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THE CAROTENOID PIGMENTS

Occurrence, Properties, Methods of Determination, and Metabolism by the Hen

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FOREWORD

This bulletin has been written as a brief review of the carotenoid pigments. The occurrence, properties, and methods of determination of this interesting class of compounds are considered, and special consideration is given to their utilization by the hen. The work has been done in the departments of Chemistry and Poultry Husbandry, cooperating, on Project No. 193.

The project was started in 1932 and several workers have aided in the accumulation of information. The following should be mentioned for their contributions: Mr. Wilbor Owens Wilson, Mr. C. L. Gish, Mr. H. F. Freeman, Mr. Ben Kropp, and Mr. William Proudfit. We are also greatly indebted to Dr. H. D. Branion of the Department of Animal Nutrition, Ontario Agricultural College, Guelph, Canada, for his fine coöperative studies on the vitamin A potency of corn.

A number of unpublished observations from these laboratories and others have been organized and included in this bulletin. Extensive use has also been made of the material presented in Zechmeister's "Carotenoide," and "Leaf Xanthophylls" by Strain. It is hoped that this work be considered in no way a complete story of the metabolism of carotenoid pigments in the fowl, but rather an interpretation of the information which is available at this time.

The wide range of distribution of the carotenoid pigments in such a wide variety of organisms points strongly to the importance of these materials biologically. In recent years chemical and physiological studies of the carotenoids have revealed numerous relationships to other classes of substances in the plant and animal world. It can be expected that relationships of even greater significance will be brought to light from time to time.

INTRODUCTION

Recognition of the fact that certain of the carotenoid pigments are responsible for the vitamin A potency of all farm feeds and the color in butter, while others are important in the color of eggs and poultry products, has resulted in a great deal of research in this field.

It has been known for sometime that the material in plants, including vegetables and fruits, which is responsible for their vitamin A potency, is not vitamin A, but is probably one or more of the closely related carotenoid pigments which may be converted into vitamin A. Chief among these is carotene, which is responsible for the coloring matter in carrots and in milk fat. There are three forms of carotene — alpha, beta and gamma. Of these the beta form is the best source of vitamin A, giving rise to twice as much vitamin A as either of the other two forms.

When these related plant pigments are ingested with the feed, the liver is instrumental in their conversion to vitamin A. The origin of vitamin A in milk and eggs is, therefore, the carotenoid pigments of the feed.

All plant pigments which are consumed, however, need not, necessarily be converted to vitamin A. There are a number of carotenoid pigments, hydroxylated carotenes (xanthophylls, or carotenols), which have no vitamin A potency. These, and/or the carotenes may be excreted unchanged or in a partially oxidized form, or in the case of the fowl, they may be deposited in the egg yolk unchanged.

It is an interesting biological phenomenon that the fowl will selectively utilize (deposit in the egg yolk, shanks or body fat) the carotenols, or hydroxy derivatives of the carotenes, while the mammal tends to utilize the pure hydrocarbons. Whereas egg yolks and the body fat of fowls contain rather large amounts of carotenols, only small concentrations of the carotenes are found.

A study of the metabolism of the carotenoid pigments in the hen seemed justified in view of the increased interest in egg yolk pigmentation, not only because of the higher food value normally associated with color, but because of certain prejudices and preferences of the buying public.

Although many biological experiments are on record which show the relative quantities of the common carotenoid pigments in poultry feeds as well as in eggs, no satisfactory, systematic quantitative study has been made showing the percentage utilization of these pigments by the hen.

THE CAROTENOID PIGMENTS[1]

W. J. PETERSON, J. S. HUGHES and L. F. PAYNE

A carotenoid, according to Bogert* *(6)*, may be defined as a nitrogen-free polyene pigment, consisting wholly or chiefly of a long acyclic chain of carbon atoms united in an uninterrupted sequence of conjugated double bonds, which system of conjugations function as the chromophore. These pigments vary in color from a bright yellow to a deep red, or even a violet, or a dark blue, the depth of shade increasing with the number of conjugations in consecutive union, and decreasing as the double bonds are saturated.

Zechmeister *(158)*, similarly characterizes the Carotenoids as follows:

(1) Yellow to deep violet-red in color.
(2) Two (or three) absorption bands in the blue or violet region of the spectrum.
(3) Solubility in the lipoids and in the typical solvents of the latter.
(4) More or less marked sensitivity toward oxygen (autoxidation and bleaching).
(5) Stability of the pigment toward alkali.
(6) Dark blue (or similar) coloration with strong sulphuric acid; also, little resistance to acids.
(7) C and H, or C, H and O as the only constituents of the molecule: the absence of nitrogen (as in fats, waxes and sterols).

The most widely distributed carotenoid pigment, carotene itself, was isolated in crystalline form, by Wackenroder *(158)*, in 1831, from the root of the carrot *(Daucus carota)*. The hydrocarbon nature of the pigment was established by Zeise[2] in 1847, and further substantiated in 1885 by Arnaud.[3] Conclusive proof came in 1907 when Willstätter and Mieg *(150)* established the formula, $C_{40}H_{56}$ for carotene. Carotene derives its name from the material from which it was first isolated, the carrot. Its wide distribution in nature probably accounts for the subsequent use of the class name, "carotenoids" to include all pigments of related chemical composition [Tswett, *(139, 140)*].

Progress in the field of carotenoid pigments is well illustrated by the fact that in 1934, when Zechmeister's treatise on the subject first appeared the formulae of only 20 of the unexceptional carotenoids had been established. Early in 1938 this list had grown to 60.

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1. Contribution No. 243 from the Department of Chemistry and No. 120 from the Department of Poultry Husbandry.

* To those particularly interested in the more recent developments in the field of carotenoid pigments, the authors wish to recommend very highly the excellent review of "Carotenoids" by M. T. Bogert in one of the two volumes (pp. 1138-1219) on Organic Chemistry edited by Doctor Gilman (1938). The review is most excellently written and considers particularly the developments in the field since Zechmeister's comprehensive review of 1934.

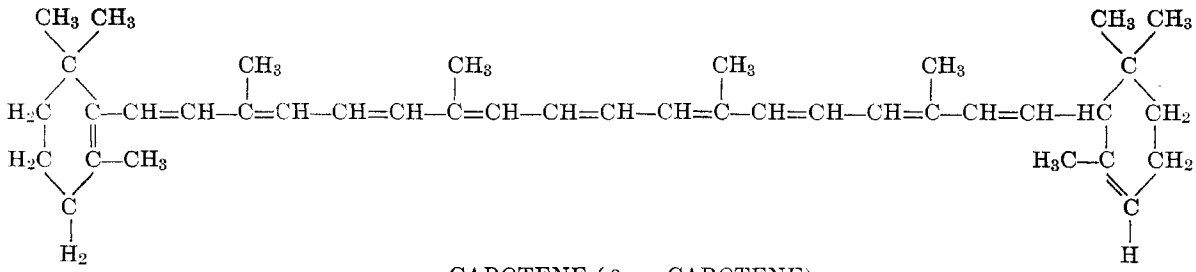
2, 3. Exact references to the papers in which these findings were first published could not be found. The facts as here presented were taken from Zechmeister's "Carotenoide" (Julius Springer, Berlin, 1934), page 113.

The carotenoids may be divided into two classes, according to their composition: the hydrocarbons (carotene, lycopene, etc., $C_{40}H_{56}$) which are readily dissolved by ether or petroleum ether, but are quite insoluble in aqueous alcohol, and the far larger class of oxygen-containing pigments, the carotenols (xanthophylls), which usually contain at least two hydroxyl groups. Like carotene, practically all carotenoid pigments have 40 carbon atoms. Although a group of compounds are included in the carotenoid classification which have less than 40 carbon atoms, these pigments are a special class, and because of the difference in their distribution in nature, need not, be considered here.

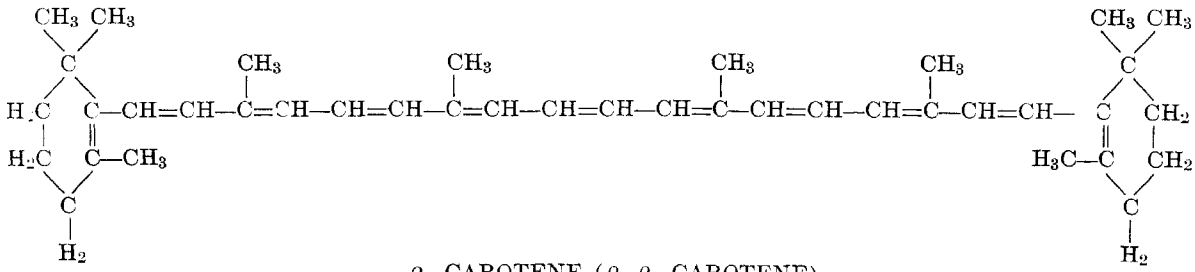
The Vitamin A-active Carotenoids

Though as many as 60 carotenoids have been reported to date, only four of the pigments of plant origin, α-, β-, γ-carotene and cryptoxanthin, have been found to possess vitamin A activity.

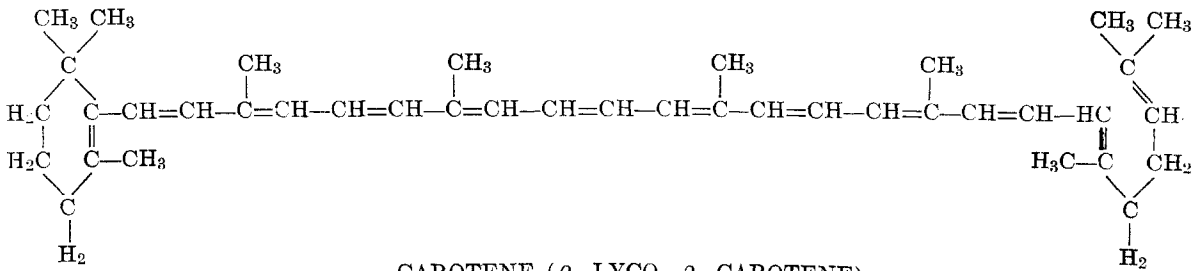
Carotene or provitamin A, $C_{40}H_{56}$, as has been pointed out, may exist in three isomeric forms known as alpha, beta and gamma carotene, Euler, Karrer, Hellstrom and Rydbom *(17)*, Karrer, Schöpp and Morf *(48)* and Kuhn and Brockmann *(61)*. The formulae of the three carotenes are as follows:



α—CAROTENE (β, α—CAROTENE)



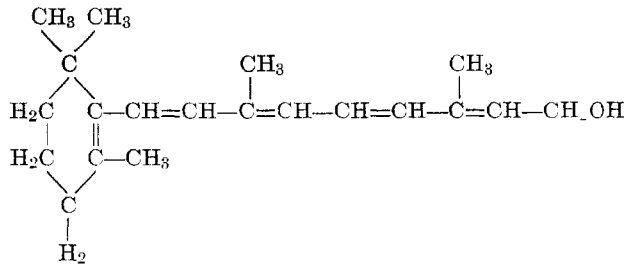
β—CAROTENE (β, β—CAROTENE)



γ—CAROTENE (β—LYCO—β—CAROTENE)

The constitution of alpha carotene was established by Karrer, Helfenstein, Wehrli, Pieper and Morf *(38)* and Karrer, Morf and Walker *(45)*; that of beta carotene by Karrer, Helfenstein, Wehrli and Wettstein *(39)* and Kuhn and Brockmann *(65)*; that of gamma carotene by Kuhn and Brockmann *(81)*. According to Winterstein *(154)* and Winterstein and Stein *(155)* there may also be a delta carotene. Palmer *(99)* has suggested that alpha should be known as B a-carotene, beta carotene as β β-carotene, and gamma carotene as β-lyco-β-carotene.

The formula for vitamin A as established by Heilbron, Heslop, Morton and Webster *(32)*, Karrer, Morf and Schöpp *(43, 44)* is as follows:

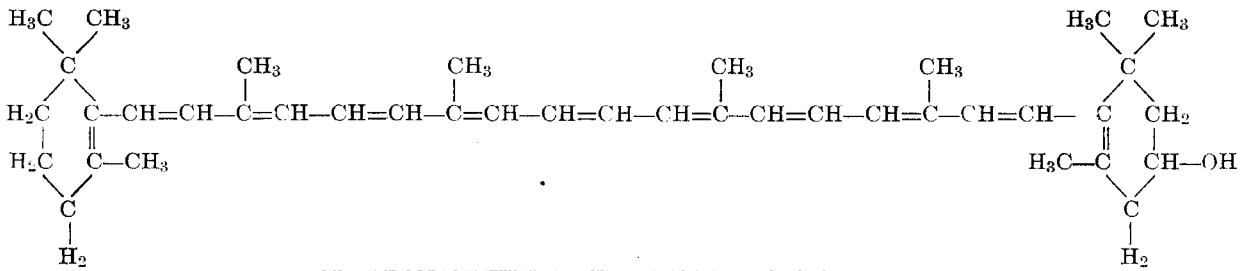


VITAMIN A

The fourth vitamin A-active carotenoid, cryptoxanthin[4], is found in rather large quantities in corn. In view of the general usage of corn as a poultry feed, and since much of the experimental work to be presented in this report concerns itself with the vitamin A potency of the pigments of corn a brief discussion of them is included here.

At present corn is known to have at least three carotenoid pigments, β-carotene, cryptoxanthin and zeaxanthin. Until as recently as 1934 only one pigment, zeaxanthin, had been isolated from corn [Karrer, Salomon and Wehrli *147*)]; Karrer, Wehrli and Helfenstein *(50)*. It had been assumed, however, [Euler, Demole, Karrer and Walker *(16)*] that corn must owe its vitamin activity to the presence of carotenes. Kuhn and Grundmann *(69)* have shown, however, that fresh corn does not contain more than traces of carotenes but does contain considerable amounts of cryptoxanthin. The structure of this pigment, which is 3-hydroxy β-carotene, as established by Kuhn and Grundmann *(68, 89)* is as follows:

4. The name *cryptoxanthol* is suggested to replace *cryptoxanthin*. The name *cryptoxanthin* is not suitable, as the suffix "-in" is by international agreement reserved for substances of unknown chemical nature. Nor is *cryptoxanthine* suitable, since the suffix "-ine" is by international agreement reserved for nitrogenous bases. The suffix "-ene" in analogy with carotene cannot be used, as "-ene" is reserved for unsaturated hydrocarbons. Since the substance in question contains a hydroxyl group, the name *cryptoxanthol* seems suitable and free from objection. The authors are indebted to G. F. Marrian, Department of Biochemistry, University of Toronto, for this suggestion. Palmer (1934) (*99*) has suggested the generic name *β-carotene-β-xanthinol* and the chemical name *β-5'β-carotenol* for *cryptoxanthin*. The suggestion made here has only the advantage of brevity and association with the original name given to this pigment. Similarly the name *zeaxanthol* is suggested to replace the name *zeaxanthin*.



CRYPTOXANTHIN (3—HYDROXY β—CAROTENE)

The relative proportion of these three pigments as found in fresh corn by these workers is shown in Table I.

It would appear that the "carotene" fractions of Euler *et al. (16)* consisted chiefly of cryptoxanthin.

Kuhn and Grundmann *(89)* found that on a vitamin-A free diet, rats which had maintained a constant weight for some time and had no appreciable amounts of vitamin A in their livers, increased in weight from 120 gm. to 148 gm. in 20 days when fed 1 mg. of cryptoxanthin daily and stored vitamin A in their livers. Only traces of unchanged cryptoxanthin were present in the livers.

Cryptoxanthin, $C_{40}H_{55}OH$, which has only one hydroxl group, is difficult to distinguish from p-carotene. Its absorption spectrum is identical with that of β-carotene and zeaxanthin, as shown in Table II. This can probably be accounted for by the fact that all three pigments are closely related structurally cryptoxanthin being a mono-hydroxy derivative of β-carotene, while zeaxanthin is a dihydroxy derivative.

Kuhn and Grundmann *(68)* stated "cryptoxanthin may be easily mistaken for B-carotene because its absorption spectrum is practically identical with it and zeaxanthin." Zechmeister *(158)* also

TABLE I.—CAROTENOID PIGMENTS IN CORN (Kuhn and Grundmann)

	Milligrams per 100 grams, fresh material.		
	Carotene.	Cryptoxanthin.	Zeaxanthin.
Italian	0.07	0.46	1.27
Hungarian (No. 1)	.05	.46	1.45
Hungarian (No. 2)	.05	.70	1.38
Grits	.05	.25	0.54

TABLE II.—ABSORPTION MAXIMA OF THE PIGMENTS OF CORN

SOLVENT.	Cryptoxanthin.			β-carotene			Zeaxanthin.		
CS_2	519	483	452	521	485.5		519	483	450
$CHCl_3$	497	463	433	497	466		494	462	429
C_2H_5OH (abs)	486	452	424	.....			483	451	423
Petroleum ether (b. 70.80)	485.5	452	424	483.5	452	426	483	451	
Hexane	484	451	423	482	451		.....		

stated that the absorption maxima of β-carotene and cryptoxanthin were almost identical. Incidentally, Kuhn and Grundmann *(68)* pointed out that if β-carotene and cryptoxanthin are present together in petroleum ether solution, washing with 95 percent methanol will remove cryptoxanthin but not β-carotene.

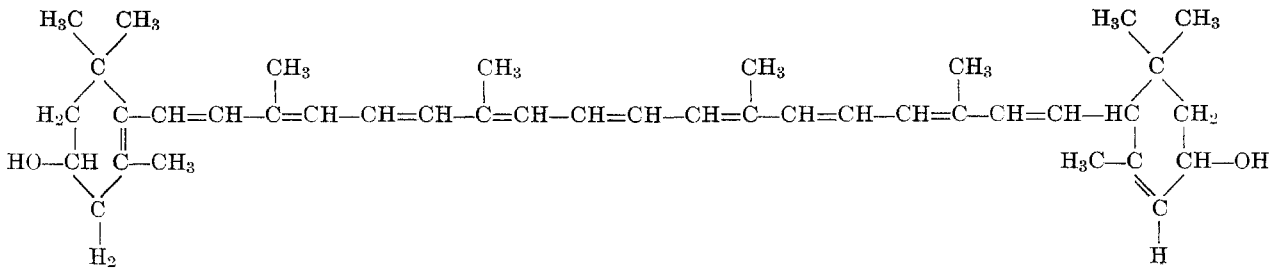
Kuhn and Grundmann *(68)* have succeeded in separating β-carotene, cryptoxanthin and zeaxanthin by adsorption on calcium carbonate or Al_2O_3 from a petroleum ether solution. By chromatographic analysis on activated Al_2O_3 zeaxanthin is strongly adsorbed and remains near the top of the column, cryptoxanthin locates itself near the center, and carotene, being only slightly adsorbed, proceeds to the bottom of the column.

The above treatment has not yet been developed to a stage where it can be successfully used in routine analysis.

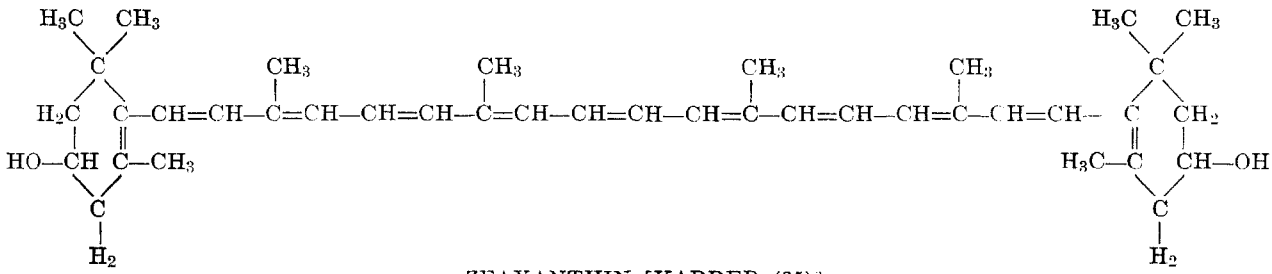
The presence of a β-ionone ring in combination with four conjugated double bonds appears to be the criterion for vitamin A activity. Of the derivatives of β-carotene, only those in which one-half of the molecule remains unchanged possess biological activity, semi-β-carotenone, $C_{40}H_{56}O_2$, formed by the opening of only one of the two-ring systems, semi-β-carotenone-oxime, β-oxycarotene, $C_{40}H_{58}O_2$, and dehydro-β-semi-carotenone. β-Carotenone with the two ring systems opened is inactive. [Kuhn and Brockmann *(62)*] Karrer, Euler and Solmssen *(36)* have shown that the addition of two hydroxyl groups to the β-ionone ring of α-carotene results in a biologically inactive compound. Kuhn and Brockmann *(64)* found that, β-carotene was twice as active as α- or γ-carotene. Theoretically, β-carotene on scission should give rise to two molecules of vitamin A whereas α- or γ-carotene could yield only one molecule, so that the demands of theory appear substantiated. Similarly, since cryptoxanthin contains only one unaltered β-ionone ring, one would expect the pigment to have only one-half the vitamin A potency of β-carotene. Zeaxanthin possessed no activity [Kuhn and Grundmann *(69)*].

The Xanthophylls (Carotenols)

Though a great many xanthophylls are known, only two of them, lutein and zeaxanthin, are found in plants in sufficient quantities to receive consideration here. The formulae of these two pigments may be written as follows:



LUTEIN [KARRER, ZUBRYS AND MORF (52)]



ZEAXANTHIN [KARRER (35)]

Xanthophyll (one of the carotenols), along with chlorophylls a and b, and carotene is found in every green plant. It is always present in green plant parts in greater concentration than carotene, and may be present either in free or esterified forms. To date no plant material has been found which will provide xanthophyll in practically pure form with the exclusion of other carotenoids, as is the case with carotene as obtained from carrots.

Xanthophyll differs from carotene in solubility. It dissolves readily in alcohol and ether, but only slightly in petroleum ether. Zechmeister *(158)* reports that to dissolve one gram about 700 c.c. of boiling or 5 liters of cold methyl alcohol are necessary. Xanthophyll dissolves readily in chloroform but slowly in carbon disulfide or benzene and not at all in glycerine. Schertz *(122)* reports a solubility of 0.0095 g. per liter of petroleum ether at 25° *C.*

Xanthophyll, like carotene, is quite stable to alkali, but is much more sensitive to acids than is carotene [Kuhn, Winterstein and Lederer *(82)*]. Willstätter and Page *(151)* point out that the stability of xanthophyll is limited, having demonstrated that xanthophyll dissolved in methyl alcoholic potash can be only slowly and incompletely regenerated.

It should also be pointed out that esters of xanthophyll have the same absorption spectrum as the free xanthophyll, but do differ in the solubilities. The esters, similar to the polyene hydrocarbons, are much more soluble in petroleum ether than in alcohols, and consequently are found in the upper layer when attempts are made to separate them with petroleum ether and dilute methanol.

There has been, and continues to be, in the literature, considerable debate as to the homogeneity of plant xanthophyll. It has been repeatedly pointed out that leaf xanthophyll consists of many components, similar to one another. Tswett *(138, 139, 140)* in his early studies using the adsorption technique, was convinced that at least three, and perhaps four xanthophylls were present in leaf pigments. Palmer and Eckles *(100)* and Kylin *(83)* have made similar observations.

In a more recent work, Strain *(134)*, using an improved adsorption technique, found that leaves contain not less than 12 xanthophylls. From an aqueous alcoholic solution of xanthophylls he obtained the following fractions: (1) three or four pigments resembling cryptoxanthin which passed through the column readily, (2) lutein, more than half the recoverable xanthophyll, (3) an optically inactive pigment, isolutein, (4) zeaxanthin (found in all leaves examined), (5) flavoxanthin, maxima at 4510 and 4220 Å. U.; (6) a pigment soluble in 60 percent methyl alcohol, and (7) several other xanthophylls.

Willstätter and Escher *(149)* were successful in obtaining a crystalline pigment from egg-yolks which in most of its properties coincided with leaf xanthophyll. A xanthophyll preparation from cow and sheep dung has also been reported, which is similar to egg lutein [Fischer *(23)*, Karrer and Helfenstein *(37)*].

Kuhn, Winterstein and Lederer *(82)*, in a more recent study report that the pigment of egg yolk is not a single pigment, but consists for the most part of "lutein" $C_{40}H_{56}O_2$, and another pigment, zeaxanthin, $C_{40}H_{56}O_2$. The latter pigment was discovered by Karrer, Salomon and Wehrli *(47)* to be the main pigment in corn.

Lutein (leaf xanthophyll) can be differentiated from zeaxanthin (corn xanthophyll) by its greater solubility in boiling methanol (1:700 as compared with 1:1550). The melting point of lutein, 193° C., is also lower than that of zeaxanthin, 207°. A comparison of the absorption maxima of lutein (Table III) with those reported for zeaxanthin in Table II shows that these two pigments differ in this respect also.

Lutein is extremely sensitive to mineral acids, or to traces of moderately strong organic acids, which raise its rotatory power and lower the melting point.

Lutein is strongly dextrorotatory: $([a]_{Cd} = 160°$ (in $CHCl_3$), or 145° (in acetic acid).

Zeaxanthin is difficultly soluble in methanol, petroleum ether and ligroin, and quite soluble in carbon disulfide, benzene, chloroform, carbon tetrachloride, pyridine and ethyl acetate. In regard to its solubility Zechmeister *(158)* presents the startling fact that if 100 milligrams of zeaxanthin is suspended in 5-10 c.c. of acetic acid, the addition of 5 c.c. of hexane will clarify the solution. This is surprising in view of the practical insolubility of the pigment in hexane alone. Zeaxanthin is optically inactive.

Zeaxanthin in the presence of atmospheric oxygen is readily oxidized. The speed of oxidation however is less than that of leaf xanthophyll.

Both lutein and zeaxanthin are inactive as provitamin A. It has been reported, however, that the action of PBr_3 on zeaxanthin produces a vitamin A active product [Euler, Karrer and Zubrys *(20)*].

TABLE III.—ABSORPTION MAXIMA OF LUTEIN

SOLVENT.	Wave length, u. u.		
CS_2	508	475	445
Petroleum ether	477.5	447.5	
Ethyl alcohol	476	446.5	420

SEPARATION AND QUANTITATIVE DETERMINATION OF THE CAROTENOIDS

Determination

All methods which have been described for the determination of the carotenoids are based upon the discovery of Borodin *(7)* in 1883 that the carotenoids may be separated into alcohol soluble and petroleum soluble fractions. Methods for the quantitative determination were also reported by Arnaud *(1)* in 1887, and by Monteverde and Lubimenko *(93)* in 1913, but the method which has been used most widely as a starting point in the development of new techniques is that of Willstätter and Stoll *(152)*. In the latter method the technique in brief is somewhat as follows:

The fresh plant material is ground finely in a mortar with quartz sand and 40 percent acetone. The ground material is then filtered and washed with 30 percent acetone until the filtrate comes through clear. The extracted material is finally washed with pure acetone, removed from the filter, macerated once again under pure acetone and filtered a second time. The combined acetone extract is then treated with ether and the acetone completely removed by washing with water. The chlorophylls are saponified with methyl alcoholic potash, which is removed from the ether solution by washing with water. The ether extract is evaporated to dryness with vacuum, the residue taken up in petroleum ether, and the extract poured into a separatory funnel. Xanthophyll is then removed from the carotene by washing first with 85 percent methanol, then 90 percent and finally with 92 percent methanol until the washings are colorless. The xanthophyll which is present in the alcohol phase is then brought into ether solution. Both the carotene and xanthophyll solutions are then washed free of methanol with water, dried, brought to volume and the concentrations determined colorimetrically, using a 0.2 percent solution of potassium dichromate as the colorimetric standard. Willstätter and Stoll *(156)* report color matches as follows:

	Millimeter depth of solution		
Carotene (0.0268 g. in 1 liter of peteroleum ether)	100	50	25
Dichromate (0.2%)	101	41	19
Xanthophyll (0.0284 g. in 1 liter of ether)	100	50	25
Dichromate (0.2%)	72	27	14

Euler, Demole, Karrer and Walker *(16)* have also found similar deviations in the comparison of carotene and xanthophyll solutions with potassium dichromate.

Numerous modifications of the Willstätter and Stoll method for the determination of carotenoids have appeared: Coward *(13)*, makes the first step the decomposition of chlorophyll; Schertz *(120)* uses diethyl ether in addition to acetone in the extraction; Smith and Smith *(128)* use pyridine in the original extraction; Schertz *(120)*

has described a spectrophotometric method for the estimation of carotene concentration; Kuhn and Brockmann *(59)* have described the use of petroleum ether and methyl alcohol in the extraction of plant tissue. The latter workers use a solution of 14.5 grams of azobenzene in 100 c.c. of 96 percent ethyl alcohol as the standard. This solution is said to deviate only slightly from Beer's Law for most concentrations of pigment. The pigment concentrations in one c.c. of petroleum ether solution which are equivalent in color value to the azobenzene solution described are given in Table IV.

Russell, Taylor and Chichester *(118)* have described a method for extraction of plant tissue in which petroleum ether is used directly for dry materials, while fresh plant tissue is triturated with sand under acetone, the pigments being subsequently transferred to petroleum ether. Chlorophyll and xanthophyll were removed by the usual technique. As a colorimetric standard these workers used a 0.036 percent potassium dichromate solution, which they found to be equivalent to 0.00206 mg. of carotene per c.c.

Guilbert *(30)* has reported a method for the determination of carotene in forage which, it is claimed gives consistently reproducible results, comparable to those obtained by the acetone-ether extraction method of Schertz *(120)*. The main features of the Guilbert method are as follows:

The sample is digested for 0.5 hour with a saturated solution of potassium hydroxide in ethyl alcohol. Ethyl ether is added to the digestion mixture and the chlorophyllins and flavones are separated by washing with water. The ether solution containing carotene and xanthophyll is evaporated on a steam or water bath to remove the ether. The residue is extracted with petroleum ether, and xanthophyll is removed by the usual method with 90 percent methyl alcohol. The petroleum ether solution, containing the carotene, is brought to volume and compared in a colorimeter against Sprague's *(129)* dye standard.

TABLE IV.—COLOR VALUES OF VARIOUS CAROTENOIDS EQUIVALENT TO A SOLUTION OF 14.5 MG. OF AZOBENZENE IN 100 C.C. OF 96 PERCENT ALCOHOL, [Kuhn and Brockmann *(59)*]

Mg. in 1 c.c. of petroleum ether.	Pigment.	Formula.
0.00235	α-carotene	$C_{40}H_{56}$
0.00235	β-carotene	$C_{40}H_{56}$
0.00242	cryptoxanthin	$C_{40}H_{56}O$
0.00252	lutein	$C_{40}H_{56}O_2$
0.00252	zeaxanthin	$C_{40}H_{56}O_2$
0.0027	taraxanthin	$C_{40}H_{56}O_4$
0.0027	violaxanthin	$C_{40}H_{56}O_4$
0.0046	helenien	$C_{72}H_{116}O_4$
0.0046	physalien	$C_{72}H_{116}O_4$

In a number of preliminary determinations in this laboratory on commercially dehydrated alfalfa meal, using the Guilbert method, consistently reproducible results were obtained only when special precautions were taken. It was found necessary to purify the ether shortly before using in order to remove ether oxides. To avoid losses of carotene during evaporation of the ether from the carotene-xanthophyll solution, it was found advisable to remove the ether by vacuum distillation, or distillation with a stream of nitrogen at a temperature of less than 40° C.

A modification of the Guilbert method which has been used in the authors' laboratories *(107)* considerably shorter, eliminates several possibilities of carotene loss in manipulation, and gives results which are readily reproducible. The original ether extraction of the Guilbert method has been eliminated entirely. Instead, petroleum ether (b. p. 40-60°) is used. This obviates the necessity of carrying on a single solvent evaporation during the course of the determination, and excludes the possibility of carotene decomposition which might occur during the ether evaporation required in the original method. The method is considerably shortened, inasmuch as the chlorophyllins, flavones, alkali, and xanthophyll can be removed directly from the petroleum ether exactly as Guilbert describes their removal from ether and petroleum ether, respectively. The method in detail is as follows:

Weigh out the samples (1-5 grams), transfer to Erlenmeyer flasks, and add 20 c.c. of a freshly prepared, saturated solution of KOH in ethyl alcohol to each gram of sample. Fit the flasks with reflux condensers, and boil the contents on a steam bath or hot plate for 30 minutes. If portions of the sample collect on the sides of the flask, wash down with alcohol from a wash bottle. Cool the contents of the flask, then pour them into a sinter-glass filter funnel, applying a vacuum only until most of the solvent has come through. The residue is then washed alternately with 25 c.c. portions of Skellysolve and absolute alcohol until the filtrate comes through clear. The suction should at no time be applied unless the sediment is partially covered by solvent. After the addition of each wash portion of solvent, more complete extraction may sometimes be obtained by stirring the sediment on the funnel plate with a stirring rod before applying suction.

Pour gently about 100 c.c. of distilled water through the alcohol-Skellysolve solution in the separatory funnel. Draw off the alkaline alcohol-water solution from the bottom of the funnel, and reëxtract three times by shaking gently with 30 c.c. portions of Skellysolve, using two other separatory funnels. Combine the Skellysolve extracts and wash them with 50 c.c. portions of distilled water until free from alkali, as indicated by the absence of color in the wash water when treated with phenolphthalein.

Remove xanthophyll from the Skellysolve solution by extracting with 25 c.c. portions of 90 percent methyl alcohol until the wash alcohol comes off colorless. This may require from six to twelve

washings, depending on the amount of xanthophyll in the sample. Wash the Skellysolve solution containing the carotene twice with 50 c.c. of distilled water to remove the alcohol and filter into a volumetric flask through filter paper upon which is placed a small amount of anhydrous Na_2SO_4. After making the carotene solution up to volume, determine the concentration either by the spectrophotometric method of Peterson, Hughes and Freeman *(107)* or the colorimetric method of Fraps *(24)*.

In the Fraps method *(24, 25, 94)* the amount of carotene in the sample is estimated by comparing it colorimetrically against 0.1 percent potassium dichromate. Put the solution of the sample in the left-hand cup of the colorimeter and set the scale at 0.5 cm., 1 cm., 2 cm., or 4 cm., according to the amount of color present. Vary the depth of the dichromate solution in the right-hand cup until the density of color in both cups is equal, and make eight independent readings, recording them in millimeters. Average the readings. Make the dichromate readings between 4 mm. and 12 mm. on the colorimeter. If a reading below 4 mm. cannot be avoided read it, but repeat the analysis with a larger sample.

By use of Table V transform the millimeter depth of 0.10 percent dichromate into parts per million of carotene. Then calculate the parts per million of carotene actually in the sample by the use of the following formula:

$$(1)\quad P = \frac{\text{parts per million of carotene (from table)} \times \text{c.c of solution}}{\text{gms. sample} \times \textit{cm.} \text{ depth of sample solution.}}$$

Report carotene to 0.1 parts per million.

TABLE V.—TABLE FOR CALCULATION OF CAROTENE

Mm. 0.1 percent $K_2Cr_2O_7$.	Parts per mil. carot.	Mm. 0.1 percent $K_2Cr_2O_7$.	Parts per mil. carot.	Mm. 0.1 percent $K_2Cr_2O_7$.	Parts per mil. carot.
1.0	0.5	4.8	2.9	8.6	5.3
1.2	0.7	5.0	3.1	8.8	5.4
1.4	0.8	5.2	3.2	9.0	5.6
1.6	0.9	5.4	3.4	9.2	5.8
1.8	1.0	5.6	3.5	9.4	5.9
2.0	1.2	5.8	3.6	9.6	6.0
2.2	1.4	6.0	3.8	9.8	6.1
2.4	1.5	6.2	3.9	10.0	6.3
2.6	1.6	6.4	4.0	10.2	6.5
2.8	1.7	6.6	4.1	10.4	6.7
3.0	1.8	6.8	4.2	10.6	6.8
3.2	2.0	7.0	4.3	10.8	6.9
3.4	2.1	7.2	4.5	11.0	7.1
3.6	2.2	7.4	4.6	11.2	7.3
3.8	2.3	7.6	4.7	11.4	7.4
4.0	2.5	7.8	4.8	11.6	7.5
4.2	2.6	8.0	4.9	11.8	7.6
4.4	2.7	8.2	5.0	12.0	7.8
4.6	2.8	8.4	5.2	.....	.....

Method for standardizing 0.1 percent potassium dichromate for carotene: Dissolve1 tube (0.1 gm.) of SMA carotene in about 2 c.c. of chloroform and precipitate with 20 c.c. of methanol. Filter and wash with a few drops of methanol and dry in a desiccator with diminished pressure for about 1 hour. Very carefully weigh out 10 mgs. of this purified carotene and dissolve in the smallest possible amount of chloroform. Dissolve in 100 c.c. of petroleum ether. Take 10 c.c. and make up to 100 c.c. with petroleum ether. This will give a 0.001 percent carotene solution. Put this carotene solution in the left-hand cup of a colorimeter and set the depth at five mm. Vary the depth of the right-hand cup of the colorimeter, which contains the 0.1 percent $K_2Cr_2O_7$ to be standardized, until the colors match in intensity. The right-hand side of the colorimeter should read 8.3 mm. If it does not read 8.3 mm., adjust the dichromate solution by adding more potassium dichromate, or more water, until it does read 8.3 mm.

If the carotene solution is set at 4 mm., the potassium dichromate reading should be 6.5 mm.

The spectrophotometric method may also be used. For each determination optical density measurements are made at wave lengths 4550, 4700, and 4800 Å. Using the absorption coefficients calculated for β-carotene in petroleum ether at these wave lengths, the carotene concentration is determined at each wave length from the equation $c=D/kb$, where b is the thickness in centimeters of the layer of solution, c is the concentration in grams per liter of the carotene, D is the optical density (read directly from the spectrophotometer), and k is the extinction coefficient (frequently designated as the specific transmissive index or absorption index). The extinction coefficients for β-carotene in various solvents are recorded in Table VI.

The carotene concentration obtained should be identical for each of the three wave lengths. Thus, for each analysis, the purity of the carotene in solution is definitely established and the complete re-

TABLE VI.—EXTINCTION COEFFICIENTS FOR β-CAROTENE

WAVE LENGTH.	80 percent ethyl alcohol, 20 percent ethyl ether, Miller (*91*, *92*).	Petroleum ether (b. p. 40-60°), Seibert.*	Skellysolve B (b. p. 60-70°), Authors.†
4,500	247	243	238
4,550	243	231	227
4,700	210	207	200
4,800	221	212	212

* These coefficients were obtained from H. F. Siebert of the S. M. A. Corporation, Cleveland, Ohio, and checked by the authors.

† Skellysolve B, a special commercial grade of petroleum ether, can be successfully substituted for petroleum ether (b. p. 40-60°) in the modified method described.

moval of other pigments ensured. When variations in the concentration calculated for the various wave lengths are within the normal limits of error for spectrophotometric measurements, the average of the three values is taken as the final figure for the carotene concentration.

The dye standard recommended by Guilbert (1934) *(30)* may also be used [Sprague *(129)*]. In this method naphthol yellow (3.06 grams) and Orange G crystals (0.45 gram) are dissolved in distilled water and made up to 1 liter. The dissolving of the naphthol yellow is facilitated by first adding water to form a thick paste, then grinding it in an agate mortar. The standard is prepared by diluting 5 c.c. of the stock solution to 1 liter. On the basis of SMA carotene (m.p. 166° to 168° C.), the value of the dye is 2.63 mg. of carotene per liter.

$$\frac{\text{Depth of standard}}{\text{Colorimeter reading}} \times \frac{100}{\text{amount of sample in grams}} \times \frac{\text{total volume of unknown}}{1000} \times \text{value of standard in mg.}$$

of carotene per liter = mg. of carotene per 100 grams of sample.

Buxton and Dombrow (11) have introduced a number of improvements to the modified Guilbert method previously described which show promise. These workers found that purified technical heptane as an extraction solvent was preferable to petroleum ether. The instrument employed was a modified Bausch & Lomb visual spectrophotometer, equipped with a Duboscq colorimeter arrangement and a rotating sector. A brief description of the experimental method follows:

Weigh accurately into a 250-ml. digestion flask 5 grams (more or less, depending on the relative potency) of dehydrated alfalfa meal. Add 75 ml. of 10 percent ethanolic potassium hydroxide and reflux on a hot plate or steam bath for 30 minutes. Agitate occasionally in order to facilitate digestion. Cool the contents of the flask, add 100 ml. of purified technical heptane, shake thoroughly, allowing the suspended material to settle, and decant the liquid portion into a 500-ml. separatory funnel. Reextract the residue with 50-ml. portions of heptane until the resultant solution is colorless (three extractions are usually sufficient). Combine the heptane extracts and wash free from chlorophyllins, flavones, alkali and xanthophylls by shaking thoroughly with 90 percent methanol (five washes are generally sufficient), and reëxtract the first methanol portion with 50 ml. of heptane. Examine the last washing for free alkali by testing a few millileters with phenolphthalein. Distill the heptane portion to a small volume under a vacuum in the presence of nitrogen gas. The concentrated carotene solution is then made to volume (50 ml.) with heptane and is ready for examination in the

visual spectrophotometer. The intensity of absorption at 4500 Å. is determined by taking the average of several readings.

To determine the percentage of carotene in the sample of alfalfa meal, the $E^{1\%}_{1\ cm.}$ 4500 Å.(heptane) = 2380 (the extinction coefficient for pure B-carotene as determined by using the medium-sized quartz Bausch & Lomb spectrophotometer) is determined. By using the following equation it is possible to calculate the carotene for a 1 percent solution:

$(S \times F/R \times C)$ = gamma of carotene for a 1 percent solution where
S = the screen factor
F = the conversion factor for pure β-carotene in heptane
R = the reading expressed in centimeters
C = the concentration

The results can be conveniently expressed as gamma of carotene per gram of alfalfa meal. Other methods for the determination of carotene have been suggested *(54, 11 1, 112, 114, 156)*. Ferrari and Bailey *(22)* have proposed a method for flour.

In routine analysis for carotene it is frequently necessary to modify the techniques described, depending upon the nature of the material.

It has been found, for example, that though digestion for one-half hour with alcoholic potash separates 98-100 percent of the available carotene in the case of dry feeds which have been run through the Wiley mill, only 50-80 percent of the total carotene is removed in the case of fresh green grasses, silage, etc. In these cases the residue is ground from the original digest with sea-sand in a mortar and is refluxed once again in alcoholic potash for 15 minutes and determined as usual. It is always wise, therefore, when working with strange material to repeat the digestion of the thoroughly macerated residue to determine whether all the carotene has been removed by one extraction.

In the present work it, was frequently necessary to determine the exact quantities of a pigment present' in feed or eggs when extinction coefficients for the pigment or pigments in question were not available. This was the case with corn, and with eggs from hens which had received corn as the sole source of carotenoid pigments. It was probable that in either case the petroleum phasic fractions contained both carotene and cryptoxanthin while the alcohol soluble pigment was probably mostly zeaxanthin.

Since the quantitative separation of carotene from cryptoxanthin by means of an adsorption column offered considerable difficulty, the carotene and cryptoxanthin were not separated, but the petroleum ether soluble fraction was obtained and the pigment concentration determined as described in a previous paper by Peterson, Hughes and Freeman *(107)*. Since the absorption spectra of β-carotene and cryptoxanthin, as previously discussed, are identical, optical densities of the petroleum solutions were read at the maxima normally used for β-carotene. The authors were unable to find in the liter-

ature extinction coefficients for cryptoxanthin[5] in any solvent. Pure crystalline cryptoxanthin was unavailable for the determination of extinction coefficients in this laboratory. Consequently the coefficients for β-carotene were used throughout for the determination of total pigment content in this fraction. It is possible that this would introduce a slight error in the results. A study of the colorimetric comparisons (Table IV) of β-carotene and cryptoxanthin solutions with a solution of azobenzene in 100 c.c. of 96 percent ethyl alcohol as presented by Kuhn and Brockmann *(59)*, however, would indicate that this error would probably not exceed 3-5 percent.

In this study zeaxanthin was determined in the following manner: The aqueous alcoholic potash residues and the 90 percent methyl alcoholic washes from the carotene determination were exhaustively extracted with diethyl ether. The combined ether extracts were washed thoroughly with distilled water, dried with anhydrous sodium sulphate, and brought to a convenient volume. The optical density was then determined spectrophotometrically at 4525 Å. Since the extinction coefficient for zeaxanthin at this wave length for ether was not available it was necessary to determine it indirectly. Ether solutions of zeaxanthin from several typical corn samples were obtained and their optical densities determined at 4525 Å. The ether solutions were then evaporated and taken up in CS_2, and their respective concentrations determined. using the coefficients reported by Kuhn and Smakula *(78)*. $\kappa = \frac{2.30}{c.d} \cdot \log_{10} I_o/I$, where c is the concentration in moles per liter and d is the thickness in centimeters of the layer of solution. These workers report $\kappa = 29.2 \times 10^4$ at 4830 Å. and 26.4×10^4 at 5170 Å. the absorption maxima of zenxanthin in CS_2. Several zeaxanthin fractions were also taken up in CHC_{l3} and their concentrations determined using the $E^{1\%}_{1\ cm} = 1500$ at 4600 Å., where $E^{1\%}_{1\ cm.} = \log I_o/I$ for a 1 cm. layer of a 1 percent solution. The concentrations thus determined were used to determine k at 4525 Å. from the equation $K = D/c.b$ where b is the thickness in centimeters of the layer of solution, c is the concentration in grams per liter of the zeaxanthin, D is the optical density (read directly from the spectrophotometer), and k is the extinction coefficient. The average k thus determined was 238, and was the value used in most of the determinations. It is not implied that this value would be identical with that obtained with pure zeaxanthin in ether solution. The accuracy of the determined coefficient would depend on the accuracy of the coefficients reported for CS_2 and $CHCl_3$, the purity of the corn zeaxanthin obtained in ether solution by the method employed, and on the completeness of the transfer of pigment from the ether solution to the other solvents used.

5. Since this bulletin was prepared for publication, the book "Leaf Xanthophylls" (Carnegie Institution of Washington, Washington, 1938, Publication No. 490) by Harold Strain has been published, in which are reported coefficients for most of the known xanthophylls.

Adsorption Columns

With the possible exception of spectroscopy, no development has been so helpful in the study of the closely related carotenoids as has chromatography, or the separation of pigments by their adsorption on a column. For this discorery we are indebted to Tswett *(138)*, who found that if a carotenoid mixture in a solution of carbon disulphide, benzene, or petroleum ether was poured through an evenly packed column (10-15 cm. x 1-2 cm.) of calcium carbonate, inulin, or sucrose, the pigments separated into well-defined zones by virtue of the preferential adsorption of the adsorptive material used for the different pigments. By pouring more of the pure solvent through the column it was frequently possible to accomplish a wider separation of the zones so that they might be separated mechanically and taken up in the proper solvent, or as was frequently the case, one of the less strongly adsorbed zones could be washed through entirely without contamination.

Adsorption methods have since been used to advantage and improved techniques have been developed by a great many workers in the field, notably, Palmer *(96, 98)*; Palmer and Eckles *(100)*; Vegezzi *(141)*; Lipmaa *(84)*; Kuhn and Lederer *(73)*; Karrer and Walker *(49)*; Kuhn, Winterstein and Lederer *(82)*; and Kuhn and Brockmann *(55)*.

Much work has been done [Strain *(132)*] particularly on the adsorption of the petroleum-phasic carotenoids. Metallic oxides, charcoal, fuller's earth, bisulfites and zinc chloride have been used. Manganese oxide, lead peroxide and chromic oxide have a tendency to oxidize the adsorbed pigments. Fuller's earth has been reported to destroy much of the adsorbed carotene, while charcoal adsorbs a fraction of the pigment so strongly, that the amounts of liberated carotene are very small. Acid adsorbents destroy most of the adsorbed pigment.

Strain *(132)* who, in recent years, has done outstanding work in the development of adsorption methods, finds that of numerous adsorbents studied, a special brand of magnesium oxide possesses the greatest number of desirable properties. This oxide exhibits a very high resolving power for different carotenes, so that each carotene separates as a single and distinct band or zone on the magnesium oxide Tswett column. In regard to this adsorbent we quote Strain (139) as follows:

"The adsorption capacity of magnesium oxide depends, to a large extent, upon its method of preparation. Thus, oxide prepared by rapid calcination of the basic carbonate is only a moderately good adsorbent, while the oxide prepared from magnesium hydroxide under suitable conditions is a most satisfactory adsorbent. The California Chemical Corporation, Newark, Calif., has very kindly made a series of preparations of magnesium oxide from which a highly active one has been selected, which permits a ready separation of the carotenes with a minimum of decomposition, and from which the adsorbed carotene can be easily eluted with petroleum ether and ethanol. This magnesium oxide is sold under the trade name of Micron Brand magnesium oxide No. 2641. We are indebted to Mr. Max Y. Seaton and the California Chemical

Corporation for their generous cooperation in the manufacture of the magnesium oxide preparations."

In the preparation of a Tswett adsorption column, a pyrex glass tubing of appropriate size is constricted at one end and a large wad of cotton pushed into place in the constricted portion of the tubing. The adsorbent is then added in small portions and each portion packed uniformly and firmly, particularly at the edges, before another portion is added. The tube is then attached to a suction flask.

Miller *(91)* has pointed out certain precautions which must be observed in carrying out chromatographic analyses. These have been found very useful and are presented here in detail:

(1) The original sample must be fairly free from impurities, the purification being carried out in an inert atmosphere to minimize the formation of oxidation products.

(2) The adsorbent as well as the carotenoid solution must be free from moisture.

(3) The adsorbent should be finely powdered, enabling close packing of the particles and consequently the formation of a firm layer of the adsorbent.

(4) The solution in the funnel containing the chromatogram must be protected from exposure to oxygen. If the formation of oxidation products is not prevented or removed as formed, the resulting carotenoid preparation after elutriation will fail to crystallize.

(5) It is necessary to wash the chromatogram to ensure complete separation of the components. As washing proceeds, the different zones move down but retain their relative position to one another.

The tight packing of adsorbents in columns frequently results in a very slow filtration rate. To overcome this Strain *(133, 134, 135)* recommends that the adsorbent be mixed thoroughly with a heat-treated siliceous earth (Hyflo Super Cel, manufactured by Johns-Manville). This mixture (usually 1:1) permits an even filtration, and the siliceous earth does not adsorb carotene.

Mackinney *(85)*, who has used the adsorption technique for the isolation of carotene from a large variety of different natural sources, has found that the beta-isomer is the principal constituent. Similar studies in these laboratories have also shown this to be true, especially with fresh materials, or those which have not been subject to the effects of excessive heat, light, and weathering. The changes brought about in the latter case will be discussed in another part of this bulletin.

In the use of a column for the separation of closely related pigments it is frequently necessary to fractionate by adsorption certain questionable fractions. That is to say, if four zones are obtained by the first adsorption, 1, 2, 3, and 4, it may be necessary to combine fractions 2 and 3 and pour them through a second column for more complete separation. In some cases even a third column may be necessary.

It is apparent that pigments owe their differences in behavior on an adsorbent to certain differences in the chemical structure of the molecules. It has been found, for example, that the most poorly adsorbed carotenoids are the polyene hydrocarbons α–, β- and γ-caro-

tene, and lycopene. In the latter group it is evident that the number of double bonds has an effect. Lycopene, with 13 double bonds is most strongly adsorbed while γ-carotene, which has 12 double bonds, is less strongly adsorbed. Next in order is β-carotene with 11 and then a-carotene, also with 11. In the case of α- and β-carotene, however, the difference in behavior is apparently influenced simply by the change in position of only one double bond.

Winterstein and Stein *(155)* have made a study of the relationship of chemical composition, structure, and behavior on a column. Winterstein *(154)* has prepared a table showing the order of adsorption of the more important carotenoids.

Adsorption Series of the Carotenoids From a Solution of Petroleum Ether

[Winterstein (*154*)]

Most strongly adsorbed	Fucoxanthin	$C_{40}H_{56}O_6$	alcohols	$CaCO_3$
	Violaxanthin	$C_{40}H_{56}O_4$		
	Taraxanthin	$C_{40}H_{56}O_4$		
	Flavoxanthin	$C_{40}H_{56}O_3$		
	Zeaxanthin	$C_{40}H_{56}O_2$		
	Lutein	$C_{40}H_{56}O_2$		
	Rhodoxanthin	$C_{40}H_{50}O_2$	ketone	Al_2O_3
	Physalien	$C_{72}H_{116}O_4$	esters	
	Helenien	$C_{72}H_{116}O_4$		
	Lycopene	$C_{40}H_{56}$	hydrocarbons	
	γ-carotene	$C_{40}H_{56}$		
	β-carotene	$C_{40}H_{56}$		
Most weakly adsorbed	α-carotene	$C_{40}H_{56}$		

Decreasing ↓ adsorption affinity

This series may now be extended and modified to include the new xanthophylls which have been separated on columns of magnesia and siliceous earth (1:1) by Strain *(134)*. In Table VII are shown the results of two typical adsorption experiments carried out by Strain on one-gram samples of leaf xanthophyll. Percentage yields of the various yellow pigments present have been calculated. (Colorless crystals present in the original xanthophyll mixture were not considered a part of the total.)

TABLE VII.—DISTRIBUTION OF XANTHOPHYLLS IN A LEAF XANTHOPHYLL MIXTURE

BAND.	Substance.	Percent.	Percent.
I	Mixture	10.9	
II	Mixture	4.1	
III	Mixture	.8	
IV	Neoxanthin	16.2	18.0
V	Flavoxanthin c	4.5	1.4
VI	Flavoxanthin b	4.0	3.6
VII	Violaxanthin b	5.3	20.0
VIII	Zeaxanthin	1.7	4.5
IX	Isolutein	1.0	1.8
X	Lutein	51.3	50.5
XI	Cryptoxanthin	Trace	Trace
Percolate	Coloress crystals	80 mg. 60	

It will be observed that in contrast to the adsorption studies which have been reported by Kuhn, Winterstein and Lederer *(82)*, lutein is less strongly adsorbed than zeaxanthin. Though these workers use a different adsorbent and solvent in their work, it has been reported by Zechmeister, Beres, and Ujhelyi *(159)* that under the same conditions, lutein was the least strongly adsorbed. Strain *(134)* has repeated these experiments and found that satisfactory separations of lutein and zeaxanthin were very difficult to obtain on columns of calcium carbonate when carbon disulfide was used as the solvent. The statement of Tischer *(137)* that lutein may be adsorbed above zeaxanthin on columns of alumina is probably in error.

By the use of columns of very small diameter (about 1 mm.), Strain claims to have been able to separate as little as 0.0015 mg. of carotene from carrot roots into α- and β-carotene. He has also accomplished separations of equally small portions of xanthophylls, the pigments having been identified by readsorption with known xanthophylls.

Absorption Spectra

Considering the important part that spectroscopic observations have played in the study of plant pigments, a brief discussion of this subject seems justified. There is probably no property of the carotenoids, with the possible exception of their behavior on adsorption, which is more readily determined with small amounts of material than the absorption spectra, or wave length at maximum absorption.

The characteristic bands of the carotenoids have been used extensively for their identification and for the determination of contaminating substances. The older work may be criticized for the all too frequent failure to include such important details as stratum thickness, concentration, solvent, etc. The importance of these factors cannot be overemphasized. With the development of highly refined adsorption techniques, it has also become evident that pigment extracts obtained by the older methods rarely contained a single pure pigment, and that, therefore, the absorption data reported for them were usually in error. With the more recent developments in knowledge concerning the use of selective solvents and subsequent separation of these pigment fractions by adsorption techniques, the spectrophotometer has become an invaluable tool in the identification and quantitative determination of carotenoid pigments.

The absorption spectra of carotenoids are typical, and easily differentiated from those of any other class of pigments. Carotene and xanthophyll, for example, have two bands in the blue and indigo blue region of the spectrum. In the older literature, it was common practice to report the position of the edges of the absorption bands. In the more recent literature, however, it has become common practice to report the points of maximum absorption (extinction or absorption maxima), which are independent of concentration or stratum thickness.

In the present work we have determined wave lengths of the absorption maxima of the pigments studied with the Bausch and Lomb No. 2750 visual photometer and No. 2700 spectrometer. The absorption of light is usually represented by a curve by plotting the logarithm of the specific absorption coefficient against the wave length. However, the method preferred is the one usually used by Miller *(91, 92)* in which the specific absorption coefficient *(a)* rather than its logarithm is plotted against the wave length, Thus, from Beer's Law:

$$I_x = I_o \, 10^{-acx}$$

$$a = \log_{10} \frac{I_o/I_x}{cx}$$

or

$$a = \frac{D}{cx}, \text{ where D, optical density} = \log_{10} I_o/I_x$$

I_o = intensity of light transmitted by the solvent cell.
I_x = intensity of light transmitted by the solution cell.
x = thickness of the solution layer in centimeters.
c = concentration of the carotenoids in grams per liter.

For a more thorough discussion of the nomenclature and different units employed for expression of the absorption spectra of pigments it is suggested that the reader consult the publications of Miller *(91, 92)*, McNicholas *(89)*, Smakula *(126)*, Smith *(127)*, Strain *(132-135)*, Weigert *(146)*, and Zscheile *(157)*, Kuhn and Smakula *(78)*, and Pummerer, Rebmann, and Reindel *(110)*.

In Table VIII are recorded the absorption maxima of some of the more common carotenoids in various solvents. These were obtained from Zechmeister *(158)* and Strain *(134)*. It is worthy of note that those pigments which are scarcely distinguishable spectroscopically do not differ essentially in the number and location of the chromophoric unions, though they may differ in the number of hydroxyl groups which they contain and in their behaviours on adsorption. An interesting example of this is the case of the three carotenoids of corn, β-carotene, cryptoxanthin, and zeaxanthin.

Possibiliites in the field of interpreting changes in the molecular structure of carotenoids from spectral data have not been given the consideration they deserve. It seems, for example, to be a fairly general principle that the more double bonds present in an uninterrupted conjugated series, the longer will be the wave length of maximum absorption (α–, β-carotene, and lycopene). It is also apparent that any chemical change which affects the nature of the chromophoric unions will markedly change the character of the spectrum, whereas very significant and deep-seated changes may be made in other parts of the molecule with but slight, if any, effect upon the absorption spectrum (physalien, and helenien, dipalmitates of zeaxanthin and lutein, respectively). These relationships have been adequately discussed by Kuhn and Brockmann *(56,61, 62, 63)*, Kuhn and Grundmann *(68, 69)*, and by Kuhn and Deutsch *(66)*.

Interesting are the cases of lycopinal and rhodoxanthin, both of which react to give oximes with considerable lightening of color and change of absorption maxima. It must be reasoned, therefore, that the carbonyl group of these pigments are conjugated with the double bonded system of the molecule. Hydroxylamine does not, however, bring about similar changes in the case of azafrinone, β-carotenone, or semi-B-carotenone. (See Table X.)

Work on the xanthophylls in these laboratories has been considerably handicapped in recent years because of the lack of proper specific absorption coefficients of some of the lesser known xanthophylls. Recently, however, the excellent work "Leaf Xanthophylls" by Harold H. Strain has appeared. Strain has determined the specific absorption coefficients for the common and also the lesser known carotenoids in several solvents. The values for a of various xanthophylls as interpreted from the absorption curves of Strain are shown in Table XI.

TABLE VIII.—ABSORPTION MAXIMA OF THE CAROTENOIDS IN VARIOUS SOLVENTS

CAROTENOID.	CS_2.	Petroleum ether.	Ethyl alcohol.	Chloroform.	Hexane.	Benzene.	References.
Rhodoxanthin	564, 525	524, 489	538, 496	546, 510	524, 489	542, 503	**63**
Lycopene	548–507	506–474	.....	517–480	.....	.....	**21, 39, 51, 61, 76, 90, 147, 148**
Capsanthin	543–503	505–475	.....	.....	.....	519–486	**158, 162, 163**
Capsorubin	543–503	507–474	.....	.....	.....	.....	**163**
Isocarotene	543–504	504–475	.....	.....	.....	.....	**29, 73, 117, 56, 57, 62**
β-carotenone	538–499	502–468	.....	527–489	.....	.....	
Semi-β-carotenone	538–499	501–470	.....	519–487	.....	.....	**62**
γ-carotene	533–496	495–462	.....	510–477	494–462	510–477	**61, 86**
Rubixanthin	533–496	.....	.....	.....	.....	.....	**70**
Bixin	523–489	.....	.....	503–469.5	.....	.....	**80**
β-carotene	521–485	484–451	480–452	497–466	482–451	.....	**21, 75, 85, 127, 132, 152, 153**
Fucoxanthin	.....	.....	484–459	492–457	.....	.....	**21, 33, 38, 151**
Cryptoxanthin	519–483	485–452	486–452	497–463	484–451	.....	**68, 69**
Zeaxanthin	519–483	483–451	483–451	494–462	.....	.....	**71, 79, 158, 162**
Physalien	514–481	483–451	.....	.....	.....	.....	**59, 158**
α-carotene	509–477	478–447	.....	.....	.....	.....	**49, 55, 59, 73, 75, 85, 132, 145**
Lutein	508–475	477–447	476–446	.....	.....	.....	**52, 82, 152, 162**

TABLE VIII—*Concluded*

CAROTENOID.	CS_2.	Petroleum ether.	Ethyl alcohol.	Chloroform.	Hexane.	Benzene.	References.
Helenien	.....	477–447	.....	.....	.....	.....	59, 82
Hydroxy carotene	508–475	478–448	.....	487–456	476–446	489–457	56, 57
Isolutein	503–473	.....	473–444	.....	.....	486–457	134
Taraxanthin	501–469	472–443	.....	.....	.....	.....	42, 74
Violaxanthin	500–469	472–443	472–443	482–451	.....	484–454	79, 154
Neoxanthin	493–463	466–436	467–437	476–447	.....	477–447	134
β-carotene-monoxide	486–456	.....	.....	465–437	.....	.....	19
Crocetin	478–448	.....	.....	463–435	.....	.....	46
Flavoxanthin	478–447	450–422	448–421	458–429	449–422	458.5–430	58
Azafrin	476–445	.....	.....	458–428	.....	.....	67

TABLE IX.—COMPARISON OF THE ABSORPTION MAXIMA OF SOME POLYENE-CARBONYL-COMPOUNDS (*158*)

POLYENE.	Solvent.	Absorption maxima.		
Rhodoxanthin	Hexane	524	489	458
Rhodoxanthin-dioxime	Hexane	513	479	451
Lycopinal	Petroleum ether	525	490	455
Lycopinal-oxime	Petroleum ether	503	471	442
β-Carotenone	Petroleum ether	502	468	440
β-Carotenone-dioxime	Petroleum ether	502	468	440
Semi-β-carotene	Petroleum ether	501	470	446
Semi-β-carotenone-monoxime	Petroleum ether	501	470	446
Azafrinone	Petroleum ether	454	429	
Azafrinone-oxime	Petroleum ether	454	429	

TABLE X.—SPECIFIC ABSORPTION COEFFICIENTS OF THE XANTHOPHYLLS (WAVE LENGTH IN ANGSTROM UNITS)

PIGMENT.	Solvent.	1st maximum.	Minimum.	2d maximum.
Neoxanthin	Ethanol	4370	4540	4670
	a	*229*	*122*	*224*
	CS_2	4630	4800	4930
	a	*145*	*105*	*120*
Flavoxanthin	Ethanol	4220	4385	4510
	a	*229*	*116*	*234*
Violaxanthin	Ethanol	4440	4600	4720
	a	*232*	*138*	*224*
Zeaxanthin	Ethanol	4530	4690	4810
	a	*244*	*200*	*218*
	CS_2	4820	5000	5120
	a	*180*	*150*	*165*
Lutein	Ethanol	4465	4625	4750
	a	*257*	*182*	*234*
	CS_2	4740	4920	5050
	a	*196*	*148*	*180*
	$CHCl_3$	4560	4725	4850
	a	*237*	*178*	*214*
	Dioxane	4540	4700	4825
	a	*248*	*170*	*224*
	Ether 20 percent, Ethanol 80 percent	4460	4625	4750
	a	*260*	*182*	*234*
	Ether	4450	4600	4740
	a	*260*	*182*	*234*
Isolutein	Ethanol	4440	4600	4730
	a	*240*	*166*	*222*
Cryptoxanthin	Ethanol	4520	4700	4820
	a	*254*	*209*	*226*
	CS_2	4840	5020	5120
	a	*202*	*172*	*180*

METABOLISM OF CAROTENOID PIGMENTS IN THE HEN

According to Needham *(95)* the first attempt to discover the nature and properties of the pigments of the hen's egg began in 1867, when Stradeler obtained a colored solution by extraction of the egg with an organic solvent. He interpreted the color as being due to bilirubin, but Thudicum *(136)* observed that the pigment, which was unsaponifiable, was exclusively soluble in fat solvents, and gave it the name lutein. It remained for Schunk *(124)* to isolate the pigment and show by spectroscopic study that it was identical with xanthophyll.

Palmer and Kempster *(102, 103, 104)* raised chicks from hatching to maturity on rations containing the merest traces, if not devoid of carotenoids. They concluded that natural yellow pigment of fowls, which is derived from xanthophyll of the feed, bears no important relation to growth or to the fecundity and reproduction. The same authors *(104)* demonstrated that cockerels fed a carotenoid-free ration showed only a faint trace of carotene, whereas those fed on a xanthophyll-rich diet for a short period of three days showed ample evidences of xanthophyll in body fat.

Palmer *(97)* in 1915 carried out carefully controlled feeding experiments in which a carotene-rich ration, a xanthophyll-rich ration, and a carotenoid-poor ration were fed to laying hens. A marked decline was observed in the color of the egg yolk from hens on a carotene-rich and carotenoid-poor ration, but the yolks increased very materially in color on the xanthophyll ration.

The exact function of pigments in the egg yolk is still a matter of debate. It was the common belief that when a hen started to lay, xanthophyll was removed from her shanks and beak to be placed into the egg yolk, but Palmer and Kempster *(104)* suggested that it was not a subtraction process but rather a means of diversion or excretion. Xanthophyll is excreted through the skin of the bird and is oxidized, while reproduction replaces the excretory process.

Kline, Schultze and Hart *(53)* and others have reported that xanthophyll will not serve as a source of vitamin A for growing chicks, whereas carotene will. Recent work, however, seems to indicate that xanthophyll does have some growth-promoting properties, as yet not fully understood. Virgin and Klussmann *(144)* have reported that the avian organism is able to convert xanthophyll into a provitamin, or more probably into a growth vitamin, differing from carotene.

Rydbom *(119)* found that rats on a diet free from vitamin A and receiving a prophylactic dose of 0.015 milligram of xanthophyll daily reach a state of equilibrium in about five weeks and then begin to decline. Guinea pigs, however, continued to grow on xanthophyll as well as they did on carotene.

Euler and Klussmann *(21)* studying the vitamin A content of livers of pigeons fed carotene or xanthophyll, found, that in either case, the substance with an absorption at 328-330 μ increased many times, so that xanthophyll might, from these data, be regarded as capable of yielding this growth factor.

Whatever may be the case, however important the biological significance of these pigments with respect to the avian organism may prove to be, it still remains a most interesting question as to why there is a specific utilization of the polyene alcohols, lutein and zeaxanthin, in preference to the hydrocarbons β-carotene and lycopene; a direct contrast to the deposition of carotene by mammals.

Carotene, zeaxanthin, and lutein are not the only pigments present in the egg yolk. The petroleum ether phase of egg yolk extracts is found upon chromatographic analysis to contain, besides carotene the pigment cryptoxanthin [Kuhn and Grundmann *(68, 69)*] Brockmann and Völker *(8)*, in order to explain the relatively high values obtained by Euler and Klussmann *(21)* for the carotene content of the egg yolk, first suggested the presence of cryptoxanthin as a possibility.

Gillam and Heilbron *(28)* have evaluated quantitatively the pigments of eggs from hens on a heavy maize ration. The quantities of carotene, cryptoxanthin and xanthophyll are in the order of 0.015, 0.19 and 1.79 milligrams per 100 grams of yolk, respectively. These figures may be conveniently expressed in the simple ratio 1:13:120.

Should one record similarly the respective pigment concentrations for corn, an interesting comparison would be found. Kuhn and Grundmann *(68)* have analyzed a number of varieties of corn for these same pigments. The average amounts in milligrams of carotene, cryptoxanthin, and xanthophyll, respectively, found in 100 grams of corn of various kinds are 0.057, 0.54 and 1.367. These figures expressed in simple ratio are 1:9:24.

Granting, of course, that the ratios expressed for corn and egg yolks are purely relative and may fluctuate widely, depending on the variety of corn and the supplementary diet fed, nevertheless, the gross variation in the ratios of xanthophyll to petroleum-phasic substances in egg yolks and maize allow for some very interesting speculation. Obviously, in a gram for gram comparison, corn contains a much smaller percentage of total pigment, as xanthophyll, than do egg yolks—pointing again to the remarkable capacity of the avian organism to selectively utilize xanthophylls in preference to petroleum phasic pigments, carotene, and cryptoxanthin. From these figures one might also conclude, that though the percentage utilization of carotene and cryptoxanthin is small, the latter pigment is probably more efficiently utilized than is carotene. This would appear to conform with the present status of knowledge as regards cryptoxanthin, since it is well known that its adsorption affinity, its constitution and observed properties are intermediate between that of β-carotene and xanthophyll.

THE CAROTENOID PIGMENTS OF FEEDS

Studies that were made on the metabolism of carotenoid pigments by the fowl necessitated the analysis of a great many feeds for their carotenoid content.

The results of carotene analyses on a variety of Kansas farm feeds are shown in Table XI. For comparison a similar table (Table XII) prepared by Maynard *(88)* is included.

TABLE XI.—CAROTENE CONTENT OF FEEDS

FEED.	Number of determinations.	Water, percent.	Carotene, mg. per 100.		
			High.	Low.	Average.
Prairie hay	18	7.04	5.76	.244	3.16
Silage	13	73.27	10.10	1.33	4.60
Alfalfa	2	8.00	1.97	1.50	1.73
Dehyd. alfalfa	18	.....	25.66	1.02	10.35
Dehyd. oats	9	.....	28.8	4.21	18.30
Spinach	1	.....	.....	.....	12.80
Corn gluten	4	.....	4.15	1.69	2.72
Oat grass silage	2	64.62	45.60	41.70	43.65
Greenmelk	2	67.5	18.00	15.90	16.95
Corn	14	.....	.97	.01	.28
Sudan grass	3	.....	6.48	5.35	5.83
Wheat grass	1	82.00	.....	.....	78.70
Oat grass	1	86.00	.....	.....	87.40
Straw	1	.....	.....	.....	.21
Cottonseed	1	.....	.....	.....	.03
Molasses	1	.....	.....	.....	.02

In cooperation with Prof. F. W. Atkeson of the Dairy Department, and Dr. A. E. Aldous of the Agronomy Department, the senior author determined the carotene content of 13 pasture plants *(2)*, typical of those used for pasture purposes in Kansas. Samples were taken from pure stands maintained in Department of Agronomy plots. Sampling was done by grasping bunches of grass in a manner similar to the way in which the cow might graze. The different plants varied in height from 6 to 15 inches at time of sampling. The work might be criticized for not representing actual pasturage conditions, as the plots were not grazed at any time.

After collection the samples were taken immediately to the laboratory and prepared for analysis by cutting as finely as possible with shears. Analysis for carotene reported in Table XIII was made according to previously described technique *(107, 30)*. Facilities did not make it possible to sample all the plants on the same dates.

Discussion of Results

All the pasture plants studied had a relatively high carotene content during the early summer. Seven of the plants sampled between May 22 and June 10 showed a wide range in carotene content, varying from 17.2 mg. of carotene per pound on the fresh basis to 57.2 mg., or a difference of about 300 percent. Four of the plants — little bluestem *(Andropogon scoparius),* big bluestem *(Andropogon furcatus),* Kentucky bluegrass *(Poa pratensis),* and redtop *(Agrostis alba)* — were quite similar, averaging about 18 mg. at that season of the year.

During midsummer (July) most of the plants markedly decreased in carotene content, the exceptions being alfalfa and Kentucky bluegrass. The alfalfa represented new growth after a hay crop had been cut. That sample of bluegrass was not typical of the conditions for the other plants, since it was taken on the college lawn where the grass had been clipped and watered.

TABLE XII.—CAROTENE CONTENT OF SOME FARM FEEDS[1]

FEED.	U. S. grade No.	Detns. No.	Water, percent.	Carotene per 100 grams.		
				High.	Low.	Average.
Fresh green alfalfa	.....	5	79.6	41.2	26.7	32.6
Alfalfa hay	1	6	8.6	11.7	3.4	4.5
Alfalfa hay	2	2	8.6	1.6	1.4	1.5
Alfalfa hay	3	2	8.6	1.2	0.1	0.7
Timothy hay	1	3	11.6	2.4	0.9	1.9
Timothy hay	2	1	11.6	.....	.....	0.8
Timothy hay	3	2	11.6	1.1	0.2	0.6
Fresh green Kentucky bluegrass	.....	2	68.4	62.0	42.4	52.2
Fresh green corn plant, cut for ensiling	.....	5	78.1	11.5	7.0	9.2
Corn fodder, old and dry	.....	2	9.0	0.6	0.2	0.4
Corn, ripe grain, yellow dent and flint	.....	6	11.3	1.0	0.3	0.6
Carrots, yellow, garden	.....	4	88.3	112.8	70.9	94.9

1. Meigs, E. B. Vitamin A value of plant feeds fully accounted for by their carotene content, Yearbook of Agriculture, U. S. Dept. Agr., pp. 324-326, 1935.

Samples taken in November showed very striking differences among the plants in their ability to renew growth and reëstablish high carotene values after the fall rains had come. Big bluestem and buffalo grass *(Buchloe dactyloides)* remained brown and naturally cured and were practically devoid of carotene. Most of the other grasses had a carotene content approaching early summer values. These differences in recovery of carotene content in late fall might be significant in livestock feeding, particularly when the fall grazing period is followed by a long winter feeding of rations low in carotene.

TABLE XIII.—CAROTENE CONTENT OF SOME PASTURE PLANTS AT DIFFERENT PERIODS THROUGHOUT THE GROWING SEASON

Date sample taken.	Pasture plants studied.	Carotene per 100 G.		Carotene per lb.		Moisture.
		Fresh.	Dry.	Fresh.	Dry.	
		mg.	mg.	mg.	mg.	percent.
5-22	Rye	5.5	25.1	25.1	114.0	82.3
11-28	Rye	8.4	30.6	37.9	138.8	72.7
5-23	Big bluestem	4.4	19.2	19.8	87.5	77.3
6-24	Big bluestem	7.7	22.1	34.8	100.5	65.4
7- 8	Big bluestem	3.6	8.4	16.5	38.2	56.8
7-21	Big bluestem	3.7	8.0	17.0	36.4	53.3
11- 4	Big bluestem	0.3	0.4	1.5	1.9	24.6
5-23	Canadian brome	10.4	53.3	47.0	242.0	80.6
7-11	Canadian brome	4.2	7.7	19.1	34.9	45.3
5-28	Kentucky bluegrass	4.1	10.2	18.8	46.2	59.0
6-26	Kentucky bluegrass	4.0	6.2	18.2	28.2	35.0
7-9	Kentucky bluegrass	6.3	8.0	28.8	36.4	20.9
7-20	Kentucky bluegrass	3.6	4.1	16.5	18.5	10.8
11- 4	Kentucky bluegrass	7.2	21.4	32.6	97.2	66.6
5-28	Little bluestem	3.8	12.2	17.2	55.2	68.9
6-28	Little bluestem	6.7	15.6	30.5	70.8	56.9
7- 8	Little bluestem	4.5	8.1	20.4	36.7	44.5
7-21	Little bluestem	2.7	4.3	12.3	19.7	37.8
6-10	Buffalo grass	12.6	28.2	57.2	128.1	55.4
6-23	Buffalo grass	8.6	15.9	39.3	72.1	45.5
7- 6	Buffalo grass	4.4	6.0	19.8	27.2	30.2
7-20	Buffalo grass	3.7	4.8	16.8	21.8	23.0
11- 6	Buffalo grass	0.7	0.9	3.0	4.2	29.3
6-10	Redtop	4.0	11.3	18.2	51.3	64.0
6-22	Redtop	3.7	6.8	16.6	30.8	46.2
6-26	Redtop	3.4	5.4	15.3	24.4	37.4
11- 6	Redtop	5.7	24.1	25.9	109.2	76.3
6-22	Alfalfa	8.8	29.2	39.7	132.6	70.7
7- 6	Alfalfa	8.0	22.2	36.4	100.6	63.8
7-22	Alfalfa	3.2	8.3	14.7	37.6	60.9
11- 6	Alfalfa	7.1	14.3	32.1	64.9	50.5
6-23	Smooth brome (Dakota source)	6.2	17.8	28.3	81.0	65.0
7-23	Smooth brome (Dakota source)	3.8	6.6	17.4	30.0	42.2
11- 4	Smooth brome (Dakota source)	8.6	33.4	39.0	151.6	74.3
6-25	Local brome	7.1	15.6	32.0	71.0	54.9
7-11	Local brome	4.9	8.5	22.2	38.4	42.2
11-13	Local brome	6.6	16.7	30.1	75.6	60.2
6-25	Orchard grass	6.8	15.2	30.8	68.8	55.2
7- 9	Orchard grass	6.4	12.2	29.0	55.4	47.7
7-22	Orchard grass	3.8	7.5	17.2	34.0	49.4
11-13	Orchard grass	6.4	15.4	29.2	69.9	58.3
11-28	Wheat	6.6	17.9	29.7	81.5	63.5
11-28	Barley	9.6	37.6	43.5	170.5	74.5

Although these results were obtained during a drought year general knowledge of the growth habits of the plants studied would lend credence to the data obtained. Whether the same relative decrease in carotene during midsummer would prevail under pasturage conditions is problematical.

A study of the variations in carotene concentration of oats grown on fertilized and on unfertilized plots was also made. The oats were drilled March 4,1938. They began showing through the ground two weeks later (March 18). Carotene analyses were made at frequent intervals during the early stages of growth. Similar analyses were also made on a stand of alfalfa for comparison. Results are shown in Table XIV.

TABLE XIV.—VARIATIONS IN THE CAROTENOID CONTENT OF THE OAT PLANT DURING THE EARLY STAGES OF GROWTH IN COMPARISON WITH ALFALFA

		Carotene (fresh basis) mg./100 g.	Xanthophyll (fresh basis) mg./100 g.	Moisture.
Plot I.—Unfertilized	4/22	8.69	10.0	85.3
	4/29	7.28	12.25	84.0
	5/6	5.80	.....	82.0
	5/13	5.80	.....	85.0
Plot II.—Fertilized	3/25	8.59	.....	80.0
(Ammonium sulfate)	4/1	10.01	.....	81.0
100 lbs. per acre	4/18	14.00	.....	85.0
	4/22	9.63	11.85	86.5
	4/29	9.63	17.87	87.0
	5/6	7.30	.....	84.0
	5/13	5.00	.....	89.0
Plot III.—Fertilized	4/22	10.30	13.50	86.0
(Treble superphosphate)	4/29	8.03	21.00	83.0
	5/6	8.00	.....	81.0
	5/13	4.62	.....	85.0
Plot IV.—Fertilized	4/22	12.00	13.75	84.3
(Ammonium sulfate and treble superphate)	4/29	9.16	26.63	88.0
	5/6	7.55	.....	82.0
50 lbs. of each per acre	5/13	4.25	.....	87.0
Plot. V.—Alfalfa	3/25	7.13	.....	76.0
	4/1	9.18	.....	81.0
	4/18	10.03	.....	78.0
	4/22	8.63	11.37	80.1
	4/29	8.80	17.2	78.0

It is evident that the highest pigment concentration was found about five weeks after drilling or about three weeks after the oats first showed through the ground. Samples from fertilized plots had a somewhat higher carotene content than those obtained from the unfertilized plot.

Protein analyses of samples from each of the plots averaged 83.3 percent, with practically no deviation, one from the other. Alfalfa, on the other hand, gave a value of 26.88 percent.

On April 30 quantities from each plot were cut and put into barrels and mixed with molasses and water (80 lbs. of each per ton). The yields of the various plots at this cutting were as follows:

Plot	Yield in pounds per acre
I	6,311
II	7,311
III	7,833
IV	7,180

It is evident that the fertilized plots gave a much better yield.

From time to time studies have also been made on the effect, of various periods of storage upon the vitamin A potency of typical farm feeds when stored under normal conditions. Changes in the carotene content of the feeds when they were stored in sacks in a dry, unheated feed room were as follows:

Date	Dehydrated oats Mg./100 g.	Dehydrated alfalfa Mg./100 g.
November 4, 1936	40.8 (28.5% protein)	49.0 (23.3% protein)
January 11, 1937	39.9	35.5
March 3, 1937	28.8	24.5
June 11, 1937	24.1	....

THE EFFECT OF GENE DOSAGE ON CAROTENOID PIGMENTS IN MAIZE

Because of the important part played by corn as a poultry feed, a study made by the senior author, in cooperation with Dr. A. M. Brunson, on the effect of gene dosage on the carotenoid pigments in maize *(9)* will be considered in some detail.

Studies on the effect of varying doses of genes which lend themselves to quantitative measurement have an interesting bearing on the mechanics of the action of the gene in the development of the individual. Mangelsdorf and Fraps *(87)* and Rhoades *(115)* have pointed out that endosperm tissue provides excellent material for such studies since its triploid nature allows genic dosages of 0, 1, 2, or 3 in otherwise similar material to be obtained and compared. Here are reported the chemical determinations of carotenoid pigments in maize endosperms with various dosages of the factors for yellow color.

Numerous comparisons of the feeding value of yellow corn and white corn are available in the literature of the past few years, indicating clearly that yellow corn is vitamin-A potent, while white corn is not. Steenbock and Boutwell *(130)* and Hauge and Trost *(31)* have shown that in segregating ears the yellow kernels contain growth-promoting substances while the white ones do not, thus further emphasizing the association between color and potency. Mangelsdorf and Fraps *(87)* have gone one step farther in showing that vitamin effect of the corn was proportional to the dosage of the gene for yellow endosperm color in samples of known factorial composition. All of these results are based on biological assays with laboratory animals.

The grain samples on which the analyses in the present study were made were obtained from ten sets of reciprocal double pollinations made in 1937. In each set, a plant of a variety having yellow seed and a plant of a variety having white seed were each self pollinated and crossed by the other plant. The resulting ears contained two types of kernels which could be easily separated on the basis of color. For each set, then, there were two ears having similar parentage which could be divided into four samples as follows:

White ♀ × White ♂
White ♀ × Yellow ♂
Yellow ♀ × White ♂
Yellow ♀ × Yellow ♂

Since endosperm tissue is the product of the fusion of two maternal nuclei with one pollen nucleus these four samples contained 0, 1, 2, and 3 doses, respectively, of the genes for yellow endosperm color from the yellow parent. In sets 1, 4, 5, and 10 it was evident that more than one factor for yellow endosperm was present and that the yellow parent was heterozygous for one of the factors. In

the heterozygous classes of these sets two separations of the dilute yellows were possible, although the dark-yellow class appeared uniform in color to the eye.

The petroleum and alcohol phasic pigments were determined as described under "Methods." A summary of the chemical analyses of the samples is presented in Table XV. In the white samples (0 dose of genes for yellow endosperm) of all sets there was no pigment in the petroleum-phasic fraction, indicating the complete absence of β-carotene and cryptoxanthin in white corn. This agrees well with the results of numerous biological assays of white corn which have

TABLE XV.—ANALYSES OF YELLOW PIGMENTS IN CORN OF IDENTICAL PARENTAGE WITH VARIOUS DOSES OF GENES DETERMINING ENDOSPERM COLOR

Set.	Varieties.	Petroleum-soluble fraction carotene and cryptoxanthin. Milligrams per 100 grams.				Alcohol-soluble fraction zeaxanthin. Milligrams per 100 grams.			
		0 dose	1 dose	2 doses	3 doses	0 dose	1 dose	2 doses	3 doses
1	Cassel White and Hays Golden,	.000	.132	.258	.414	.092	.140	.222	.527
2	Cassel White and Hays Golden,	.000	.081	.132	.282	.000	.067	.245	.823
3	Cassel White and Hays Golden,	.000	.138	.234	.404	.000	.233	.259	1.260
4	Cassel White and Hays Golden,	.000	.100	.320	.474	.002	.014	.166	1.054
5	Cassel White and Hays Golden,	.000	.143	.309	.853	.000	.235	.440	2.610
6	Freed White and Hays Golden,	.000	.173	.530	.830	.000	.105	.370	1.700
7	Freed White and Midland.....	.000	.170	.385	.705	.033	.213	.432	1.815
8	Freed White and Midland.....	.000	.157	.367	.740	.051	.167	.390	2.360
9	Freed White and Midland.....	.000	.160	.690	.820	.070	.125	.325	1.475
10	Freed White and Midland.....	.000	.270	.552	.945	.000	.263	.490	1.431

TABLE XVI.—AVERAGE PIGMENT CONTENT OF SAMPLES BY VARIETAL GROUPS, FROM TABLE XV

Set.	Varieties.	Petroleum-soluble fraction, carotene and cryptoxanthin. Milligrams per 100 grams.				Alcohol-soluble fraction zeaxanthin. Milligrams per 100 grams.			
		0 dose	1 dose	2 doses	3 doses	0 dose	1 dose	2 doses	3 doses
1–5	Cassel White and Hays Golden,	.000	.119	.251	.485	.019	.138	.266	1.255
	Mean ÷ dosage.....	.....	.119	.125	.162	.....	.138	.133	.418
6	Freed White and Hays Golden,	.000	.173	.530	.830	.000	.105	.370	1.700
	Mean ÷ dosage.....	.....	.173	.265	.277	.....	.105	.185	.567
7–10	Freed White and Midland.....	.000	.189	.498	.802	.038	.192	.409	1.770
	Mean ÷ dosage.....	.....	.189	.249	.267	.....	.192	.204	.590

been reported. Starting in all cases at zero, the pigment content of the petroleum-phasic fraction of each set progresses with more or less regularity through the samples representing 1, 2, and 3 doses of genetic factors for yellow endosperm. Although numerous individual deviations occur, the various dosages in each set represent essentially a straight line relationship. However, there is a slight tendency for greater than additive effect on the average, as will be mentioned later. Assuming a fairly constant ratio between β-carotene and cryptoxanthin with a consequent constant vitamin A potency per unit of pigment, these results very nearly confirm the straight line relationship found in the feeding experiment of Mangelsdorf and Fraps *(87)*.

In five of the white samples the alcohol-soluble fractions contained no pigment. In the other five white samples slight traces of pigment were found. In the two heterozygous classes with one dose or two doses of genes for yellow endosperm, the zeaxanthin content was of the same general order as for β-carotene plus cryptoxanthin. In the homozygous yellows with three doses, however, the zeaxanthin content was always appreciably above that of β-carotene and cryptoxanthin, the ratio varying from 1.27:1.00 in set 1 to 3.19 in set 8.

The 10 sets of double pollinations involve three different combinations of varieties. The averages of each combination are presented in Table XVI. Attention is directed to the approximately straight line relationship of zeaxanthin content for 0, 1, and 2 doses and the sharp increase for 3 doses. This behavior not only is approximately the same for the three averages as shown, but also is quite consistent for the individual sets if they are separately plotted.

In order better to visualize the relationships, entries for "mean ÷ dosage" have been made in Table XVI to show the effect per gene in the various dosages. In the petroleum soluble fraction there is a slight but consistent tendency to increase the effect per gene with the higher dosages, although the effect is not, far from additive. In the alcohol soluble fraction, however, there is in all cases a very sharp increase in effect per gene for the three-dose samples over that of the one- or two-dose samples. The effect per gene for three doses as compared to that for two doses is in the ratio of 3.14:1.00, 3.06:1.00, and 2.89:1.00, respectively, for the three averages shown in Table XVI. No explanation is offered as to why similar pigments should behave so differently for identical changes in factorial composition.

Marked differences are noted in the pigment content of the petroleum fractions of the homozygous yellow samples. Sample 2 with 3 doses of yellow from Hays Golden contains .282 mg./100 gms. in contrast with sample 5, also with 3 doses of yellow from the same variety, which contains .853 mg./100 gms. This suggests considerable variation among strains in vitamin-A potency, although in these results the β-carotene and the cryptoxanthin are lumped together and it is possible that variations in β-carotene which is twice as potent as cryptoxanthin might materially affect the apparent ratio. It

seems probable, however, that relatively large variations in vitamin-A potency do occur, and that a search for inbreds of high potency might result in the production of hybrids with much greater vitamin effect than that of ordinary yellow corn.

THE RELATION OF THE CAROTENOID PIGMENTS OF FEED TO THE CAROTENOID PIGMENTS OF EGG YOLK *(34)*

The work of Schunck *(124)*, Palmer and Kempster *(102, 103, 104)*, Kuhn and Grundmann *(71)*, Brockmann and Völker *(8)*, and Gillam and Heilbron *(28)* shows that the carotenoid pigments present in egg yolk consist almost entirely of xanthophyll with only a small amount of cryptoxanthin and carotene. So far no experiments have been reported which give quantitative data concerning the relation of the amounts of these carotenoid pigments in the feed to the amount of these pigments in the egg yolk. The following experiments give some data on this quantitative relationship.

In the first of these experiments the source of the pigments was kept constant while the amount in the rations varied, in the second experiment the amount of pigments in the ration was kept constant, while the source of the pigments was varied.

EXPERIMENT I

The object of this experiment, which was conducted in the spring of 1934, was to determine the amount of carotenoid pigments that would be deposited in egg yolk when definite amounts of these pigments were incorporated in the feed by the use of yellow corn. To secure this information, hens were first depleted of carotenoid pigments by feeding them a ration low in pigments (Table XVII).

TABLE XVII.—A CAROTENOID FREE RATION SUPPLEMENTED WITH VARYING AMOUNTS OF YELLOW CORN

	Lot I.	Lot II.	Lot III.	Lot IV.
Corn, yellow, ground	10*	20*	30*	.....
Rice, polished	60	50	40	70
Kraftogen (A) casein	17	17	17	17
Yeast, dried brewers'	5	5	5	5
Wood pulp, spruce	3	3	3	3
Oyster shells, ground	2	2	2	2
Bonemeal, steamed	1.5	1.5	1.5	1.5
Salt	1.0	1.0	1.0	1.0
Cod-liver oil (Nopco XX)	0.5	0.5	0.5	0.5

* Number of grams fed daily per hen.

When the yolks of the eggs produced were of a uniform light color —almost devoid of pigments—the hens were given a definite amount of carotenoid pigment by supplementing the basal ration with yellow corn. All the eggs pigmented as a result of feeding the corn were collected and the amounts of pigments contained were determined.

Four lots of four White Leghorn hens each were used in this experiment. After a three weeks' preliminary period on the basal ration, each hen in lot I was given 10 grams of ground yellow corn per day for a period of 21 days. Those in lot II were given 20 grams and those in lot III, 30 grams, while lot IV was continued on the basal ration. The ground corn was placed directly in the crop of each bird with a suitably constructed funnel. After the corn-feeding period, all the hens were continued on the basal ration for a period of 21 days, after which time the yolks of the eggs from the hens which had been fed corn (those in lots I, II, and III) were as low in pigment content as those from the hens in lot IV which had been on the basal ration all the time.

The amounts of carotenoid pigment in the basal ration and in the yellow corn were determined by the method of Schertz (1925), using the spectrophotometer for the final estimation of the pigments. In this method the pigments are divided into the alcohol phasic fraction, consisting largely of xanthophylls, and the petroleum phasic fraction, consisting largely of carotenes and cryptoxanthin. No attempt was made to separate these two fractions into the individual pigments which they contain.

The yolks produced by each hen were pooled, and samples taken for analysis. The amounts of pigment in all the eggs produced by each hen in each of the four lots during the corn-feeding period and the following 21-day period were determined. The results of the analysis are summarized in Table XVIII.

These results are in accord with the well-known fact that the alcohol phasic fraction of the carotenoid pigments is deposited in egg yolk in greater concentration than the petroleum phasic fraction. They also show that the percentage of the alcohol phasic fraction, the xanthophylls, of the feed deposited in the egg increased as the amount of corn was increased in the ration. The hens receiving 10 grams of corn per day deposited 17.4 percent of the xanthophyll in the egg, while those receiving 20 to 30 grams of corn deposited 21.4 and 23.5 percent, respectively.

The variations between individual hens were much greater with the petroleum phasic fraction than with the alcohol phasic fraction. One hen in lot A receiving 10 grams of corn daily deposited in her egg yolks 37 percent of cryptoxanthin and carotene which she received from the corn in her ration while another hen in the same lot deposited only 0.65 percent of these pigments in her yolk. No such variation was found in the case of the alcohol phasic fraction.

EXPERIMENT II

In experiment I three levels of yellow corn were fed, namely, 10, 20, and 30 grams per hen per day. In the second experiment this series was continued by feeding lot IV 40 grams of corn per day. In addition to continuing the work on yellow corn, two other sources of carotenoid pigments, dehydrated alfalfa and young green barley, were used. The barley was supplied in the form of green buttermilk, a product made by grinding the young plant, mixing the ground mass with buttermilk, and condensing the mixture in a vacuum. The alfalfa and the barley were fed in such amounts as to furnish about the same amount of the alcohol phasic fraction, the xanthophylls, as was furnished by the yellow corn.

The rations used are listed in Table XIX.

These feeds were analyzed for carotenoid pigments by the Peterson modification *(107)* of the Guilbert *(30)* method with the results shown in Table XX.

Four lots of eight White Leghorn hens each were used in this experiment. They were kept in individual hen batteries equipped with feeding cups so that accurate records of feed consumption could be obtained. The hens were fed the experimental rations for three weeks before eggs were saved for analysis. All the eggs produced the fourth week were analyzed for carotenoid pigments by the same method used in analyzing the feeds. The results of these chemical analyses are given in Table XXI.

The percent of pigments from the corn deposited in the egg yolk is the percent which would have been expected from the results in the first experiment. In the first experiment 17.4, 21.4, and 23.5 percent of the alcoholic phase of the pigment were deposited in the yolk when 10, 20, and 30 grams of corn were fed. The 25.73 percent deposited in this experiment is in line with these results. Since both

TABLE XVIII.—AVERAGE AMOUNT OF CAROTENOID PIGMENTS IN THE EGG YOLKS OF HENS RECEIVING VARYING AMOUNTS OF YELLOW CORN

Lot No.	Mgs. of pigments in the corn* fed.		Mgs. of pigments in yolk in excess of amounts** in controls.		Percent of corn pigments deposited in egg yolks.	
	Cryptoxanthin and carotene.	Xanthophyll.	Cryptoxanthin and carotene.	Xanthophyll.	Cryptoxanthin and carotene.	Xanthophyll.
A	1.13	4.47	.133	0.781	12.0	17.4
B	2.26	8.95	.144	1.910	6.4	21.4
C	3.39	13.42	.164	3.160	4.8	23.5

* One gram of corn contained 0.00538 mgs. of cryptoxanthin and carotene and 0.0213 mgs. of xanthophyll.

** The hens on the basal ration receiving no corn deposited an average of 0.03 mgs. of cryptoxanthin and carotene and 0.131 mgs. of xanthophyll in their yolk.

TABLE XIX.—A CAROTENOID FREE RATION SUPPLEMENTED WITH VARIOUS CAROTENOID RICH MATERIALS

	Lot I.	Lot II.	Lot III.	Lot IV.
White corn	41.5	41.5	41.5	0.0
Yellow corn	0.0	0.0	0.0	41.5
Ground wheat	25.5	25.5	25.5	25.5
Ground oats	10.0	10.0	10.0	10.0
Meat and bone scrap	10.0	10.0	10.0	10.0
Dried buttermilk	5.0	5.0	5.0	5.0
Alfalfa meal	0.0	0.0	3.0	0.0
Corn starch	3.0	3.0	0.0	3.0
Oyster shell	2.0	2.0	2.0	2.0
Bone meal	1.0	1.0	1.0	1.0
Salt	1.0	1.0	1.0	1.0
Cod-liver oil	1.0	1.0	1.0	1.0
Green buttermilk*	0.0	6.5	0.0	0.0

* Green buttermilk is made by condensing chopped fresh green barley with buttermilk.

TABLE XX.—CAROTENOID CONTENT OF FEEDS USED IN LOTS I, II, III, AND IV

FEED.	Mgs. carotenoid pig. in 100 gms. feed.	
	Petrol. phasic frac. (carotene) and crypt.	Alcohol phas. frac. (xanthophyll).
White Corn, Lot I	0.08	0.18
Green Barley, Lot II	0.43	0.86
Dehyd. Alfalfa, Lot III	0.49	0.92
Yellow Corn, Lot IV	0.29	0.94

TABLE XXI.—AVERAGE MGS. OF CAROTENOID PIGMENTS IN THE FEED CONSUMED BY HENS IN A SEVEN-DAY PERIOD AND IN THE EGG YOLKS PRODUCED DURING THE SAME PERIOD

SOURCE OF PIGMENT IN THE RATION.	Av. No. of eggs produced per hen.	Av. No. mgs. pig. in feed consumed.		Av. No. mgs. pig. in yolk produced.		Percent pig. depos. in egg yolk.	
		Petrol. fract.	Alcohol fract.	Petrol. fract.	Alcohol fract.	Petrol. fract.	Alcohol fract.
Green Barley	4	3.28	6.57	0.086	1.04	2.62	15.84
Dehyd. Alfalfa	4.4	3.48	6.54	0.087	1.03	2.50	15.75
Yellow Corn	5	2.28	7.38	0.161	1.90	7.07	25.73
Control	2.5	0.61	1.38	0.000	0.03	0.00	2.18

the method of feeding the corn and the method of chemical analysis were different in the two experiments, the agreement between the results indicates the reliability of the data secured.

The results of this experiment show a marked similarity in the metabolism of the pigments derived from the green leaves, alfalfa and barley. Although each of these leaves contains a high amount of the petroleum phasic fraction, which is almost pure beta carotene, very little of this fraction was deposited in the egg. Only 2.62 percent of this fraction was deposited in the case of green barley and 2.5 percent in the case of dehydrated alfalfa. The percent of the alcohol phasic fraction, xanthophylls, was almost the same for the two leaves, being 15.84 percent for green barley and 15.75 percent for dehydrated alfalfa.

A much higher percentage of each fraction of the pigment of yellow corn was deposited, that is, 7.07 percent for petroleum phase and 25.73 percent of the alcohol phase. This high deposition of the petroleum fraction of the corn is no doubt due to the high percentage of cryptoxanthin which this fraction from yellow corn contains. This pigment contains one hydroxyl group which tends to give it properties somewhat resembling the xanthophylls. Since xanthophylls are deposited in the egg yolk in fairly high concentration, but carotenes only in small amounts, one might expect cryptoxanthins from yellow corn which resembles zeaxanthin in structure to be deposited in higher concentration than carotene.

EXPERIMENT III

Thirty-two White Leghorn hens which had been kept on a feed low in carotenoid pigments until eggs of a uniform light color were produced were fed the following purified pigments: A, carotene; B, cryptoxanthin; C, zeaxanthin; D, lutein; and E, esters of cryptoxanthin and zeaxanthin. An amount of material containing approximately one milligram of each of these pigments was placed in the crop of each hen daily for seven days. All the eggs laid during the seven days of feeding these pigments, and those laid 14 days after discontinuing the pigment were kept for analysis. All the yolks from each hen were pooled separately and analyzed for petroleum phasic and alcohol phasic pigments.

The results were similar to those in the two previous experiments. The pigments of the petroleum phasic fractions did not find their way into the egg yolk when fed in the pure form. The alcohol phasic fractions, on the other hand, were deposited in the yolk. In accord with previous results, a higher percent of zeaxanthin was deposited than leaf xanthophyll (lutein). A summary of results is shown in the following table:

TABLE XXII.—AVERAGE AMOUNT OF CAROTENOID PIGMENTS IN THE EGG YOLKS OF HENS RECEIVING VARYING AMOUNTS OF CRYPTOXANTHIN, β-CAROTENE, XANTHOPHYLL AND ZEAXANTHIN

	No. of hens in each lot.	Total amount of pigment fed in mg.	Ave. No. of yolks per hen.	Total weight of yolks in gms.	Mgs. of pigment in excess of amount in control.	Percent of pigment deposited.
Leaf xanthophyll	8	7	15.6	270	.8014	11.45
Zeaxanthin.....	8	7	14.4	233	1.7046	24.35
Cryptoxanthin..	8	7	17.0	299	0.0000	0.00
β-carotene.....	8	7	14.7	255	0.0000	0.00

EXPERIMENT IV

Two lots of eight White Leghorn hens were used in this study. The hens were kept on a basal ration low in carotenoid pigments until the egg yolks were of a uniform light color. Then each of the hens in Lot A were fed 30 grams of oat grass silage per day for seven days. Those in Lot B received 10 grams of dehydrated oat grass per day, or an amount which was calculated to provide approximately as much carotene and xanthophyll as was available for the oat silage group. Eggs from a number of hens from each lot were saved for short periods just before the start of the feeding period and also for a period approximately 18 days later to serve as controls. All the eggs laid by each hen from the time three days preceding the start of feeding until the fourteenth day following the start of feeding were pooled for analysis.

A summary of the results is given in Table XXIII.

TABLE XXIII.—AVERAGE AMOUNT OF CAROTENOID PIGMENTS IN THE EGG YOLKS OF HENS RECEIVING OAT GRASS SILAGE AND DEHYDRATED OATS

Lot No.	Feed.	Mgs. of pigment in material fed.*		Mgs. of pigments in yolks in excess of amount in controls.†		Percent of pigments deposited in yolks.	
		Cryptoxanthin and carotene.	Xanthophyll.	Cryptoxanthin and carotene.	Xanthophyll.	Cryptoxanthin and carotene.	Xanthophyll.
A	Silage.....	23.3	44.1	0.389	2.60	1.67	5.9
B	Dehydrated oat grass.....	30.1	46.4	0.343	2.82	1.10	6.0

* The silage contained 11.12 mg./100 of carotene and 21.03 mg./100 of xanthophyll. The dehydrated oats contained 43 mg./100 of carotene and 66.34 mg./100 of xanthophyll.

† The hens on the basal ration deposited no cryptoxanthin and carotene and 0.45 mg./100 g. of xanthophyll in their yolks.

When this experiment was repeated, using only 15 grams of oat grass silage in Lot A and 5 grams of dehydrated oats in Lot B, the results were practically the same. Lot A deposited 2.06 percent of carotene plus cryptoxanthin in the yolks while Lot B deposited 1.28 percent. Lot A deposited 5.6 percent of xanthophyll while the hens receiving dehydrated oats deposited 7.0 percent.

There was considerable individual variation in the percentages of each pigment deposited from both feeds. The ranges were as follows:

Feed	Percentage of pigments deposited in yolks — Range — Carotene and Cryptoxanthin (percent)	Xanthophyll (percent)
Silage	0.90-3.9	1.9- 9.9
Dehydrated oats	1.03-1.80	4.0-14.4

The amounts of petroleum phasic carotenoids deposited were smaller and less variable when dehydrated oats was the source of pigment. No ready explanation for this difference suggests itself. It is significant, however, that silages have been found to contain a number of pigments in the petroleum soluble fraction which are modified xanthophylls, made petroleum soluble by acids present in the silage.

EXPERIMENT V

In the previous experiments the amount of pigment ingested by the hens studied was carefully controlled. In this experiment, however, advantage was taken of a group feeding experiment in which the effects of varying amounts of dehydrated immature oat grass in the ration were compared with dehydrated alfalfa.

Four lots of 100 White Leghorn hens each were used in the study. Lot I received 10 percent dehydrated alfalfa, and Lots II, III and IV received, respectively, 10, 15, and 20 percent of dehydrated immature grass meal. The rations consisted of a dry mash, shelled white corn, and whole wheat, all hopper fed. The mash feed was as follows:

Rations	Lots I	II	III	IV
White corn, ground	25	26	23.5	21
Wheat, ground	25	25	25	25
Oats, ground	25	25	25	25
Meat and bone scrap	7	6	3.5	1
Fish meal (sardine)	6	6	6	6
Alfalfa meal, dehydrated	10	0	0	0
Immature grass meal	0	10	15	20
Sardine oil	2	2	2	2
Salt	1	1	1	1
Protein percentage	16.55	16.51	16.51	16.51

Protein percentage, alfalfa meal, fourth cutting	23.4
Protein percentage, dehydrated immature	28.5*
Protein percentage, green oat plant meal	....
Protein percentage, meat scrap, high grade	55.0
Protein percentage, fish meal, high grade	65.0

The experiment, which commenced in October, 1936, was terminated in July, 1937, at which time yolks of 20 eggs picked at random from each lot from one day's lay were studied for color with the Bausch & Lomb H. S. B. Color Analyzer (Cat. No. 33-24-20). The instrument derives its name from the fact that it specifies a color in terms of three attributes, hue, saturation, and brilliance.

The Optical Society of America in its report of the Colorimetry Committee defines these terms as follows: *Hue* is that attribute of color in respect to which it differs from a gray of the same brilliance. *Saturation* is that attribute of color possessing hue, which determines their degree of difference from a gray of the same brilliance. *Brilliance* is that attribute of any color in respect of which it may be classed as equivalent to some member of a series of grays which range from black to white. Hue indicates whether a color is red, green, or blue; saturation whether it is strong or weak color; and brilliance whether it is light or dark.

There are 10 major hues available with the instrument, designated red, yellow-red, yellow, green-yellow, green, blue-green, blue, purple-blue, purple, and red-purple. It is customary to designate these hues by the initial letter, thus, red is R. These 10 hues are available in each of the nine brilliance steps. Thus there may be had a R_5 or a R_9, or a G_7, etc. For each hue and brilliance there are available various steps of saturation beginning with very weak colors and proceeding to the strongest, which are those included in the series with the instrument. The greatest saturation possible is controlled by the available pigments and is not uniform for all hues. Neither do all the hues reach their maximum saturation at the same brilliance level.

A color in the Munsell System is expressed in the following manner. The hue is designated by the initial letter as R, G, or B. The brilliance is designated by the numerator of a numerical fraction following the letter as $R_{5/}$, $G_{6/}$ or $B_{3/}$. The denominator expresses saturation, as $R_{5/4}$, $G_{6/8}$, $B_{3/10}$. Thus $R_{5/4}$ is a red on the brilliance level of $N_{/5}$ and four steps out from the gray axis. With the instrument used, the color was specified in the same manner, but the three attributes were each specified more exactly as, for instance, $6.7\ R\ \frac{5.2}{4.6}$ where 6.7 gives the exact position in the hue circle of 100 parts, 5.2 the exact brilliance, and 4.6 the exact saturation. For

* As indicated in these figures, the protein was very high in the dehydrated oat grass meal. The amounts of meat scrap and white corn were adjusted so as to give approximately the same protein level in each ration.

the method of attaining these results, the reader is referred to the directions for use of this instrument published by the manufacturers.

Results of this study are presented in Table XXIV. It is evident that regardless of the feed there is a large individual variation in all three attributes. This difference between individuals is doubtless due to the variations in individual consumption of feed. It has also been shown in previous experiments that even when hens are consuming exactly the same quantities of pigment, there may be a considerable difference between individuals in their capacity to utilize the pigment.

This experiment was similar to Experiment V. Three lots of 100 White Leghorn hens received as a part of the dry mash ration 12 percent greenmelk,* 12 percent oat grass silage and 5 percent alfalfa meal in lots I, II, III, respectively. To make lots I and III more nearly comparable, the ration in lot III included 12 percent condensed buttermilk. The greenmelk and the silage were fed each morning. Near the close of the experiment the hens in lot II were permitted to range on young Sudan grass to replace the silage previously fed. The mash feed was as follows:

Rations	Lots I	Lots II	Lots III
Yellow corn	20	20	20
Wheat	20	20	20
Oats	20	20	20
Bran	10	10	10
Alfalfa-leaf meal	..	..	5
Meat meal	4.5	5.0	4
Fish meal	4.5	5.0	4
Soybean oil meal	4.5	5.0	4
Starch	3.5	2.0	..
Salt	0.5	0.5	0.5
Nopco XX	0.5	0.5	0.5
Condensed milk	..	..	12.0
Greenmelk	12.0	..	..
Sudan grass and oat silage	..	12	..
Totals	100	100	100
Protein (*percent*)	18.5	19.88	18.81

The experiment, which was started in October, 1937, was terminated in July, 1938. All the eggs laid in each lot on March 1, April 1, and May 1 were collected, the yolks separated and pooled for analysis of carotenoid pigments. Results of the analyses are presented in Table XXV.

The silage-fed group produced eggs containing larger amounts of both carotenes and xanthophylls than did the greenmelk and alfalfa groups. Though studies of eggs from individual hens were not made in this case, it can probably be assumed from the preceding experiment that there was a great individual variation. In view of the methods used in sampling, however, the data presented probably give a fair picture of the average pigment distribution in eggs from hens fed as described under the conditions of the experiment.

* Greenmelk is made by condensing chopped fresh green dehydrated oats with buttermilk.

TABLE XXV.—THE PETROLEUM- AND ALCOHOL-PHASIC CAROTENOIDS OF EGG YOLKS (mg./100 g.)

DATE.	Lot I.		Lot IIa.		Lot IIIb.	
	Petr.-phasic.	Alc.-phasic.	P	A	P	A
March 1...	0.20	1.66	0.27	2.40	.08	.85
April 1.....	0.23	1.76	0.39	3.15	.08	1.00
May 1.....	0.33	2.23	0.30	3.40	.14	1.12

The Greenmelk used in Lot I contained 5.84 mg. of carotene and 12.07 mg. of xanthophyll per 100 g.

a. The silage used in Lot II contained 11.11 mg. of carotene and 21.03 mg. of xanthophyll per 100 g.

b. The dehydrated alfalfa used in Lot III contained 24.5 mg. of carotene and 46.0 mg. of xanthophyll per 100 g.

THE VITAMIN A ACTIVITY OF CRYPTOXANTHOL[6] (CRYPTOXANTHIN) WITH SOME OBSERVATIONS ON THE ABSORPTION CURVES OF THE PETROLEUM PHASIC FRACTION OF FEEDSTUFFS[7]

The cryptoxanthin and carotene contents of American corn gluten, Canadian corn gluten and Argentine yellow corn used in this study were, respectively, 2.625, 0.00, and 0.645 mg. per 100 gms.

A diagram of the absorption spectrum of the petroleum phasic fraction of the American corn gluten used in this work is shown in figure 1. These curves were determined visually on a Bausch & Lomb spectrophotometer and are subject to the normal errors of reading. The optical densities are in arbitrary units simply to compare the curve of pure β-carotene solution with that of corn gluten pigment. The matter of concentration is not involved. It will be observed that the critical points are shifted about 50 Angstroms toward the ultraviolet and that the optical density at 4800 Å is relatively much lower with respect to the optical density at 4700 Å than that of pure β-carotene. Curves of this nature have frequently been found in old feeds, or feeds which have received rather rigorous heat or sunlight treatments. It is singular that the effects of heat, light, etc., appear to give a product with a curve more nearly like that of α-carotene (Fig. 2). It might be possible for such agencies to convert a part of β-carotene into the other forms or derivatives of carotene or into hydroxy carotene, which should be the first oxidation product of carotene. Shinn, Wiseman, Kane and Cary *(125)* in their

6. A part of this work was presented at the Toronto Biochemical and Biophysical Society, February, 1938, (Can. Chem. **22**, 84).

7. The authors are pleased to acknowledge the coöperation of Dr. H. L. Branion and Dr. R. L. Martin, of Ontario Agricultural College, Guelph, Ontario, in this study.

chromatographic studies have also pointed out that carotene extracts of hays and silages exhibit absorption relations at critical wave lengths which are different from those of pure β-carotene.

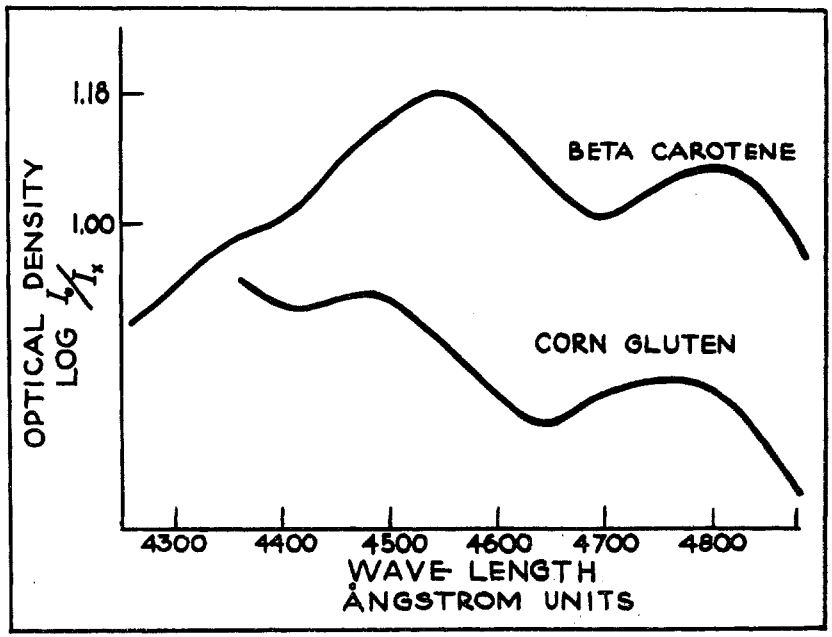


FIG. 1—Absorption spectrum of the petroleum phasic fraction of corn gluten.

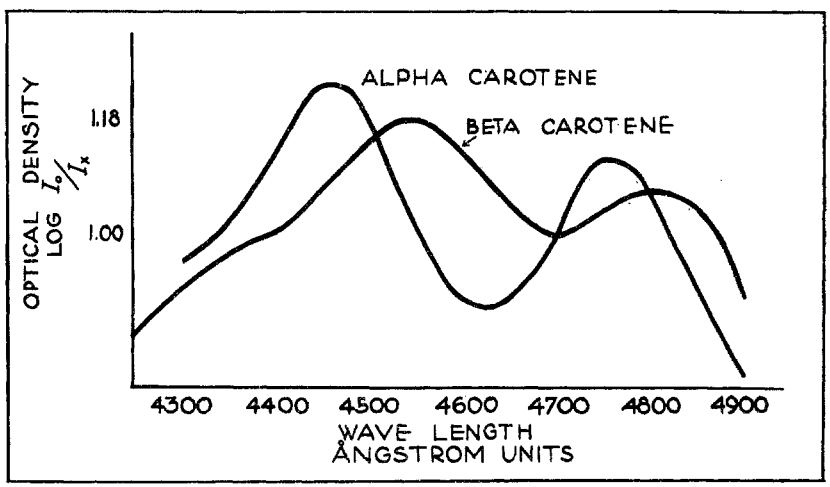


FIG. 2—Absorption spectrum of alpha and beta carotene in Skellysolve B.

A number of experiments[8] were performed to show the effects of heat and light upon β-carotenein feeds and in solution.

Dehydrated oats, the petroleum phasic fraction of which gave the normal absorption spectrum for β–carotene, still contained considerable petroleum soluble pigment after heating for 48 hours at 100° C. The absorption curve (figure 3) of this fraction, however, had lost all similarity to that of β-carotene, possessing a maximum at 4465 Å and no maximum at 4800 Å.

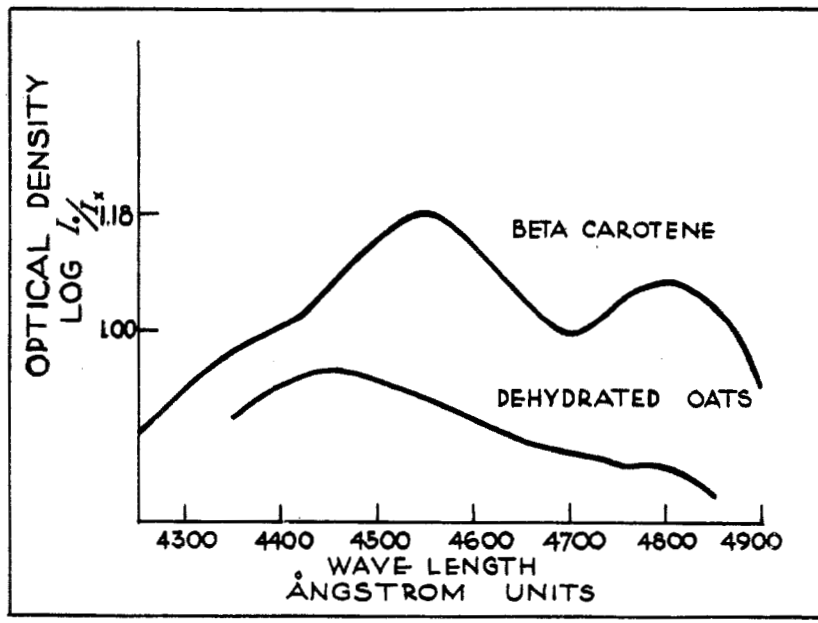


FIG. 3—Absorption spectrum of the petroleum phasic fraction of dehydrated oats after heating 48 hours at 100°.

A somewhat similar curve was obtained when a petroleum ether solution of β-carotene was exposed to sunlight until practically all the color had disappeared. As shown in figure 4 the first maximum had shifted considerably toward the ultraviolet while the optical densities at 4700 and 4800 Å were practically identical.

The most striking evidence of the effect of heat upon the β-carotene in feeds was obtained in an experiment in which six aliquot samples of dehydrated alfalfa were heated at 100° C. for different lengths of time from 0 to 7 hours (figure 5). The petroleum soluble fraction of each sample was obtained, the xanthophylls removed with 90 percent methyl alcohol and optical densities determined at 4490, 4550, 4700 and 4800 Å. It will be observed that for the un-

8. Some of these data were presented at the 93d Meeting of the American Chemical Society, Chapel Hill, North Carolina, April, 1937.

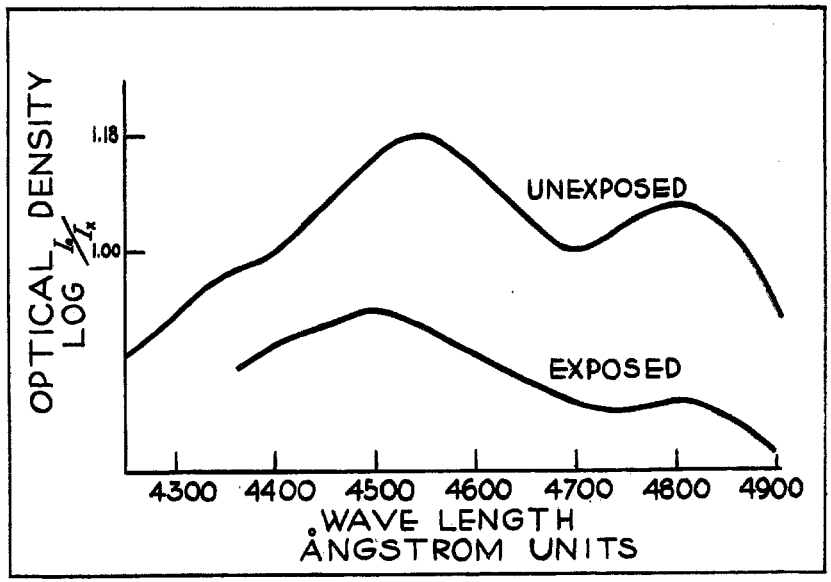


FIG. 4—Absorption spectrum of beta carotene in petroleum ether after exposure to sunlight until practically colorless.

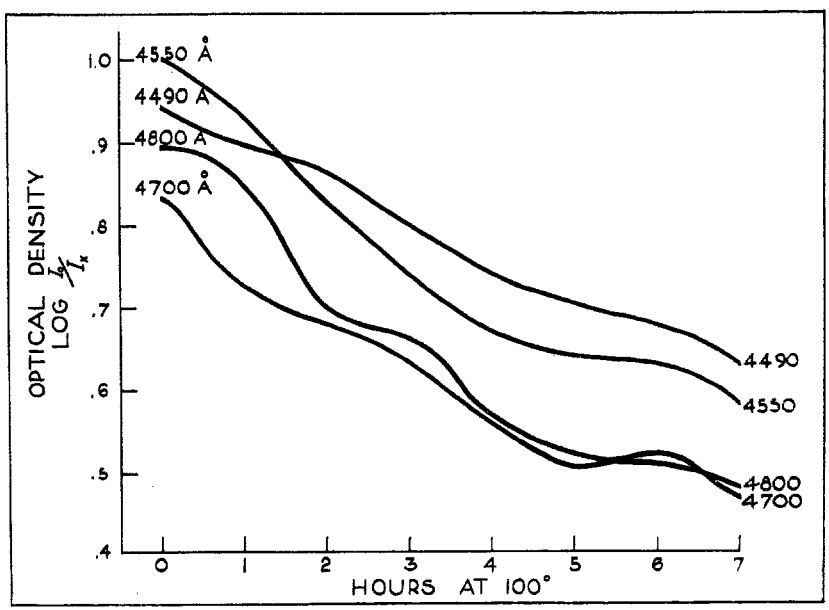


FIG. 5—Effect of heat upon the absorption maxima and minimum of petroleum fractions of dehydrated alfalfa.

heated sample the optical density at 4550 Å was considerably greater than that at 4490 and that the optical densities for all wave lengths had the same relationships to each other as would those for true β-carotene. After one and five-tenths hours heating, however, the optical densities at 4490 Å and 4550 Å became identical, while for all samples which had received more prolonged heat treatment the optical densities at 4490 Å were greater than those at 4550 Å. Interesting also is the manner in which the optical densities at 4700 and 4800 Å became almost identical after two hours heating and remained thus upon subsequent heating.

From a consideration of the results of Kline, Schultze and Hart *(53),* Frohring and Weno *(26)*, Rinprose and Norris *(116)*, Biely and Chalmers *(5)*, Record, Bethke and Wilder *(113)*, and Bearse and Miller *(3)* it can be concluded that the minimum vitamin A requirements of growing chicks is about 150 International units of vitamin A per 100 grams of ration, calculating all of the pigment in the petroleum phasic fraction as β-carotene. It is recognized, however, that most of this pigment was not carotene but cryptoxanthin. A preliminary separation indicated that about 90 percent of this pigment was cryptoxanthin. The carotene figures were converted into International units by the use of the factor 1.6 (1 International unit being equivalent to 0.6 micrograms of β-carotene).

The basal diet was a modification of that of Elvehjem and Neu *(14)* having the following composition: ground white corn 57.5, wheat middlings 25, casein 12, dried yeast 2, iodized salt 1, oyster shell 1, bone meal 1, and irradiated cholesterol 0.5 parts.[9] Spectrographic analysis showed that this control diet was vitamin A deficient but not absolutely free from the vitamin. It might be pointed out that mixed feeds which have a carotene content of less than 1 mg. per 100 gms. are very difficult to analyze with any high degree of accuracy. If a small sample (1-5 gm.) is used for analysis the color in the final extraction is too light to read accurately. If a large sample is used the efficiency of the extraction is considerably impaired. The corn gluten was added to the diet at the expense of the white corn. One group received the basal diet with 10 percent of the Canadian corn gluten feed added. It served as an additional negative control since this corn gluten contained no carotenoid pigment. Most Canadian corn gluten feed is a by-product from the manufacture of corn starch for human consumption and white corn is almost universally used. Rarely, some yellow corn may be used, but its relative proportion is so small, that to all intents it is negligible. Another group received 25 percent of yellow Argentine corn. This amount furnished 257.5 units of vitamin A per 100 grams of ration, again calculating all the pigment in the petroleum phase as beta carotene. A group receiving 1/8 percent fortified cod-liver oil was also included as a further control. This amount furnishes more

9. This was generously furnished by J. Waddell, of E. I. du Pont de Nemours & Co., New Brunswick, N. J. It contained 300 International units of vitamin D per gram.

of the vitamin than is actually required but it is an amount commonly used in practice.

Fifteen White Leghorn and fifteen Barred Plymouth Rock chicks made up each group. The chicks were individually weighed each week and the experiment continued for 10 weeks. Feed consumption was ad libitum.

The mean weekly weights and the number of birds surviving in each group are shown in Table XXVI. The birds which died all showed symptoms of vitamin A deficiency, namely, incoördination, prostration, before death. A number of these birds suffered from xerophthalmia, although it was not severe. Post-mortem examination revealed an accumulation of urates in the ureters and kidneys in the majority of cases, but only a few birds had pustules in the respiratory tract. It will be seen that 150 International units of vitamin A, as furnished by the carotenoid pigment in the corn gluten, per 100 grams of ration, calculated on the basis that this pigment was β-carotene, did not furnish sufficient vitamin A for good livability and only supported fair growth. The diet containing 250 units per 100 grams of ration, calculated on the same basis, gave much better livability, although the growth was still subnormal. It has been pointed out that about 90 percent of the carotenoid pigment in this corn gluten was cryptoxanthin. Hence it is evident that cryptoxanthin is only half as valuable as a source of vitamin A for chicks as b-carotene. This is in agreement with the theoretical consideration that cryptoxanthin on scission can only yield one molecule of the vitamin since it has only one unaltered β-ionone ring, whereas β-carotene can yield two. Ringrose and Norris *(116)* reported as a result of biological assay with chicks that the vitamin A content of American corn gluten varied from 7 to 25 units per gram. As calculated from the spectrophotometric analysis, the corn gluten used in this experiment contained about 42 units per gram, but since the bioassay showed that cryptoxanthin was only half as valuable as β-carotene (or, in other words that a unit of vitamin A is 1.2 micrograms of cryptoxanthin as against 0.6 micrograms of β-carotene) this corn gluten contained 21 units of vitamin A per gram, which is in agreement with the Cornell assay. These results emphasize the serious errors which may arise in spectrophotometric assays of carotenoid pigments if calculations are based on the assumption that all of the pigment in the petroleum ether phasic fraction is β-carotene. It would appear that such chemical assays cannot be relied upon, without bioassays, until routine methods are available by which the proportion of the various carotenes and cryptoxanthin in this fraction can be measured. Since it has been established that β-carotene predominates in green forages the method is quite valid for such feeds.

Since 257 units per 100 grams of ration, furnished by Argentine[10]

10. There is a possibility that this corn may have been South African, and it was reported as such in the preliminary communication, but subsequent investigation almost precludes such a possibility.

corn (Lot 6), gave decidedly superior results to 250 units per 100 grams of ration supplied by the American corn gluten (Lot 5), it would seem that the proportion of carotene and cryptoxanthin in different varieties of yellow corn may be somewhat variable. It might be pointed out that this corn contained about 10 units per gram, and it had undoubtedly been stored for some months. Fraps and Treichler *(66)* from bioassays, concluded that fresh yellow corn contained 7 International (5 Sherman) units per gram, which also points to the same conclusion. We are proceeding with this phase of the problem. A consideration of this lot in comparison with those receiving cod-liver oil (Lot 8) affords additional evidence that chicks utilize carotene as a source of vitamin A, just as efficiently as vitamin A itself, as shown by Record, Bethke and Wilder *(113)*. The results further suggest that the chick's requirement of vitamin A for growth may be greater than its requirement of the vitamin for livability.

The results with the Canadian corn gluten show that it contained little or no vitamin A potency. This was to be expected, as pointed out, since it was made from white corn.

It appears, therefore, that cryptoxanthin, a carotenoid pigment present in yellow corn for which the name cryptoxanthol is proposed, is only half as active as β-carotene as a source of vitamin A for chicks. There is some indication that the proportion of cryptoxanthin and carotene in yellow corns may be somewhat variable.

THE PETROLEUM-PHASIC CAROTENOIDS OF EGG YOLK

It has been known for some time that the fowl will selectively utilize (deposit in the egg yolk, body fat, or shanks) the xanthophylls, or hydroxy derivatives of carotenes, while the mammal tends to use the pure hydrocarbons. Whereas egg yolks and the body fat of fowls contain rather large amounts of xanthophylls, only small concentrations of the carotenes are found. A review of the literature and a study of several hundred pigment analyses in these laboratories reveals that the average xanthophyll: petroleum phasic carotenes ratio is about 10:1.

The presence of carotene in egg yolk was first suggested by Willstätter and Escher *(149)*. This was later confirmed by Kuhn and Brockmann *(59)* in 1932 in several varieties of hens' eggs. Gillam and Heilborn *(28)* in 1935 reported the first chromatographic study of the petroleum-phasic carotenoids of egg yolk, establishing the fact that more than one pigment was present in this fraction and also that cryptoxanthin, a petroleum soluble pigment of yellow corn, is also deposited in the yolk. When carotene, lycopene, and violaxanthin *(82)* respectively, were fed in oil solutions to White Leghorn hens, carotene and lycopene were found in traces at most, and violaxanthin not at all, in the egg yolk. Palmer and Kempster *(104)* found that annatto, which contains bixin, was without influence on

TABLE XXVI.—AVERAGE WEEKLY WEIGHTS (GMS.) AND LIVABILITY OF CHICKS FED VARYING AMOUNTS OF CRYPTOXANTHIN AS FURNISHED BY CORN GLUTEN

DIETS.	Weeks.										
	0	1	2	3	4	5	6	7	8	9	10
Vitamin A deficient	*30	30	30	30	25	17	8	2	0	0	0
1. Control	39	59	91	120	150	168	215	282	.....	.....	.....
American corn gluten	30	30	29	29	26	22	19	12	11	8	8
2. 100 units†	39	63	96	123	155	216	266	299	358	504	562
3. 150 units	30	30	29	28	28	27	25	23	21	10	8
	38	57	88	123	168	238	294	363	394	489	531
4. 200 units	30	30	30	30	30	29	28	18	18	17	16
	39	62	90	127	167	247	277	348	416	520	584
5. 250 units	30	30	30	30	29	26	25	23	22	21	20
	38	62	90	128	178	254	325	382	433	516	579
Argentine yellow corn:											
6. 257 units	30	30	29	29	29	29	29	29	29	29	29
	40	64	100	150	203	284	379	502	610	783	887
Canadian corn gluten:											
7. 0 units	30	30	30	28	26	20	12	5	2	0	0
	38	63	90	136	188	227	249	257	322	.....	.....
Fortified cod-liver oil:											
8. 375 units	30	30	30	30	30	30	30	30	30	30	30
	40	63	103	169	270	400	486	590	701	777	826

* Number of birds surviving at each weighing.

† International units of vitamin A per 100 grams of ration, calculated on the basis that all of the carotenoid pigment was β-carotene.

the color of the adipose tissue of fowls. Brown *(10)* has reported that in order for a fowl to deposit a carotenoid in the egg yolk or fat, it appears to be necessary that at least one ring of the molecule contain one, and only one, hydroxyl group. Strain *(134)* found that eggs from White Leghorn hens kept on rations of meat, corn and very small quantities of barley seedlings, contained lutein, zeaxanthin, and cryptoxanthin. When the rations were composed of wheat, bran, and milk with about 20 percent of fresh alfalfa or an equivalent quantity of freshly dried alfalfa, the eggs contained minute traces of neoxanthin, traces of flavoxanthin-like compounds, and considerable quantities of zeaxanthin, isolutein and cryptoxanthin. Strain also reports that even though hens had consumed large quantities of carotene, only traces of this pigment were found in the eggs, the carotene being composed principally of β-carotene with traces of α-carotene. Considerable quantities of a fluorescent substance were adsorbed with the cryptoxanthin. A survey of absorption spectral data accumulated in several hundred analyses of plant and animal materials performed in this laboratory revealed that the petroleum-phasic fractions of a number of substances possessed absorption curves which were not identical with that of pure β-carotene. A comparison of optical densities at the absorption maxima and minimum showed that particularly in the case of eggs, feed corn, and feeds which had been stored for long periods, the optical density was less at 4800 Å than at 4700 Å, whereas pure β-carotene has a maximum at 4800.

The purpose of this work has been to study in more detail the carotenoids of the petroleum phasic fraction of eggs under controlled conditions. In one series the source of carotenoids was dehydrated alfalfa, dehydrated oats, and fresh oat grass; in the other, corn was used. The curves are shown in figure 6. When corn is the sole source of carotene the maxima are at 4450 and 4750 Å and the minimum at 4679 Å. With green feed the maxima are at 4500 and 4750 Å and the minimum at 4600 Å.

Chromatographic studies were made of petroleum fractions of eggs from each feed, using a Strain column. In the case of leaf feed about 80 percent of the pigment passed through the column readily, while 20 percent was more strongly adsorbed. When corn was the sole source of carotene 55 percent was washed through readily, while the middle zone (20 percent) and top zone (25 percent) had to be removed by elution with alcohol. The main fraction in all cases had absorption maxima identical with the original solution.

The main pigment fraction in either case, when mixed with carotene and poured through a column, could not be distinguished from it, *i.e.,* β–carotene and the egg pigment came down as a single band. In the case of the eggs obtained when corn was the only source of carotenoid pigment, it may probably be assumed that the main fraction was identical with the modified β-carotene of the corn, the absorption curve of which has been previously discussed in another part of this bulletin; the middle fraction was probably cryptoxan-

thin, similarly modified, and the top fraction oxidized carotenes. In the case of the eggs obtained on green feeds, the absorption curve of the main fraction is not so easily explained, particularly since previous studies of the green feed chromatographically had shown the petroleum phasic fractions to consist of pure β-carotene. Either the slightly acid digestive processes of the hen are responsible for the conversion of the ingested β-carotene, or perhaps the hen has a more selective mechanism for the separation of pigments than it has been possible to produce in the laboratory. That is to say, since the fowl

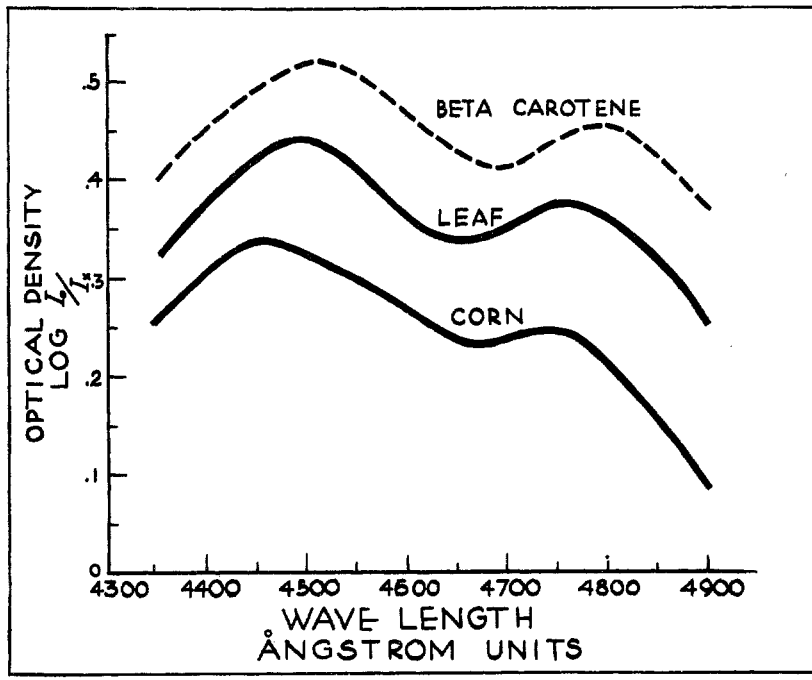


FIG. 6—Absorption spectra of the petroleum phasic carotenoids of egg yolk.

at best deposits only a very small fraction of the petroleum phasic carotenoids ingested, it is possible that most green feeds do contain a small amount of this modified beta carotene, which though it cannot be separated from β-carotene by the usual adsorption techniques, can be selectively utilized by the fowl. Evidence for this view is the fact that it has not been possible to introduce β-carotene into the egg by feeding.

A consideration which cannot be disregarded here is the interesting observation of Zechmeister and Tuzson *(164)* that carotenoids readily undergo certain isomerization processes in certain solvents, particularly when heated. This change is usually accompanied by a decrease in color intensity and a movement of the extinction max-

ima toward the shorter wave lengths. These workers claim that in no case is the phenomenon caused by the adsorption process, as has been put forth by Gillam and co-workers (29). If the work of Zechmeister and Tuzson is correct, then it might be countered that the absorption curves of the pigment fractions of eggs studied in this report were the result of the heat and solvent effects during extraction. This hardly seems likely in view of the fact that similarly conducted extractions on fresh plant materials have usually given petroleum fractions whose absorption maxima were identical with that of β-carotene. It is suggested that these observations could well be explained by the original presence of pseudo-a-carotene in the eggs.

THE VITAMIN A POTENCY OF THE PETROLEUM SOLUBLE PIGMENTS OF EGGS *(109)*

In regard to the vitamin A potency of the various pigment fractions of egg yolks, it may be said that though bioassays have not been conducted in this case, a great deal concerning vitamin A potency may be surmised from a knowledge of the relation of chemical structure of carotenoids to their absorption spectra and behaviour on an adsorption column. It is known, for example, that when the absorption bands of two carotenoids practically coincide, the number and location of the chromophoric unions must likewise be essentially identical. One is impressed with the fact that absorption spectra of these pigments so nearly approached that of α-carotene. Also it is well known that the opening of an ionone ring tends to shift the absorption maxima markedly toward the longer wave lengths, so it may be assumed that in this case the ionone rings are intact. It is known also that the introduction of hydroxyl groups into the ionone rings does not change the position of the absorption bands, but does increase their adsorbability on a column. For these reasons then, it seems likely that the vitamin A potency of these fractions, though less than that of β-carotene, would not be less than that of α-carotene. In this connection it may be pointed out that Kuhn and Brockmann *(58, 57)* have described a pigment (β-hydroxy carotene) obtained by the oxidation of β-carotene, which is completely soluble in petroleum ether, has maxima at 4460 and 4760 Å and has one-half the vitamin A potency of β-carotene. This compound has the formula $C_{40}H_{58}O_3$. The complete structure is not known, but it has been established that one β-ionone ring is intact, and that one of the oxygen atoms is that of an hydroxyl group. It is possible that this pigment is identical with the more strongly adsorbed egg pigment obtained from green-fed hens.

It has also been possible to demonstrate that 1-20 percent of the carotenols of eggs may be present in esterified form. In the cases studied in this laboratory, an average of 8 percent has been found.

It is important, therefore, in performing carotene analyses, to saponify in some stage of the procedure, since zeaxanthin, cryptoxanthin, and lutein esters are petroleum soluble and may give an apparent carotene analysis which is several hundred percent too high.

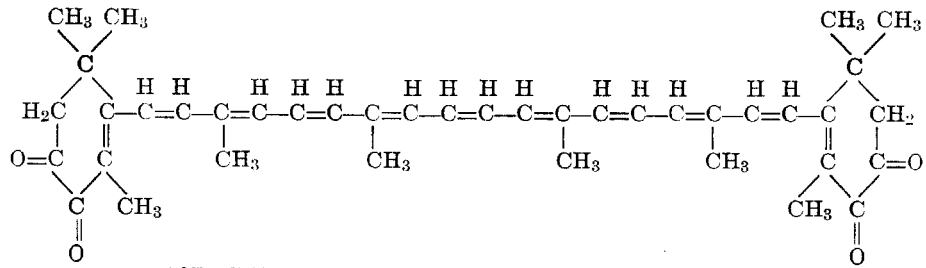
SUMMARY

The principal petroleum-phasic carotenoid of egg-yolk, as obtained by the usual method for determining carotene, has absorption maxima and a minimum different from that of β-carotene. Saponification and petroleum extraction of egg-yolks from hens receiving either fresh oats, dehydrated oat grass or dehydrated alfalfa give petroleum fractions all of which have maxima at 4500 and 4750 Å. U. with a minimum at 4600 Å. U. Adsorption on a column of MgO (50 percent) and siliceous earth (50 percent) gives two fractions: *A* (80 percent), which passes readily through the column, and *B*, which is more strongly adsorbed and can be removed by eluting with alcohol. When corn is the sole source of carotene, the petroleum-phasic fraction possesses maxima at 4450 and 4750 Å. U., and a minimum at, 4675 Å. U. Adsorption on a Strain column gives three fractions, *A* (55 percent), *B* (20 percent), and *C* (25 percent). *A* passes through the column readily, *B* is more strongly adsorbed, while *C* remains near the top of the column. Regardless of the carotene source, the absorption maxima and minimum of each of the component fractions are very nearly identical with that of the original solution before its separation on a column. Petroleum fractions obtained without saponification may contain from 1 to 20 percent of the total carotenols present in esterified form.

OLIVE-COLORED YOLKS

Thus far we have given consideration only to those pigments which may appear in the egg under normal conditions of feeding. Experienced poultrymen, however, have frequently been troubled by the occurrence of off-colored, or olive yolks during certain seasons of the year. Very little is known regarding the factors resulting in the production of olive yolks. Payne *(105)* observed that hens which had free access to Shepherd's Purse *(Capsella Bursa-pastoris)* or Field Penny-cress *(Thlaspi arvense)* produced olive-colored yolks within a short time. Berry *(4)* reported that if a flock, during the winter months, has an alfalfa range so large that the birds do not consume all aboveground parts of the plants, the eggs produced will practically all grade very low in quality. These eggs will not only be very dark in color, approaching an olive shade, but will be mottled and nearly black in some spots. The observation of Berry's is substantiated by the fact that eggs of this type are found in large numbers in the early spring when the eggs are candled at collecting points.

A review of the literature reveals that a variety of green pigments, other than those related to chlorophyll, occur in nature. Green to



ASTACIN [KARRER, LOEWE AND HUBNER (*41*)]

blue-black chromoproteins of crustacea, have been described by Verne *(149)* and Chatton, Lwoff and Parat *(12)*. The pigments in these cases, in contrast to the carotenoids of plants appear to be polyenes coupled with protein to give a water-soluble deeply colored complex. Verne *(143)* has reported that the blue-black native pigment of the lobster is a protein coupled with a carotenoid having the formula $C_{40}H_{56}$. Compounds of this class have been reported under various names, chief of which are vitellorubin, tetronerythrin, crustaceorubin, zoonerythrin, astroviridin, etc. In a recent work Kuhn and Lederer *(77)*, studying the pigment of the lobster *(Astacus gammarus* L.) gave the name "astacin" to the polyene portion.

The shell of the lobster contains a blue-black, the eggs, a green chromolipoid. Both pigments *(168)* are water soluble. Hot water, dilute HC1, acetone, or alcohol decomposes the protein combination and the color changes to red. After removal of the protein, the astacin is still not free, but in ester form. In the case of the "ovoester" obtained from lobster eggs the pigment is hypophasic when distributed between petroleum ether and 90 percent methanol, *i.e.,* the pigment dissolves in the alcohol phase, while preparations from the shell or hypodermis are epiphasic. Hydrolysis with alkali converts all the ester to astacin. (Table XXVII.)

TABLE XXVII.—PIGMENTS OF THE LOBSTER (NORWAY) ACCORDING TO KUHN AND LEDERER (77) (THE YIELDS REPRESENT AMOUNTS FROM 500 G.)

Shell.	Hypodermis.	Eggs.
Brown-black Chromoprotein	Red lipochrome (insoluble in water)	Green chromoprotein (soluble in water)
HCl ↓ acetone	↓ Extracted with acetone.	↓ acetone
Red astacin-ester (epiphasic)	Red astacin-ester (epiphasic)	Red astacin-ovoester (hypophasic)
↓ NaOH	↓ NaOH	↓ NaOH
Astacin (3-4 mg.)	Astacin (7-8 mg.)	Astacin (2-3 mg.)

It cannot be stated with certainty that the olive color of yolks is in any way related to astacin or its ester-protein complexes. It seems significant, however, that repeated attempts in these laboratories to isolate the green pigment of eggs with organic solvents resulted in the complete disappearance of the green and the appearance of the normal yellow yolk pigment. This behaviour is typical of the blue-green pigments of crustacea.

Another possibility lies in the fact that carotenoid pigments react with certain organic and mineral acids to give blue compounds. This has been frequently used in the differentiation of xanthophylls. Strain *(135)* points out that monoxy- and dioxyxanthophylls do not

form colored products when their solutions in ether are treated with concentrated hydrochloric acid, whereas most of the xanthophylls which contain more than two atoms of oxygen form blue solutions in the acid layer. Inasmuch as olive-colored yolks are most frequently obtained during seasons of growth when it would seem entirely possible that plants would not have their normal distribution of pigments, a study of this possibility seemed warranted.

When dehydrated oats and solutions of carotenoid pigments were treated with a variety of organic and mineral acids and fed to hens for a period of 10 days, the eggs subsequently produced were normal in color. However, this is not considered final proof that the olive color of yolks is not associated with the blue-green colors obtained with acids on carotenoids. It is possible that if the pigments are related, a much more subtle control of conditions would be necessary.

Water, ether, alcohol and acetone extracts of a grass silage were fed. This silage had repeatedly been shown to give a fairly high percentage of olive-colored yolks when fed in original form. Though the results were negative in the sense that olive-colored yolks were not obtained, the hens receiving the ether extract produced eggs which were repeatedly set aside as "off-colored" by an expert candler.

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