor when cut. The stems became red-orange in a very few seconds after cutting. The juice came rapidly to the cut surface and reddened almost immediately. This juice was filtered and passed over Magnesol, giving one pink and one orange band. These were eluted readily by acidified 95% ethyl alcohol and the absorption spectrum of the pink solution is shown in Figure 6. A similarity to the onion pigment is noticeable in the visible region, but the ultraviolet absorption is very intense and is probably due largely to impurities. The pink solution turned brown on storage in air at room temperature for a few hours.

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STARCH ANALYSIS

Determination of Amylose in Starch

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Congo red is used colorimetrically for the determination of amylose in starch. Adsorption of the dye is independent of the chain length of the polysaccharide over a wide range. The extinction coefficient of the adsorbed dye is the same for both amylose and dextrin. The results are discussed and compared with those obtained from the usual iodine sorption and colorimetric methods.

THERE ARE SEVERAL METHODS for the determination of amylose in starch. Some of these methods (*4*, *8*) are based on analysis of the hydrolysis products, which procedure is often time-consuming. Iodine has been used colorimetrically (*1*, *6*, *7*). The latter method is based on the fact that amylose binds iodine, whereas amylopectin does not. Optical measurements of the iodine-starch mixtures are usually made at a fixed wave length, in the vicinity of 610 mμ, which is approximately the absorption maximum of the mixture. Iodine interaction depends not only on the degree of branching, but also on chain length, thereby leading to conflicting analytical results (*5*). It seems desirable to obtain another indicator which does not depend on chain length, although responding to changes in the degree of branching in the starch. Congo red appears to be a suitable adsorption indicator for this purpose.

Experimental

Materials. A histological grade of Congo red (National Aniline Division, Allied Chemical and Dye Corp.) was used, without further purification. Buffers were prepared from reagent grade potassium hydrogen phthalate and so-