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CAROTENOIDS IN CORN GLUTEN

by

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INTRODUCTION

The endosperm protein, or gluten, of corn is obtained in large quantities as a by-product in the manufacture of starch. Although potentially a cheap source of protein for industrial use, the material has received but little commercial exploitation other than its use in feeds. One of the principal reasons for this lack of commercial utilization has been the difficulty encountered in purifying the crude gluten. This difficulty may be ascribed to (a) lack of homogeneity in the protein itself, (b) the presence of considerable amounts of fine-granule starch not separable by the customary mechanical methods, and (c) the fact that the color of yellow corn, consisting of carotenoids, appears to have an affinity for the protein, giving it an undesirable yellow cast.

So far as is known, the only purification process now in use commercially is that used to obtain zein. Advantage is taken of the fact that this protein is soluble in aqueous alcohol to separate it from all other materials except the color and the sterols. Presumably these are then removed by treatment with a solvent such as benzene or gasoline. This process gives a pure material, eminently suitable for many uses. However, the numerous technical difficulties inherent in any such solvent extraction procedure lift the protein out of the low price class wherein lies the largest market. It is evident that there is still a need for a cheaper purification procedure.

This problem of the purification of the corn proteins has been included as part of a general research program dealing with the milling of corn. An examination of the literature indicated that much work of a fundamental nature was necessary to furnish a logical basis for the projected studies. Of the corn proteins, only zein has been adequately characterized. A great deal is known about the pure carotenes, but the nature of the transformations which they undergo under the influence of various reagents has not been satisfactorily established.

Of this preliminary work only that relating to the carotenoids is included here. This work was intended to furnish a foundation for possible solutions to the specific problem of color removal. In particular it was desired:

1. To determine if the carotenoids are the only coloring matter present in the gluten.

2. To determine, by means of chromatographic analysis, if the carotenoids are altered when the gluten is processed by present commercial methods.

3. To investigate briefly any hitherto untried methods of approach to the bleaching problem.

The first and last of these objectives were relatively easy of attainment, but the study of the isomerized pigments proved to be much more complex than was anticipated. Reference to the literature indicated that some of the pigments were extremely labile in character. Experiment showed that others were almost identical in many of their properties.

Attempts to use the customary methods of carotenoid analysis for the isolation of these pigments revealed that extensive modifications of these methods were necessary before they could be applied in the present work. This phase of the problem resolved itself into a general study of the isolation and properties of isomerized pigments. Suitable modifications in existing methods were devised and purified zeaxanthin, the chief corn pigment, was isomerized under controlled conditions in order to aid in the identification of some of the many new pigments which were found present in treated corn gluten.

REVIEW OF LITERATURE

Since the carotenoid pigments invariably occur in minute quantities, their chemical constitution could be satisfactorily investigated only with the advent of the newer techniques of microchemistry, especially Tswett's method of chromatographic analysis which was rediscovered by Kuhn, Grundmann, Zechmeister and Palmer. The basic formula of the carotenoid molecule is now definitely known to consist of an aliphatic carbon chain of conjugated double bonds terminated at each end by a six-membered carbon ring. The known carotenoids differ chiefly in the type of substituents found on the terminal rings, hydroxylated derivatives constituting the xanthophyll class and unsubstituted hydrocarbons the carotene class.

The literature on carotenoids has been admirably summarized in a number of recent publications. The chapter on carotenoids (8) in Gilman's "Treatise on Organic Chemistry" is valuable for its discussion of the theoretical aspects. Zechmeister's manual (26) on chromatographic adsorption and Strain's monograph (24) on the leaf xanthophylls are particularly useful for their descriptions of experimental techniques. All three give quite complete references to the original literature. Palmer's book (20) completely describes the earlier work on this subject.

Carotenoids in Corn

Only three carotenoids--zeaxanthin, cryptoxanthin, and beta-carotene--are known to be present in yellow corn. A number of workers including Holmes and Leicester (11), Randolph and Hand (22), Myburgh (19), and Clarke and Gring (4) have determined the quantities of these pigments by colorimetric methods without actually isolating crystalline pigments. Karrer (12, 13), using classical chemical methods, was the first to isolate the main pigment of corn--zeaxanthin.

A few years later Kuhn and Grundmann (15) showed by means of the chromatograph that cryptoxanthin (and possibly a trace of beta-carotene) were the only other pigments present. From a kilogram of Italian corn they isolated 14 milligrams of zeaxanthin and 1.4 milligrams of cryptoxanthin. Buxton (1) has recently confirmed the presence of these pigments although it is not clear whether he actually obtained the beta-carotene in the solid form.

Chemical Changes of Carotenoids

Prior to the general use of the chromatograph for separating pigments, it had been known that the carotenoids were readily altered by a variety of agents. Completely oxygenated and halogenated derivatives had been prepared, and the fact that acids, heat and light would cause changes had been noted, although the products were not identified.

In one respect the chemical reactivity of the carotenoids is of prime importance since by some unknown reaction the beta-carotene molecule splits in two with the simultaneous addition of water at the point of rupture, thus forming two molecules of vitamin A.

Oxidation

In his book on chromatographic analysis, Zechmeister has summarized the known products which result when peroxides or chromic trioxide react with the various carotenes. In general, the first point of attack is at the rings. Hydroxyl, aldehyde or ketone groups can be formed, after which the rings may be ruptured.

The addition of oxygen from the air may occur in either of two distinctive modes. The first is a slow addition, requiring days or even weeks for completion depending upon the physical state of the carotenoid. At completion the amount of oxygen added corresponds with the number of double bonds. No study of any of the possible intermediate products has been reported in the literature.

Karrer (13) found for zeaxanthin crystals in contact with air a percentage increase in weight of only 6.5% after twenty days, and a 36.7% increase after 120 days. Strain (24) adsorbed zeaxanthin on cotton and after exposure to air extracted and chromatographed the pigment. He states that a number of bands developed, but did not carry the experiment further. Kuhn and Brockmann (14) state that air-oxidized beta-carotene is partly converted into substances which pass into the methyl alcohol phase in partition. The fraction left in the petroleum ether phase is much more strongly adsorbed than is the unchanged beta-carotene. Other investigators have noted the presence of traces of strongly adsorbed pigment layers in the chromatogramm, but dismiss them with the statement that they were probably oxidized material.

The second mode of oxygen addition occurs in the presence of an enzyme. In contrast to the slowness of the above reaction, carotenoids are decolorized almost instantly, in the presence of this enzyme. Haas and Bohn (9) have patented the use of such an enzyme made from soybeans for the bleaching of bread dough.

Quite recently Sumner and Dounce (25) have investigated this enzyme. They conclude that it is an aerobic oxidase and show that it will oxidize the double bonds in unsaturated oils (except castor oil) as well as those of the carotenoids. The enzyme is active between the pH values of 5.9 and 7.1, with a slight optimum at 6.5. It is readily destroyed by heat.

From his work with etiolated seedlings, Strain concludes that they contain enzymes which bleach the carotenoids, but he did not continue with a study of the oxidized products. However, his experiments emphasize the necessity of suitable precautions against such enzymes in any work with the carotenoid pigments. As may be concluded from the persistence of the color in corn, it does not contain the enzyme.

Changes caused by chromatographic adsorption

Since the chromatograph is such a delicate tool, it offers the easiest and often the only method by which partly isomerized colored products can be isolated. Consequently it is of fundamental importance that the adsorption process itself does not alter the pigment. A number of investigators claim that adsorption does cause partial isomerization of the pigments. For example, both Shinn and coworkers (23) and Strain (24) claim that certain adsorbents, particularly strongly active ones, will cause isomerization. No experimental evidence is offered.

It is, of course, true that adsorbents contaminated with oxygen, oxidizing materials or acid will cause destruction of the pigment, but such conditions may be easily remedied. The work of Holmes and Corbett (10) on pure vitamin A is the only experimental evidence found to indicate that such isomerization may take place. According to their results all of the commonly used adsorbents, with the exception of iron-free silica gel, will give rise to colored products from vitamin A in the presence of some of the usual solvents. Such color production was occasioned by activated alumina, magnesium oxide, and especially iron-containing materials, in the presence of benzene, cyclohexane, or ether.

Since the carotenoids are much more stable than vitamin A it does not follow that the above results are directly applicable to these pigments, but it does suggest that highly active adsorbents should not be used with strongly adsorbed pigments. Such a combination has the further disadvantage that elution is hindered and drastic conditions are required. It is highly probable that most of the apparent isomerization ascribed to the chromatographic process is in reality due to other factors, as described in the following paragraphs.

Isomerization due to heat and light

There is no general agreement as to the stability of carotenoid solutions exposed to light. While Clausen and McCord (5) state that solutions fade somewhat even in a few hours, McNicholas (17) found that the absorption spectra of a carotene solution exposed to diffuse sunlight changed but little during the first month. After this time the bands became quite broad or disappeared entirely. Alcohol solutions of xanthophylls are particularly stable, according to Strain.

The peculiar susceptibility of the carotenoids to heat has long been recognized, but it is only in the past few years that the exact nature of the reaction has received an adequate explanation. It has now been definitely established that all carotenoids, upon solution, undergo a reversible isomerization process with the formation of an equilibrium mixture containing one or more new pigments. The process is quite slow at icebox temperatures but rapid in warm solutions. The isomerized pigments can be separated by the chromatograph and usually crystallized, after which each, if dissolved and heated, will again give the original equilibrium mixture.

Gillam and coworkers (6, 7) were originally misled by this phenomenon and believed that the adsorption process was the causative factor. Using pure beta-carotene, they found that repeated adsorption would finally result in the formation of a new pigment, forming a band below the original. The new material was isolated in crystalline form, and when subjected to repeated adsorption it changed back into the original beta-carotene.

Zechmeister's (27, 28, 29) later work showed that the formation of the new pigment is not caused by the adsorption process but rather by the heating required to concentrate the carotene solution between each run. For example, he refluxed pure zeaxanthin in benzene and upon chromatographic adsorption of the cooled solution found that three bands of pigment were in evidence. Of these the lowest and largest was zeaxanthin in unaltered condition. The other pigments, after elution and crystallization, both gave unsharp melting points lower than zeaxanthin and absorption maxima at shorter wave lengths. The most strongly adsorbed layer, neozeaxanthin A, is strongly dextrorotatory in contrast to the original levorotatory zeaxanthin.

A summary of Zechmeister's work includes the following additional facts:

1. The isomerization reaction is catalyzed by a trace of iodine.

2. The reverse reaction is more rapid than the forward reaction.

3. With hypophasic pigments (zeaxanthin) the new bands are above the original; with epiphasic pigments (cryptoxanthin and beta-carotene) they are below.

4. The tendency toward isomerization increases in the order beta-carotene, cryptoxanthin, zeaxanthin.

5. Alumina cannot be used to separate zeaxanthin and its isomers.

6. The equilibrium zeaxanthin mixture gives only one band of pigment after esterification.

7. A solution of any of the zeaxanthin pigments should not be kept longer than one day if appreciable isomerization is to be avoided.

8. Isomerized pigments are more soluble than the parent pigments.

Upon publication of these results, Gillam (2) repeated and extended his previous work on beta-carotene, with complete confirmation of Zechmeister's conclusions. In a study of the temperature coefficient of the reaction, he found even at -2° C. a slight tendency toward isomerization, the reaction proceeding quite rapidly above 40° C. If heat is used in the crystallization process, only beta-carotene can be isolated even from a solution originally containing only the isomerized pigment. Crystalline beta-carotene is perfectly stable. Both Strain (24) and Quackenbush, Steenbock and Peterson (21) have published results which in general tend to confirm the work of Zechmeister on the xanthophylls.

No experiments have as yet been recorded dealing with other properties of these synthetic pigments. A knowledge of their resistance to oxygen and acids would be of considerable value. It is probable that this isomerization reaction may play a role in the function of carotenes in living plants.

In view of the readiness with which these new pigments form, particularly in the case of xanthophylls, it is surprising that no mention of them has been made previously. Many of the methods used to prepare pigment solutions involve the use either of heat or of time sufficient to allow for their formation, yet in the literature there is no indication of their presence. The adsorbents used may have been so strong that they were not separated from the parent pigment, or they may have been lost in the mother liquors, since they are more soluble.

Isomerization due to acid

When xanthophylls are heated with acids the pigments undergo extensive change. With concentrated acid, such as glacial acetic, the pigment may be completely decolorized, as shown by Karrer (13). Milder conditions alter the pigments to a less extent; with lutein, much of the pigment becomes epiphasic in the petroleum ether-methyl alcohol partition.

Although Kuhn and Brockmann (14) claim that no noteworthy change in adsorption in the chromatograph results from acid treatment, later work indicates that such is the case. Working with lutein from silage, Quackenbush, Steenbock and Peterson (21) showed that at least three and possibly five acid isomerized pigment layers were indicated by the chromatogramm. None of the altered pigments could be crystallized. In a similar experiment on lutein, Strain (24) showed that the altered pigment is only weakly adsorbed in the column. He was able to crystallize the color which passed through the column but does not offer any proof that the material was homogeneous. The effect of dilute acid on either zeaxanthin or cryptoxanthin does not seem to have been recorded. Beta-carotene apparently is not altered by mild acid treatment (14).

Summary

The table on the following page summarizes the properties of the corn carotenoids.

Properties of Corn Carotenoids

Pigment	Melting Point	Optical Rotation	Absorption Maxima*	Investigator
Zeaxanthin	215.5°	-40°	496; 463	Zechmeister, Strain
Cryptoxanthin	169°	± 0°	496; 463	Zechmeister, Strain
Beta-carotene	182°	0°	496; 463	Gillam
Neozeaxanthin A	106°	+120°	489; 457½	Zechmeister
Neozeaxanthin B	93°	± 0°	489; 457½	"
Neozeaxanthin C	154°	—	485; 455	"
Neocryptoxanthin	—	—	490; 459	"
Neo-beta-carotene	166°	—	477; 446**	Gillam
Zeaxanthin, partly oxidized	Gives a number of bands.			Strain
Cryptoxanthin, partly oxidized	No record in literature.			----
Beta-carotene, partly oxidized	Peroxides & CrO_3 give $-OH$, $-CHO$, or $>CO$ groups, followed by ring breakage to give azafrinone aldehyde.			Zechmeister
Zeaxanthin + acid	Decolorizes upon heating with glacial acetic acid.			Karrer
Cryptoxanthin + acid	No record in literature.			----
Beta-carotene + acid	No change on mild treatment.			Kuhn and Brockmann

*In benzene; **in hexane.

EXPERIMENTAL METHODS

Precautions

The usual precautions, summarized by Miller (18), that must be taken in working with carotenoid solutions were carefully observed. All solutions were kept in stoppered bottles under an atmosphere of nitrogen away from strong sunlight. Solutions kept longer than a few hours were stored in the icebox. Low-boiling petroleum ether was used in all of the operations to help prevent oxidation. Erlenmeyer or other narrow-mouthed containers were always employed where possible.

Crystallization Procedure

The above procedure was also observed in the crystallization process. Solutions were always kept at 0° C. when allowed to stand.

The use of Emich filtering sticks of porous alundum discs sealed in glass tubing made it feasible to recrystallize repeatedly very small amounts of material without transfer from the original container. Since these filtering sticks are most easily used with pressure instead of suction, nitrogen gas was used as the pressure source in filtrations requiring more than a few minutes.

This method of filtration was of particular advantage in the removal of sterol impurities, as solutions could easily be filtered either in an ice-salt mixture or at the temperature of a CO_2-acetone solution.

Zeaxanthin pigments were crystallized either from alcohol-water mixtures or by adding Skelly A to acetone or acetone-benzene solutions. The purification of cryptoxanthin pigments is a difficult problem if heating is to be avoided. In some cases the white impurities were most easily removed by filtering an acetone solution cooled with carbon dioxide and acetone. In other cases they were removed by their insolubility first in ice cold Skelly A and then in cold methyl alcohol. The color loss in such operations was usually large. The purified pigment was crystallized from benzene-methyl alcohol mixtures or from alcohol and water. As repeatedly observed in the literature, impurities present in more than traces made crystallization impossible.

All melting points were taken with the customary electrically heated aluminum block, the sample being held between two cover glasses and a microscope used for observation. The apparatus was checked and gave correct readings throughout the entire range. Although this method is the only one which can be used with minute amounts of material as isolated in this work, none of the melting points of the carotenoids recorded in the literature seem to have been taken in this fashion. It has been noted by a number of workers that the carotenoids decompose rather than melt. Consequently the observed melting point may vary considerably with the method used. One test with zeaxanthin gave identical values by the present method and with open capillary tubes in an oil bath. Under the microscope it was observed that a slight softening always occurred from 2 to 5 degrees below the actual point of fusion.

As noted by Kuhn, Winterstein and Lederer (16), repeated recrystallization has little effect in raising the melting point. Experiment showed that the melting point was not raised by a two-hour drying period in a vacuum at room temperature, so all samples were tested directly as filtered.

Absorption Spectra Measurements

All absorption spectra data recorded in the following pages was obtained by Mr. Baldwin using the micro-spectrograph constructed by himself and Dr. Bear. Since only the points of maximum absorption were desired, it was not necessary to use solutions of known concentration. A small amount of pigment was dissolved in benzene in the absorption cell and the light-absorbing properties of the solution recorded immediately on a photographic plate.

Equipment suitable for determining the optical rotation of colored solutions was not available.

Chromatographic Technique

Fundamentally the procedure for chromatographic analysis is quite simple: a solution of the material is passed through a column containing the adsorbent and further additions of solvent are made until the various components are separated as well defined bands, or in some cases until bands successively wash through the column. In the first case, the bands are mechanically separated, and the adsorbed material eluted with an appropriate solvent. A brief generalized description of the process is given by Cassidy (3). Strain (24) and particularly Zechmeister (26) give detailed directions for the complete analysis. The methods used in the present work follow the recommendations of these authors in all but a few details.

Column

The simplest type of column--test tubes of varying sizes to which were fused small pieces of glass tubing as outlets--proved entirely adequate for containing the adsorbent. A cork ring supporting a pierced porcelain plate which was covered with a layer of asbestos supported the column of adsorbent material. With this arrangement it was possible either to push out the developed column by means of a thin rod or to collect separate fractions of the liquid as it passed through the column.

It was found that the column could be filled most easily by tamping successive portions of adsorbent with the column under a slight vacuum from the water pump, as suggested by Zechmeister. For a number of reasons it was found expedient to force the solution through the column under pressure rather than to use suction. In preliminary experiments it was noted that the viscosity of the liquid used greatly influenced the rate of flow. Thus, petroleum ether (boiling point 28-38°C.) solutions passed through the column markedly faster than did benzene solutions.

Obviously the use of suction with petroleum ether would cause large losses of solvent, with attendant fire hazard. Since the use of compressed nitrogen as a source of pressure would also serve to exclude air, the apparatus was designed to operate under pressure, 20 to 30 cm. of mercury being adequate with the adsorbents used. The apparatus was so arranged that additions of solvent could be made without release of pressure on the column. More uniform bands were obtained if the column was first treated with solvent until a steady state was reached.

Solvents

Petroleum ether, either alone or mixed with benzene, was used exclusively. The use of these low boiling solvents possessed the additional advantage that upon elution they could be readily evaporated at room temperature. The solvent used in developing a chromatogramm was always washed, dried with Na_2SO_4, and fractionally distilled before re-use.

Adsorbents

Calcium carbonate and aluminum oxide exclusively were used as adsorbents. Although Zechmeister highly recommends calcium carbonate, Strain claims that none of the preparations he used showed good adsorptive properties. In confirmation of the latter's findings, it was discovered that the C.P. powdered material on hand gave broad, ill-defined bands.

A much better carbonate, giving sharply defined bands and a higher adsorbing capacity, was made by precipitation as follows: 226 grams of white, anhydrous calcium chloride, 8-mesh, was dissolved in 300 cubic centimeters of distilled water and the solution filtered through filter cell to remove the slight flocculent precipitate. Then 230 grams of anhydrous C.P. sodium carbonate was dissolved in a liter of distilled water in a large container which could be stoppered.

The filtered calcium solution was slowly poured beneath the carbonate solution in a manner analogous to the Kjeldahl technique, after which the container was stoppered and shaken vigorously. This caused the formation of a solid gelatinous mass, which, after adding 500 to 1000 cc. of water and shaking for about five minutes, suddenly crystallized. The precipitate was filtered, washed thoroughly, re-suspended in a liter of water and shaken. Finally it was again filtered and washed with water followed by 95% alcohol. The final product, dried at 105-110° C. over night, is a very fine powder, and to give a better flow of liquid it was mixed with an equal weight of Hyflo Super Cel, as recommended by Strain.

Ordinary powdered C.P. aluminum oxide of two different brands possessed good adsorbent properties, but Hydralo crushed to pass through an 80 mesh sieve was used because it gave more rapid flow of liquid.

Activation and Deactivation

Heating the calcium carbonate at temperatures above 110-120° C. did not noticeably enhance its adsorptive properties. The alumina was activated by heating in air for 10-20 hours at 300-500° C. This activated alumina appeared to cause some decomposition of the solvent, resulting in the formation of non-volatile liquids. However, repeated adsorption of a pigment on this material never occasioned the formation of new pigment bands, although it naturally made the isolation of the pure pigment impracticable.

For a milder adsorbent the activated material was moistened with water and dried at 105° C. This deactivated material had no effect on the solvent.

Recovery

The calcium carbonate could be recovered and used repeatedly with no change in properties. Thorough washing with alcohol and drying at 105° C. was sufficient. Since elution with the alumina was often incomplete, this material was always washed with alcohol and then heated at about 400°.

Development

Experience showed that no pigment band could be considered homogeneous unless it was treated with a developing solution sufficiently strong to cause it to move slowly an appreciable distance down the column. For the more strongly adsorbed pigments, the addition of 1-5% acetone to the solvent gave a convenient range of developing solutions. Skelly A - benzene mixtures were suitable for the more weakly adsorbed pigments.

Where there were a number of bands not differing widely in adsorption affinity, each band had to be adsorbed twice more in a separate column to remove the last trace of adjacent pigments.

Elution

When possible, pure acetone was used to recover the adsorbed pigments. Epiphasic pigments were transferred to Skelly A after elution. All solutions were evaporated below room temperature.

Micro-chromatograph

The identity of two pigments or the homogeneity of a single pigment was easily determined with a micro-chromatograph having a column two centimeters high and one centimeter in diameter. It was found that neither calcium carbonate nor deactivated alumina was altered in adsorptive capacity by washing with pure acetone. This fact made it possible to use these small columns indefinitely. After each run it was necessary only to elute with a few drops of acetone, wash the column with Skelly A, and proceed with the next test.

Remarks

With crude pigment solutions, the size of column needed is governed largely by the amount of white impurities present. When such impurities have the same adsorption coefficient as the pigment, they cause the color band to become broad and often indefinite. Long, narrow columns are theoretically to be preferred, especially if many of the pigments are present in small amounts as in the present case. However, the use of such columns is very time-consuming. Strain states that as long as sixteen hours is necessary to run one analysis with the long column he employed.

It was found more convenient to use broader, shorter columns (adsorbent 10 cm. high and 4.3 cm. in diameter) for a preliminary rough separation and to re-work individual bands into smaller columns. The first separation required from three to five hours, while adsorptions of single pigments could be made in as little as one-half hour.

Preparation of Solutions

From the literature survey it is evident that isomerized pigments must be isolated under carefully circumscribed conditions if one is to be certain that the pigments were present in the original material and did not arise as artifacts during the course of the analysis. This is especially true in the case of the heat-labile isomers described by Zechmeister.

An ideal procedure would fulfill the following requirements:

1. Give complete recovery of pigments in minimum amounts of solvent.
2. Remove impurities which might hinder adsorption.
3. Avoid heat, acid, and oxidation.
4. Be sufficiently rapid to eliminate or reduce the formation of the heat-labile isomers during the process.
5. Require no special large-scale equipment.

Examination of the published methods for preparing carotenoid solutions suitable for chromatographic adsorption indicated that they fell short of meeting all of these requirements. The procedure used by Kuhn and Grundmann in their work on corn is typical of the one usually employed. It involves:

1. Extraction of the pigments with alcohol and petroleum ether.
2. Transfer of the pigments into the petroleum ether by dilution with water.

3. Separation of the xanthophylls by partition.

4. Saponification of the petroleum ether phase at slightly elevated temperature.

5. Adsorption of the purified pigment solutions on activated adsorbents.

The authors do not state the volumes of solutions required, the time necessary, nor the difficulties encountered.

The following experiments were made to determine if all the above steps were essential, and, if so, to discover how they could be performed so as to conform to the conditions outlined.

The material used was a commercial gluten, air-dried directly after being separated from the starch. It was first determined that about 300 grams of gluten was the largest amount that could be handled with the available equipment. The color from this gluten was extracted with 500 c.c. of 95% alcohol, followed by 300 c.c. of Skelly A and a final 500 c.c. portion of alcohol. The pigment in this solution could not be completely recovered by diluting with salt water (3 liters) and extracting with workable amounts of Skelly A. To keep the volume of solvent at a minimum, the diluted solution was first extracted with 500-700 c.c. of Skelly A and the remaining pigment recovered by further extraction with 300-500 c.c. of benzene. The combined pigment solutions were washed to remove alcohol and dried with sodium sulfate.

A solution prepared in this manner passed through $CaCO_3$ without any adsorption. On activated alumina the bands were broad and held poorly. By washing the solution with dilute alkali or by passing it through a column of calcium hydroxide (40 grams) diluted with Hyflo Super Cel (50 grams), the adsorption on alumina was improved although there was still no adsorption on $CaCO_3$. With alkali washing, color was lost in the aqueous layer and the tendency for emulsions to form made the process tedious. Using solid calcium hydroxide, the acidic substances formed a gelatinous precipitate which caused the flow of liquid to become very slow. A small amount of pigment was adsorbed with this precipitate at the top of the column.

A solution purified with calcium hydroxide was adsorbed on alumina (two columns 4.3 cm. in diameter and containing a 6 cm. layer of activated and a 1.3 cm. layer of deactivated material) and gave the following bands:

5 mm. - yellow (zeaxanthin and derivatives)
6 mm. - little color
14 mm. - broad orange band with evidence of a purple band at the bottom edge
14 mm. - light pink
1 mm. - yellow
3 mm. - pink

Upon elution and evaporation of the pigment solution from the top layer, there was left 2-3 c.c. of oil. Elution was not complete unless the alcohol was acidified slightly with acetic acid. The crude pigment was not adsorbed by $CaCO_3$ nor did readsorption on deactivated alumina decrease the amount of impurities. Further tests showed that, while the impurities were quite insoluble in cold methanol, the oily precipitate carried down considerable color with it. Partition experiments indicated that the oily material was readily separated by this method and that adsorption of the color on $CaCO_3$ was then successful. At least six pigment bands were evident.

The broad orange cryptoxanthin layer was highly contaminated both with the sterols present in the corn and the oil formed by decomposition of the solvent. The latter could not be completely removed either by precipitation at low temperatures or by readsorption on deactivated alumina. The small lower layers were likewise contaminated with large amounts of oil.

In another experiment the pigment solution was passed through a 4 cm. column of deactivated alumina to remove the zeaxanthin fraction. It was then saponified by shaking overnight at room temperature with 10 grams of KOH dissolved in 50 c.c. of methanol.

The problem of removing the soaps from this mixture without loss of color proved to be very troublesome. It was never completely solved. Even careful addition of water gave a lower aqueous layer which contained a considerable fraction of the total color. As much as twelve liters of water and dilute alcohol (30-60%) were required for the complete removal of the soap, and the tendency for emulsion formation was pronounced. The solution also contained colloidal material which was filterable only after drying with sodium sulfate.

After much experimenting, it was discovered that the process could be materially shortened by use of the centrifuge. Concentrated salt solution was first added to the saponification solution and the mixture allowed to stand in the separatory funnel until the liquid separated into three layers--a clear top solvent layer, a middle layer of precipitated soaps, and the lower layer of salt-alcohol solution. The viscous soap layer, containing much of the color, was transferred to centrifuge bottles and part of the color recovered by repeated centrifuging with benzene and Skelly A. Further purification of the combined pigment solution now gave no difficulty.

In further experiments with this method, it was found that treatment of the separated soap mixture, centrifuged to remove most of the water, with 50% caustic solution would give a still greater recovery of the pigment. With this modification the soaps became more compact upon centrifuging and seemed to have less affinity for the color.

The use of concentrated alkali was also valuable in removing the other colloidal material present. By vigorously shaking the cloudy solvent solution with a small portion of concentrated caustic, the colloidal material largely gathered in the alkali layer and the remainder agglomerated and no longer passed through filter paper.

In the experiment being described, the first method using the customary water extraction was employed to remove soaps.

The cryptoxanthin pigment was now readily adsorbed on deactivated alumina as a sharply defined 1 mm. orange band. Although the eluted pigment was easily separated from the sterols, only a trace of impure cryptoxanthin (M.P. 130-140°) could be isolated. From later experiments it is probable that this band contained other pigments as well as cryptoxanthin. The lower carotene pigment bands were adsorbed only on activated Hydralo.

These experiments indicate that none of the steps in the procedure used by Kuhn and Grundmann may be omitted, particularly if crystalline pigments are desired. The outline of the procedure to be used in subsequent experiments is as follows:

1. Extraction. 300 grams of gluten extracted with one liter of 95% alcohol and 300 c.c. of Skelly A.

2. Recovery of pigments. The pigments in the extraction

liquor are recovered by addition of 3 liters of salt water and extraction first with 500-800 c.c. of Skelly A followed by extraction with 200-400 c.c. of benzene. The two are not combined.

3. Partition. The zeaxanthin pigments are removed from the Skelly A by extraction with 90% methanol and recovered with the original benzene extract. The benzene solution is purified by alkali washing and adsorbed on $CaCO_3$.

4. Petroleum ether phase. This is saponified by shaking overnight with KOH in methanol. The soaps are removed by the centrifuge method and the purified pigment solution adsorbed on deactivated Hydralo.

EXPERIMENTAL RESULTS

The wet milling process for corn consists of first softening the kernels by heating for two days at 50° C. in the presence of dilute aqueous sulfur dioxide solution, and secondly of grinding the softened kernels and separating the major components--starch, gluten, germ, and hull--by flotation processes. The starch which contaminates the gluten may be removed by hydrolysis with dilute HCl at an elevated temperature.

A general picture of the changes which the carotenoids undergo could be obtained by isolation of these pigments in whole untreated corn, in the gluten recovered after softening the kernel, in the gluten after removal of starch by acid hydrolysis, and in the oil obtained as a residue in the solvent extraction process.

Carotenoids in Whole Corn

Five hundred grams of Black Yellow Dent corn, 1938 crop, was ground in a Wiley mill with 30 mesh screen. The sample was added rapidly and the first half coming through the screen, consisting almost exclusively of starch and germ, was discarded. The horny yellow gluten was further ground to pass through a 60 mesh screen. The pigments were extracted and worked up according to the method outlined, except that the Skelly A solution was not saponified.

The zeaxanthin fraction, developed with Skelly A containing 5% acetone in a column of $CaCO_3$ 4.3 cm. in diameter and 9 cm. high, gave the following bands:

27 mm. - white
1 mm. - yellow
2 mm. - white
1 mm. - yellow
8 mm. - white
11 mm. - orange

In addition there was a very faint line below the top two. Readsorption of these upper layers, which contained only a small amount of color, gave a band of zeaxanthin, typical behavior of the neozeaxanthin pigments. The zeaxanthin weighed approximately 4 mg. and melted at 188° C. All weighings were made on an ordinary analytical balance and are only approximations. The recrystallized product melted at 192.3°, a second adsorption on $CaCO_3$ failing to raise this melting point. Absorption maxima were at 490 and 458 mμ.

The cryptoxanthin fraction, developed with Skelly A containing 2% acetone on a column of activated Hydralo 3.4 cm. in diameter and 6 cm. high, gave the following bands:

2 mm. - clear
2 mm. - yellow

3.5 mm. - clear
20 mm. - pink

The 2 mm. yellow band was epiphasic, showing that it was cryptoxanthin and not zeaxanthin.

Carotenoids in Gluten after Softening the Kernel

Three thousand grams of Black Yellow Dent corn, 1938 crop, was softened by the usual procedure and milled in the laboratory milling apparatus. There was isolated 520 grams of moist gluten containing approximately 30% solids. This was covered with 1200 c.c. of 95% alcohol, filtered after standing one hour and washed with 800 c.c. of alcohol. The Skelly A was omitted since only the zeaxanthin pigments were to be isolated. In other experiments not recorded here where both alcohol and Skelly A were used, the extracted starch-protein mixture was practically white in color.

The zeaxanthin pigments were recovered by partition between 85% ethanol and Skelly A and transferred to the original benzene extract. After purification by alkali washing, this was adsorbed on two columns of $CaCO_3$, giving a large number of thin pigment bands above the main zeaxanthin layer. In addition some color passed through the columns which gave one salmon pink layer when adsorbed on deactivated Hydralo. The combined upper layers were readsorbed.

Column:--$CaCO_3$ (10 parts C.P. powdered plus 1 part of special), 4.3 cm. in diameter, 8.5 cm. high, developed with benzene-Skelly A (1:4).

11 mm. - light tan, no sharply defined layers
0.5 mm.- thin yellow brown line (AO)
1 mm. - white
1.5 mm.- orange (B)
1 mm. - white
1 mm. - orange (C)
1 mm. - white
2 mm. - orange yellow (D)
0.5 mm.- white
1 mm. - light yellow (E)
2 mm. - three thin lines; orange, yellow, pink
6 mm. - not quite white
0.5 mm.- trace of zeaxanthin

When the pigment in band AO was readsorbed it gave a lower band of zeaxanthin--typical behavior of the neozeaxanthins. The pigments in bands D and E faded on storage under nitrogen --typical behavior of acid-isomerized pigments. None of the pigments were obtained in crystalline form.

The main zeaxanthin layers were eluted and the pigment recrystallized from 10 c.c. of acetone-benzene mixture by adding 30 c.c. of Skelly A. Melting-point, 192-193° C. A second readsorption and two recrystallizations did not raise this melting point. Final yield, 5 mg.

Carotenoids in Gluten after Acid Hydrolysis to Remove Starch

The material used in this experiment was a commercial product---Regular Maco manufactured by the American Maize-Products Company. The details of the procedure for removing the starch are not known, except that acid hydrolysis at elevated temperature was used. Two hundred grams of the material were extracted and worked up as outlined. The residue was light brown in color.

Zeaxanthin pigments:---Preliminary working-up processes in this part of the experiment required only ten hours.

Column:---$CaCO_3$, 4.3 cm. in diameter, 10 cm. high, developed with a 1:1 mixture of benzene and Skelly A containing 2% acetone.

2 mm. - clear
3 mm. - orange band, edges indefinite
2 mm. - not quite clear
2 mm. - neozeaxanthin A
8 mm. - yellow
1 mm. - neozeaxanthin B
2 mm. - clear
2 mm. - yellow, faint
2 mm. - clear
2 mm. - yellow, faint
3 mm. - clear

2 mm. - pink with yellow borders
10 mm.- containing traces of two faint lines
1 mm. - clear
9 mm. - zeaxanthin

In addition, some color passed through without forming any bands. Only the neozeaxanthins A and B, the pink band, and the zeaxanthin were recovered. Mixed microchromatographs with pure neozeaxanthins A and B confirmed the identity of these two pigments.

Repeated adsorption of the pink band above the zeaxanthin showed that it was a mixture of two pigments--pink above and yellow below--with practically identical adsorption coefficients. With the small amount of pigment, a separation of the two was not possible. From the 9 mm. orange layer was recovered 5.5 mg. of zeaxanthin, which, after one recrystallization, melted at 199-200° C. and had absorption maxima at 492 and 463.5 mμ.

The color which passed through this first column was adsorbed on a smaller column of deactivated Hydralo to give two pigment bands, salmon pink and orange. Readsorption of the salmon band on $CaCO_3$ showed that it consisted of at least five pigments. The main band, a brick pink, was purified by further adsorption to give less than one milligram of an oil. The pigment did not give sharp absorption maxima but there appeared to be a slight maximum at 483 mμ. Adsorption of the orange band on $CaCO_3$ also showed that a number of pigments were present in addition to the main orange-yellow band. From this band a trace of impure cryptoxanthin (M.P. up to 130° C.) was isolated. Comparison with other experiments indicated that this band was probably a mixture.

Cryptoxanthin pigments:--The Skelly A - methanol saponification mixture was worked up as follows: The methanol layer which contained most of the color was first separated and the Skelly A solution further extracted with a liter of methanol containing 1% KOH to remove the larger part of the remaining color. The soaps in the methanol solution were removed by the usual centrifuge process and the pigment adsorbed on deactivated Hydralo. This was done in lieu of evaporation and the column was not developed. A purple band was evident below the main orange band.

The sterols accompanying the eluted pigments were removed by precipitation from a small volume of methanol. The purified pigment mixture, dissolved in Skelly A, could now be adsorbed on $CaCO_3$. Samples of C.P. calcium hydroxide, C.P. calcium oxide, and a technical lime all gave very weak adsorption.

Column:--4.3 cm. in diameter, 9.5 cm. high, developed with Skelly A - benzene (4:1).

4 mm. - four thin orange lines

1 mm. - zeaxanthin, with a faint pink band just below it.

14 mm. - almost clear
3 mm. - orange, cryptoxanthin, not separated from
9 mm. - light yellow

The pigment corresponding to the purple band on alumina washed through the column. The 3 mm. orange band gave a solid pigment upon elution and evaporation. After one recrystallization from benzene plus methanol, the pigment weighed 3.7 mg. and melted at 145-148° C. The crystals were oblong plates characteristic of cryptoxanthin. The third, fourth and fifth recrystallizations from benzene and methanol gave crystals which melted constantly at 155-158°. Readsorption of the combined mother liquor and crystals, and three recrystallizations of the pigment from the top orange layer did not raise this melting point. Adsorption maxima were at 488 and 460 mμ, indicating that the pigment was not pure. The 9 mm. yellow band in the original column gave an oil upon elution. It was probably a mixture of neocryptoxanthin and acid-isomerized zeaxanthin.

Carotenoids in "Xanthophyll Oil"

A sample of "xanthophyll oil"--the residue left in the commercial solvent extraction process for obtaining zein from corn gluten--was obtained from the Corn Products Refining Company.

Preliminary experiments indicated that the blood-red, viscous oil was entirely soluble in Skelly A, acetone and benzene, and insoluble in methanol. Solutions in Skelly A or acetone gave no precipitate after standing in ice over night. At the temperature of CO_2 they became semi-solid. Mixtures of benzene and methanol removed considerable color without apparently dissolving much of the oil. The presence of large amounts of soap-like material was indicated.

One hundred c.c. of the oil was diluted with 200 c.c. of benzene and 300 c.c. of methanol in a separatory funnel and cooled in ice for two hours. The lower oil layer was then drawn off. The upper layer was diluted with 800 c.c. of Skelly A and the bulk of the zeaxanthin pigments removed by partition, using 1800 c.c. of 90% methanol. The presence of the soap-like material caused loss of color in purifying the benzene solution into which the pigments were transferred. One-half of the purified benzene solution was adsorbed on $CaCO_3$.

Column:--4.3 cm. in diameter, 10 cm. high, developed with 500 c.c. of 1:1 Skelly A - benzene containing 2.5% acetone.

34 mm. - white at top, ill-defined yellow at bottom
1 mm. - clear
3 mm. - orange, neozeaxanthin A
1 mm. - clear
20 mm. - orange, neozeaxanthin B

2 mm. - clear
9 mm. - orange (C)
1 mm. - clear
2 mm. - orange (D)
1 mm. - clear
1 mm. - light yellow

Below this the column contained some zeaxanthin, most of which had passed through. In addition, there were two bands of pigment below the zeaxanthin which were not saved.

The neozeaxanthin A band, purified by readsorption, did not crystallize from a mixture of acetone and Skelly A, but precipitated in semi-colloidal condition from alcohol and water. Part of this material melted about 90° C. and the rest between 175 and 186°. Under the microscope, the presence of white impurities was evident. A microchromatograph showed that the pigment was homogeneous. Mixtures with pure neozeaxanthin B gave two bands of pigment, and with neozeaxanthin A, only one.

The neozeaxanthin B pigment band crystallized slowly from an ice cold acetone - Skelly A solution as small nodules. The solid material, 4.6 mg., was apparently not changed by recrystallization, the melting point remaining at 116-117° C. with preliminary softening. A mixture with pure neozeaxanthin B gave only one main pigment band upon microadsorption.

The 9 mm. orange band (C) with absorption maxima at 480 and 453 m could not be crystallized. The small amount of color in band (D) gave a faint absorption maxima at 484 mμ. The zeaxanthin in the solution which passed through the first column was recovered on a second column. The material (13.8 mg.) melted at 192-194° C., readsorption and recrystallization failing to change the melting point. The material gave only one main band of pigment in a mixed microchromatograph with the zeaxanthin isolated from Chinese Lantern plants.

The cryptoxanthin fraction, after saponification and removal of the soaps, was adsorbed on alumina.

Column:--4.3 cm. in diameter, 2.5 cm. of deactivated and 8.5 cm. of activated alumina, developed with 700 c.c. of Skelly A containing 1% acetone.

10 mm. - zeaxanthin at top, not quite clear below
10 mm. - orange, more intense at bottom
2 mm. - clear
3 mm. - pink (C)
62 mm. - very light yellow
3 mm. - clear
2 mm. - pink

The color in the pink band (C) showed a slight absorption maxima around 486 mμ. The sterol impurities in the cryptoxanthin pigment band were removed by successive precipitation from cold Skelly A, benzene-methanol, and acetone at the temperature of solid CO_2. About one-half of the color precipitated as a solid from a small amount of benzene-methanol solution. Three recrystallizations raised the melting point of the material from 159-166° C. to 177°. More careful examination of the crystals revealed that two pigments were present.

In addition to the characteristic oblong plates of cryptoxanthin there were long needles, usually in the form of small rosettes. The mixture gave only one pigment band on deactivated alumina but two well-separated bands on $Ca(OH)_2$. Three further recrystallizations served to remove the remaining cryptoxanthin and gave a pigment (about 1 mg.) melting constantly at 179-181° C. A micro-adsorption on $Ca(OH)_2$ now gave only one yellow band. Mixed with pure cryptoxanthin the microchromatograph showed two pigment bands with the yellow below the orange cryptoxanthin band. The absorption maxima of the pigment were at 487 and 456 mμ.

The oil left from the first separation with benzene and methanol was saponified and adsorbed on alumina after removal of the soaps by washing with water. Much of the color was lost in the soap layer.

Column:--deactivated Hydralo, 3.4 cm. in diameter, 8.5 cm. high, developed with benzene - Skelly A (1:4).

5 mm. - zeaxanthin at top, clear below

3 mm. - orange

1 mm. - not quite clear
1 mm. - pink
50 mm. - clear
15 mm. - pink

The pigment from the lower 15 mm. layer of beta-carotene was dissolved in a small amount of benzene-methanol solution and allowed to stand overnight in an ice bath to precipitate a small amount of white material. The solution was evaporated and redissolved in about 1 c.c. of benzene and 2-3 c.c. of methanol. On standing in an ice bath the beta-carotene slowly precipitated as characteristic diamond-shaped crystals along with some white material. The white material was readily soluble in a small amount of Skelly A. The crystals (less than 1 mg.) of beta-carotene melted at 175-177° C. Three recrystallizations did not change this melting point. Absorption maxima of the pigment were at 492 and 464 mμ.

Zeaxanthin and Cryptoxanthin from Chinese Lantern Plants*

Chinese lantern plants, stored in a dry dark place for about six months, were de-stemmed and the whole flower, including the pods and the paper-like lantern part, thoroughly macerated by passage through a meat grinder. Seventy four grams of material were obtained. This was extracted during two days with a total of 400 c.c. of Skelly A, to part of which was added 50 c.c. of absolute alcohol, and with

*Physalis Alkekengi

275 c.c. of benzene to which was added 200 c.c. of acetone. The extracted residue was brown in color. The solvent was evaporated at room temperature, the residue saponified over night and the soaps removed by the centrifuge method, giving 800 c.c. of benzene solution containing the color.

Column:---4.3 cm. in diameter, 3.8 cm. activated Hydralo, 5.2 cm. deactivated Hydralo, developed with 2% and 5% acetone in Skelly A.

3-4 mm. - bright red, zeaxanthin
1 mm. - clear
11 mm. - yellow, cryptoxanthin (orange-red in benzene)

Numerous faint bands were present in the activated adsorbent.

Elution of the zeaxanthin layer with alcohol and benzene left the alumina yellow. The crude zeaxanthin was washed with about 100 c.c. of Skelly A and then with 10 c.c. of benzene-acetone mixture. The residue melted at 204-205° C. Recrystallization from acetone-benzene by addition of Skelly A did not change the melting point. Yield, 53 mg. Absorption maxima were at 493 and 461 (?) mμ. From the mother liquors 18 mg. of material melting at 190-197° were obtained. A microchromatograph showed that the main fraction was homogeneous.

The impurities contaminating the cryptoxanthin (abs. max. 493½; 464) were removed by precipitation from acetone cooled with CO_2. After four recrystallizations from benzene-methanol solution, the pigment (1-2 mg.) melted at 163-164° C. About 1 mg. of less pure pigment, melting at 160° after four crystallizations, was recovered from the mother liquor. Due to mechanical losses the yield of cryptoxanthin was low.

Effect of Heat upon Zeaxanthin

The zeaxanthin isolated from the xanthophyll oil (8.4 mg.) was heated to reflux under nitrogen for 1¼ hours. The benzene solution (10 c.c.) was diluted with 40 cc. of benzene and chromatographed immediately on $CaCO_3$.

Column:—4.3 cm. in diameter, 10.5 cm. high, developed with benzene, benzene plus Skelly A, and 2.5% acetone in benzene-Skelly A (1:1), total 800 c.c.

1-2 mm. - clear
1 mm. - bright orange, neozeaxanthin A
1 mm. - clear
1 mm. - lighter orange, neozeaxanthin B
1 mm. - clear
1 mm. - yellow
18 mm. - clear
24 mm. - zeaxanthin

The top lines were eluted together, separated by adsorption on a smaller column 3 cm. in diameter and the two orange bands purified by adsorption in individual columns. In all cases a small band of zeaxanthin, well below the main pigment band, was evident. The purified neozeaxanthin A (1 mg.) did not crystallize from acetone-Skelly A solution and came down as an oil upon evaporating the solution to dryness. Evaporation of an ethanol solution gave a solid pigment without definite crystalline form. This material melted at 105-106° and had absorption maxima at 484 and 457 mμ.

The neozeaxanthin B pigment precipitated as a solid (1 mg.) when water was added to an acetone-Skelly A solution. It melted at 108-109° and showed absorption maxima at 480 and 453 mμ. Three and eight-tenths milligrams of crude zeaxanthin were recovered, melting at 192-194° after recrystallization.

Effect of Air upon Zeaxanthin

Zeaxanthin (6.8 mg.) isolated from Chinese Lantern plants was dissolved in 4 c.c. of 95% ethanol and 4 c.c. of benzene. A fine stream of air was bubbled through the solution at room temperature for two days. There was no apparent decrease in color intensity.

Column:--$CaCO_3$, 3.4 cm. in diameter, 7.5 cm. high, developed with benzene-Skelly A (1:1) containing 1% acetone.

1 mm. - orange
2 mm. - clear
4 mm. - light yellow
5 mm. - clear
10 mm. - faint yellow

15 mm. - clear

15 mm. - zeaxanthin

The two yellow bands were faint and had indefinite boundaries. From the 10 mm. band containing about 0.3 mg. of pigment, a very small amount of zeaxanthin (mixed microchromatograph and melting point at 195-198°) was recovered. The top orange band proved to be a mixture of two pigments when adsorbed on a smaller column. At least one of these pigments appeared to be a neozeaxanthin, although the microchromatographic test was not too clear. Five and one-half milligrams of unchanged zeaxanthin were recovered also.

Effect of Acid upon Zeaxanthin

Zeaxanthin (8.8 mg.) isolated from Chinese Lantern plants was put in a test tube with 4 c.c. of benzene and 8 c.c. of 95% ethanol, 1% in acetic acid. After displacing the air with nitrogen the tube was stoppered and heated over night at 52° C. The pigment was then transferred to 60 c.c. of benzene which was washed with dilute alkali to remove the acetic acid and alcohol.

Column:--$CaCO_3$, 3.4 cm. in diameter, 8.5 cm. high, developed with benzene-Skelly A (1:1) containing 1% acetone.

2 mm. - orange

.5 mm. - clear

1 mm. - yellow

2 mm. - clear
2 mm. - yellow
40 mm. - a faint band near the top, clear below
20 mm. - zeaxanthin

The amount of pigment in the center bands was negligible. Purification of the top orange band gave a pigment melting at 104-105° C., neozeaxanthin A. In a similar experiment with 30.9 mg. of zeaxanthin, 17.3 mg. of unchanged pigment were recovered.

Zeaxanthin (17.3 mg.) was then treated in a similar fashion, this time in the presence of 20 c.c. of 95% ethanol, 1 c.c. of 1 N HCl, and 5 c.c. of benzene. The benzene solution was adsorbed on $CaCO_3$ and gave the following chromatogramm:

4 mm. - two orange bands
2 mm. - clear
10 mm. - slightly colored but no definite bands
30 mm. - zeaxanthin
30 mm. - faint traces of two thin yellow bands
2 mm. - pink

Some color passed through the column without forming a band, and a yellow band below the pink also passed into the filtrate upon development. The solution of the former was adsorbed on activated Hydralo as a purple band. The eluted pigment weighed 51 mg. and was reduced to 17 mg. by evaporation overnight under a water-pump vacuum. The pigment showed a faint absorption maximum at about 488 mμ.

The yellow band which passed through was adsorbed on deactivated Hydralo. Since the pigment behaved similarly to cryptoxanthin in the chromatograph, a mixed adsorption with pure cryptoxanthin was made. Only one band was shown by the microchromatograph using deactivated alumina. The pigment showed a faint absorption maximum around 486 mμ.

The pink band below the zeaxanthin gave a slight absorption maximum at 483 mμ. Nine and one-tenth milligrams of crude zeaxanthin were recovered. The two orange bands at the top of the column were readsorbed on a smaller column two centimeters in diameter:

10 mm. - 3 thin lines, orange, yellow, and lemon
10 mm. - neozeaxanthin A
2 mm. - clear
7 mm. - neozeaxanthin B
3 mm. - clear
2 mm. - faint pink band

The neozeaxanthin A (3.3 mg.) melted at 100-103° C.; the neozeaxanthin B (2.1 mg.) at 90-108°. Neither was purified by readsorption.

The experiment was repeated with identical results. Further tests on the yellow band which resembled cryptoxanthin in behavior showed that it was also epiphasic to the same degree as the latter. Its behavior in the chromatograph was rechecked, this time on $CaCO_3$. About 0.5 mg. of cryptoxanthin from Chinese Lantern plants was dissolved in Skelly A and adsorbed on a column of $CaCO_3$ 2 cm. in diameter. There appeared only one 2.5 mm. orange band. The pigment was recovered, combined with the yellow pigment (about 1 mg.) from zeaxanthin, and readsorbed on another column 2 cm. in diameter. Thorough development with benzene-Skelly A (1:4) did not cause any separation of the two bands. The final chromatogramm was a 4.5 mm. band, the upper 2.5 mm. being the characteristic orange color of cryptoxanthin and the lower 2 mm. being a light yellow in color. This yellow fraction of acid-isomerized zeaxanthin thus has an almost identical adsorption affinity with the cryptoxanthin.

None of the acid-isomerized pigments except neozeaxanthins A and B showed any tendency to crystallize. Evaporation of solutions in acetone, Skelly A, and ethanol, in which solvents the pigments are readily soluble, always left an oil. When these oils were kept in the dark in cork-stoppered containers in an atmosphere of nitrogen, they faded to almost colorless oils in the course of about a week. Under identical conditions, the crystalline pigments were stable indefinitely.

Bleaching Gluten with Enzymes

The gluten used in this experiment was a suspension of the material obtained in a laboratory milling run on waxy maize corn. The suspension, about 10% solids, was washed by centrifuging to remove any SO_2 and the yellow gluten resuspended in distilled water. Two soybeans were dehulled, ground in a mortar and pestle and added to 50 c.c. portions of the suspension. Air was bubbled through the liquid for three hours. Results are tabulated in the following table:

Buffer Added	pH	Bleaching
Material as washed	4.9	None
2 drops glacial acetic acid	4.4	None
Few crystals of $(NH_4)_2SO_4$	4.8	None
Few crystals of CH_3COONH_4	5.1	Slow, complete
Few Crystals of CH_3COONa	5.2	Rapid, complete
2 drops concentrated NH_4OH	7.0	Very rapid, complete
2 drops concentrated NH_4OH	7.0	None; no enzyme added

The bleached gluten was almost pure white.

DISCUSSION AND CONCLUSIONS

The necessity for rapidity of analysis because of the labile nature of the isomerized carotenoids, the need for preliminary purification of the pigment solutions, and the repeated chromatographic adsorption necessary to purify individual pigments made a quantitative isolation of the pigments impossible. However, since the loss of color in similar experiments was approximately the same, it is possible to make a rough estimate of the amount as well as of the nature of the changes which the carotenoids undergo as the gluten is subjected to the various commercial processes.

Most apparent is the fact that little oxidation of the pigment, at least to colored derivatives, takes place during these processes. All but one or two of the principal new pigment bands revealed by the chromatogramm have been identified as arising either from heat or acid treatment. In some cases this identity was established by mixed adsorption or by melting points; in others, by the color and position of the pigment band in the chromatogramm and by absorption spectra measurements. The control experiment with pure zeaxanthin showed that the neozeaxanthins A and B which were isolated from gluten were actually present therein, and did not form during the isolation processes.

It may further be concluded that much of the pigment present in the corn remains unchanged when the kernels are softened by heating in aqueous solution. However, over half the zeaxanthin is transformed into other pigments when the gluten is subject to acid hydrolysis for starch removal. The behavior of the cryptoxanthin has not been definitely established, but the fact that relatively large amounts of this pigment may be isolated from acid hydrolyzed gluten suggests that it, and the beta-carotene, are rather resistant to isomerization.

Since the color in gluten obtained directly after the softening process could be completely extracted by solvents, and since this gluten could also be bleached with enzymes, it is evident that no large amounts of non-carotenoid pigment are present in this product. Further research is needed to determine the origin of the non-solvent extracted color present in acid treated gluten.

The experiments on the acid isomerization of zeaxanthin have a bearing on the vitamin A analysis of corn or corn products. Since cryptoxanthin is the chief vitamin A precursor in corn, it is customary to analyze for this pigment as a measure of the vitamin A content. Such analysis usually includes only partition of the extracted pigments between alcohol and petroleum ether, followed by a measurement of the light absorption of the pigment in the petroleum ether phase at one wave length. The experiments made here indicate that such measurements would give fictitiously high values of pro-vitamin in corn products which have been in contact with acid. Further, even the isolation of an apparently homogeneous orange pigment band, corresponding to cryptoxanthin, especially on an active adsorbent such as alumina, would not be sufficient evidence that the pigment was pure cryptoxanthin. Only actual isolation of the crystalline carotenoid would seem to be conclusive.

The experiments also have some interest from a more theoretical standpoint. The tests made with purified zeaxanthin indicate that isomerization reactions, particularly strong acid isomerization, lead to the formation of a large number of new derivatives.

One explanation for the large number of pigments arising from acid treatment would be that both neozeaxanthins A and B are transformed to new derivatives independently of zeaxanthin itself. If such were the case, the new derivatives should occur in groups of three. The fact that three of the acid-isomerized pigments appear as pink (or purple) bands in the chromatogramm is very suggestive in this respect.

The experiments bring out very clearly the necessity for using mild adsorbents in the separation of isomerized pigments. They show that alumina, even when deactivated, is quite unsuited for any final separation of the isomerized pigments, although it can be used to advantage in the isolation of unaltered pigments.

The literature pertaining to the chromatographic process is practically devoid of any explanations for the phenomenon. That the process is, in a large measure, an acid-base reaction in non-aqueous media has apparently never been suggested, although the fundamental role of the hydrogen and hydroxyl ions in the aqueous chromatograph is well known. If it is assumed that the hydrogen ion also plays a major role in the non-aqueous chromatograph, then a number of facts are readily explainable.

First, it is apparent that the solvents used are essentially neutral (not acidic or basic) in character, and that the adsorbents are alkaline or acid adsorbing in nature. A crude experiment with powdered sodium carbonate indicated that it had some adsorptive power for the zeaxanthins.

The role of alcohol as a developing and eluting agent follows from its acidic nature. Other substances which are acidic in non-aqueous media such as benzene, acetone, and acetic acid, cause development or elution in proportion to their acid strength. Acetic acid is a very powerful eluent.

The position of the different carotenoids in the column can be explained on the basis of their varying acidity. Thus a dihydroxy derivative (zeaxanthin) is more acidic and more strongly adsorbed than is a mono-hydroxy derivative (cryptoxanthin), while an almost neutral substance (beta-carotene) is only weakly adsorbed. The marked drop in adsorption affinity which occurs when the xanthophylls are esterified would be expected.

The adsorption of two materials, often differing radically in chemical structure as in the case of cryptoxanthin and the sterols, at the same place in the column may be explained by assuming that both have the same "acid strength".

The deleterious effect of water also receives some explanation. Since it is capable of both acidic and basic reaction, it would partly "neutralize" the adsorbent.

The difficulty of completely extracting the carotenoids, particularly the xanthophylls, from soaps or soap solutions suggests that the pigments are actually chromatographically adsorbed on the colloidal soap molecules.

SUMMARY

1. Existing methods for the isolation of carotenoid pigments have been modified to make them more suitable for the isolation of the isomerized pigment derivatives from corn gluten. Greater recovery of pigments from saponification mixtures may be obtained by the use of concentrated aqueous alkali and centrifuging.

2. Closely related pigments are separable only on mild adsorbents. Deactivated alumina is not suited for such separations. Calcium carbonate showing the necessary selective adsorption has been made by precipitation. The presence of acidic and saponifiable materials hinders adsorption, particularly on mild adsorbents.

3. Acetone is of value as a developing agent. Adsorbents treated with acetone are not deactivated, and the complete removal of pigments with this solvent can be used to advantage with the microchromatograph.

4. The original pigment of the corn kernel is almost wholly carotenoid in nature.

5. Processing corn gluten under conditions which involve exposure to heat, air, and acid transform the carotenoids partly into heat and acid isomerized derivatives. No large amounts of colored, partly oxidized pigments are present.

6. Neozeaxanthin B, prepared according to Zechmeister's procedure, differed markedly both in melting point and in absorption spectra from the values he records.

7. A brief study of the acid isomerization products of zeaxanthin has been made. Dilute acetic acid results mainly in the formation of neozeaxanthin A. Dilute hydrochloric acid causes the formation of at least ten isomerized pigments. With the exception of neozeaxanthins A and B, these are oils. One of the pigments was found to be almost identical with cryptoxanthin both in partition and adsorption affinity.

8. The presence of beta-carotene in corn has been confirmed by the isolation of the material in crystalline form.

9. The suggestion is made that the chromatographic adsorption of carotenoids is fundamentally an acid-base reaction occurring in non-aqueous media.

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