THE CAROTENOID PIGMENTS OF BRITISH COLUMBIA PILCHARD OIL.

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by
Arthur M. Eastham
and
Kenneth A. West

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Head of the Dept. of Chemistry.

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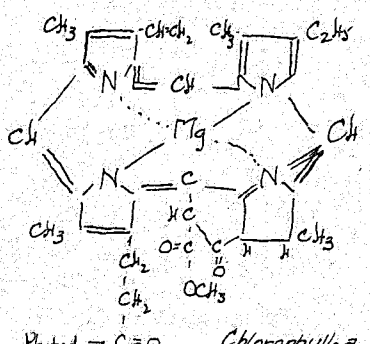
Since the pigments of plants have always attracted the attention of man, it is not surprising to find that they have been subjected to a great deal of scientific investigation. The fascinating changes of colour which take place in the plant during the spring and fall, and the beautiful colours of flowers and fruits at maturity have undoubtedly stimulated much of this research, but it is significant, that very few problems are ever very thoroughly attacked unless they are of industrial or physiological interest.

From earliest times, man has used paints and dyes and until the modern era nature had to be the source of such coloring materials. When however, with the development of organic chemistry, it became possible to synthesize natural materials, the synthesis of colouring matters was not overlooked, and since the first step in such synthesis is the determination of the structural formulae, it is not surprising to find that the structures of many of the natural pigments have been known for a considerable period of time.

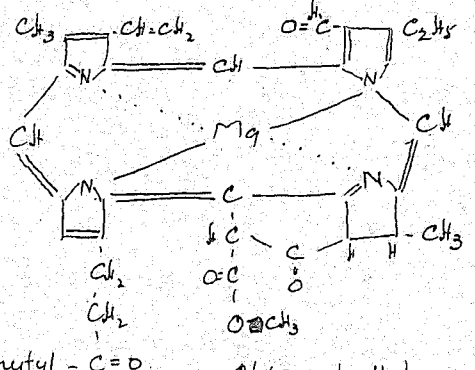
The physiological aspect of pigmentation is of more recent origin, but nevertheless it has been perhaps the greatest single stimulus to the study of the plant pigments which we have had. It might be said that, on the basis of distribution, there are three main classes of plant pigments, namely the anthocyanins, the chlorophylls and the carotenoids, and of these at least two, the chlorophylls and the carotenoids are of distinct physiological importance. Probably the most outstanding of all the processes carried on by

living material is photosynthesis, the process by which organic compounds are formed in the plant from carbon dioxide and water, and a process which is completely dependent on the green chlorophyll pigments. The carotenoids, which until recently have undergone but little investigation, have recently with the discovery of their close relationships to vitamin A, come very much to the fore and it is doubtful if any single field of biochemistry ~~has~~ has received much more concentrated attention over a period of five or six years.

The three main classes of pigments have chemically and physiologically, but little in common. The chlorophylls, we know from the brilliant researches of Willstätter and his students and more recently of H. Fischer and his coworkers, to have the following structure:



Phytyl - C=O Chlorophyll-a



Phytyl - C=O Chlorophyll-b

The carotenoids, some of which, for reasons yet unknown, are always found associated with the chlorophylls in the plastids, are hydrocarbons, or derivatives of hydrocarbons, which consist of long chains of carbon atoms joined by a series of conjugated double bonds. The anthocyanins, and we may include here for the moment

the related pigments, xanthones, flavones and madder reds, are aromatic compounds and have as the base for their molecules, such compounds as benzene, napthalene and anthracene. They are, unlike the other two groups, chiefly water soluble, and are responsible for most of the blues and violets as well as some of the reds and yellows found in nature. The natural carotenoids are all yellow to red in colour, and unlike the other groups, are not confined to plants although plants seem to be their original source. As yet there is no evidence of animals having synthesized any carotenoids, although they unquestionably do change plant carotenoids to typically animal carotenoids such as astacin, which is found in so many of the lower animal forms.

As has been mentioned above, the physiological importance of the group has been one of the reasons for the recent rapid development of our knowledge of the carotenoids. Another factor, however, which has influenced the development of the field is the development of methods of investigation. The great difficulties in handling these pigments compared to those of, for example the anthocyanins, have had a definite retarding effect upon the accumulation of accurate data, but it is interesting to note that some of these new methods when now applied to the supposedly thoroughly investigated anthocyanin group suggest that these pigments might be advantageously reinvestigated in the light of our newer knowledge.

It would be well perhaps, before going any further, to consider

the chemical and physical properties of carotenoids, and the methods which are used in their separation and identification.

A carotenoid may be defined as a nitrogen-free polyene pigment consisting wholly or chiefly of a long acyclic chain of carbon atoms united in an uniterrupted sequence of conjugated double bonds. The colour varies from bright yellow to deep red but may even be violet or dark blue. In general, the depth of shade increases with the number of consecutive conjugated double bonds.

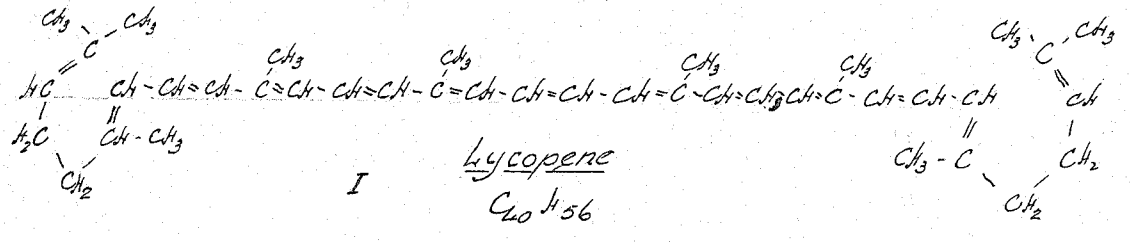
The existing nomenclature of these compounds is unfortunately rather confusing. Due to the presence in the literature of large numbers of historical names for the compounds of this group, attempts at standardizing the naming of the pigments has met with only partial success. The basic hydrocarbons which were formerly known under the one name "carotene" may now be divided into at least six compounds, all of which are characteristically soluble in benzine and insoluble in ether, and all of which have the general formula $C_{40}H_{56}$. In naming these compounds it has become common practice to call the principle members of the hydrocarbon group "carotenes" to conform with typical hydrocarbon nomenclature. There are recognized at present four carotenes, designated a-, β-, γ-, & δ-. An isomer of these, which is of considerable importance, is lycopene, the pigment of ripe tomatoes.

From these basic hydrocarbons are probably derived the oxygenated carotenoids, which may have as their substituents, the groups -OH, C = O, -COOH, -CHO & OCH_3. Formerly they were all classed as

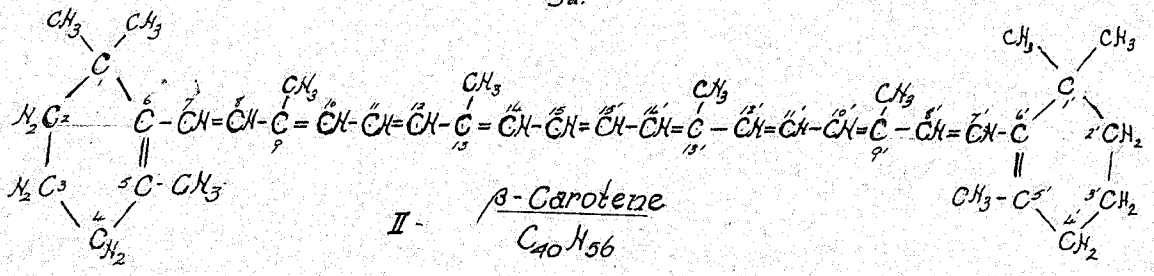
"xanthophylls", although some authors preferred to use this term for pigments containing hydroxyl groups only. An attempt is now being made to call the hydroxy-carotenoids, carotinols to conform with the international system of alcohol nomenclature and the name "xanthophyll" would then be reserved for the 3.3' dihydroxy-a-carotene, which is so common in green plants, and which goes also by the name of lutein.

Under this system, the name "carotenone" should be applied to the keto-derivatives of the hydrocarbons, but it has unfortunately been already applied to other compounds and is therefore not available.

The numbering systems used in naming carotinoids are several, but throughout this review, the system of Karrer will be adopted. It is illustrated in the following examples:

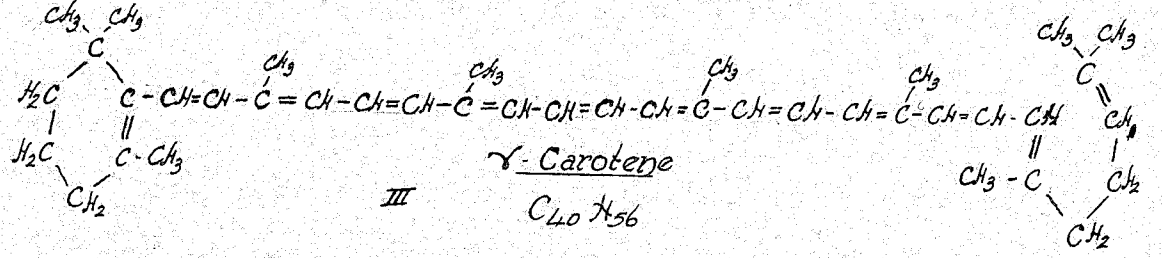


I Lycopene $C_{40}H_{56}$



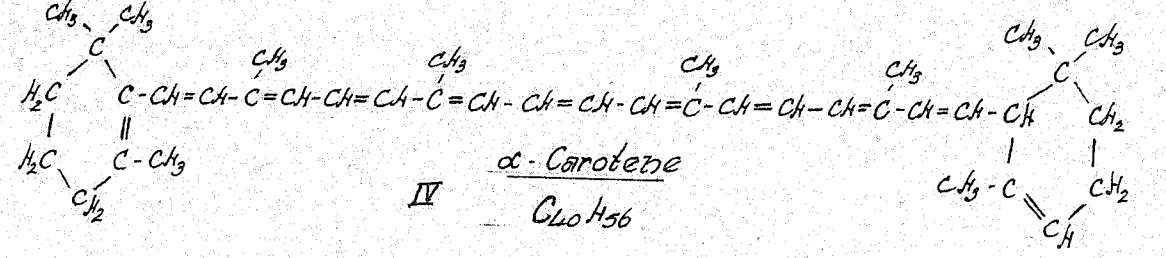
II - β-Carotene

$C_{40}H_{56}$



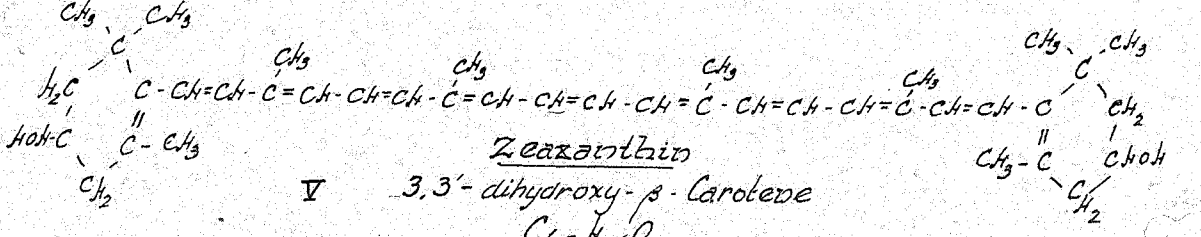
III γ-Carotene

$C_{40}H_{56}$



IV α-Carotene

$C_{40}H_{56}$



V Zeaxanthin

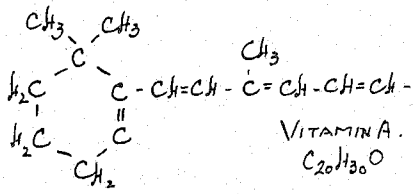
3,3'-dihydroxy-β-Carotene

$C_{40}H_{56}O_2$

In nature the forty carbon atom, or C_{40} carotenoids, are most common and will therefore be considered here almost entirely. Formula 1 above represents the structure of lycopene. It is a straight chain compound in which the β-ionone rings at each end are unclosed. β-carotene (Formula II) has the same structure with the exception that both β-ionone rings are closed.

γ-carotene differs from these two pigments in having only one of the rings closed and α-carotene, although having both rings closed, has a shift in the position of the double bond of one ring from 5.6 to 4.5.

β-Carotene shows strong vitamin A activity, α- and γ-carotenes less. The formula of Karrer for the vitamin A molecule is here given for purposes of comparison



VITAMIN A.
$C_{20}H_{30}O$

The close relationship between this and the vitamin A active carotenoids is obvious. If β-carotene were hydrolysed between the central (15.15) carbon atoms to produce two primary alcohols, two molecules of vitamin A would be formed. In the case of a- and γ-carotenes it is found that the vitamin A activity is only half that of β-carotene and a glance at the formulae will show the reason. In a- and γ-carotene only one of the β-ionone rings is identical with that of vitamin A, the difference in the other ring being one case a shift in the double bond and in the other, an unclosed ring. Cryptoxanthin, having the structure 3-hydroxy-β-

-carotene is also active as a source of the vitamin, but Zeaxanthin, 3,3¹ -dihydroxy-β-carotene is inactive showing that the presence of a hydroxyl group, in the 3-position of the β -ionone ring at least, is sufficient to destroy the activity.

The methods of handling, detecting, separating and identifying pigments of the carotenoid group are not numberous. The compounds are very sensitive to physical conditions especially in solution. They are usually destroyed by temperatures of above 55 degrees C and are sensitive to oxidation under atmospheric pressures. Whenever possible, especially in concentrated solutions, they should be handled under nitrogen.

Probably the most important tool in the hands of those studying carotenoids is the method of Tswett chromatographic analysis. It has been well described in the literature, but the review by Cook (1) would be worth reading by those interested.

The method is based upon adsorbtion of the pigments from solution by chemical compounds such as alumina, magnesia, calcium hydroxide or calcium carbonate. A glass tube of desired dimensions is connected with a suction flask and then packed tightly and evenly with the adsorbent, which may be mixed with a filter-aid of some inert material to increase the rate of flow. A solution of the pigment or pigments is then introduced at the top of the column and allowed to percolate down. This solution for best adsorbtion should be fairly concentrated and should be made up in a solvent in which the pigment is not very soluble.

For the xanthophylls, benzine is often satisfactory, but carbon disulphide and ether are often used. The pigments are adsorbed on the column in zones which may be sharp or quite diffuse. These zones or bands are then sharpened by washing with fresh solvent and are then usually cut apart mechanically to separate them.

The pigment separation thus obtained is very satisfactory. In the case of Petalozanthin, ~~which will be discussed elsewhere~~, a new pigment was discovered by means of chromatogrphic analysis. When all other methods of separation had indicated Petaloxanthin to be the same as Antheraxanthin, a mixture of the two pigments showed two bands on the column. It can also be noted here that this method has been applied to anthocyanins and has suggested that some of the earlier work in this field might be profitably reinvestigated. Karrer applied the method to the purification of vitamin A and through its aid was able to finally isolate this compound, from the unsaponifiable residue of fish liver oil, in a pure crystalline state.

The only disadvantage of the method is the possibility of isomerization of the pigments in the process (2, 3.). For this reason it is important to choose the adsorbent to be used with some care. Charcoal has many disadvantages; it is too strong an adsorbent and makes elution of the pigment difficult, it is an oxidizing agent and can produce decomposition of the pigment, and finally its colour makes it impossible to see the bands. A rather strong adsorbent which does not cause much decomposition is alumina which can be obtained in various degrees of activation. Strain (4)

found the most suitable adsorbent for the carotenes to be a specially prepared magnesium oxide.

Rather closely related to the chromatographic analysis method, is a method of detecting mixtures which has been used with some success and which goes by the name of "capillary analysis". When a strip of filter paper is placed with one end in a solution of pigments, it is found that the solvent rises in the filter paper and the pigments form definite zones on the paper. The relative position of the bands together with their breadth and colour, are characteristic of certain pigments. Kylin (5) in his investigations of the carotenoids of higher plants and algae used this method extensively. His results however, are not sufficiently accurate or fundamental to allow their interpretation in terms of our present knowledge and it can be said that this method is very limited in its application.

Vying with the chromatograph in value to the investigator is the spectroscope. By means of these two tools he can give almost positive identification to a pigment although it is usual not to consider identification complete and unquestionable unless the pigment is obtained in pure crystalline form, so that melting point determinations and analyses can be made upon it. However, because of the very small amounts of pigment that may be available and because only small amounts are required for spectroscopic examination, this method has been of inestimable value.

The absorbtion curves of nearly all the pigments have been accurately worked out and are recorded in the literature. It is usual now to record simply the maxima of the absorbtion bands and the solvent in which they are measured. Much of the early work has been of little value because the workers have failed to record important data such as solvent employed. The solvent used is of particular importance because it can cause shifts of at least 40 mu in the position of any one band.

Solubility tests constitute the final important method in the identification of carotenoid pigments. The so called Kuhn and Brockmann micro method is essentially the distribution of pigments between two mutually immiscible solvents, and of these themost important distribution is that between petroleum ether and aqueous methanol. By use of this technique, the basic hydrocarbons and pigment waxes (carotenoid esters) can be separated from the free oxygenated carotinoids because, whereas the former are preferentially soluble in benzine, the alcohols, ketones, and acids are retained almost entirely in the alcohol layer. An exception to this general classification is cryptoxanthin, which probably because of its low oxygen content, is fairly soluble in both phases but goes if anywhere to the benzine layer. After the above separation has been made, the petrol ether layer may be further subdivided by saponifying which breaks down the esters yielding the free pigments and then redistributing them between the same two solvents. Thus the presence of esters may be detected. The pigment extracts must however be watched for the presence of oils and other interfering

substances which may distribute themselves in such a way as to change the solvent properties of one of the phases, and thereby destroy the value of the separation.

A considerable number of colour reactions of carotenoids are reported and were widely used by the pioneer investigators. We know now however, that their lack of specificity has been the cause of much trouble, and that though they may be of value when coupled with other evidence, they should not be relied on. We will mention only the reactions with sulphuric acid and with the chloroform solution of antimony trichloride. Concentrated sulphuric acid is sometimes used to detect the presence of polyene pigments because in their presence it produces a blue colour, which seems to depend upon the number of double bonds in sequence, for its shade. Kuhn and Winterstein, working with the compound $C_6H_5-(CH = CH)_n-C_6H_5$ found that the colour produced depended upon the value of n, thus -- for n = 1 or 2, no colour; n = 3, yellow orange; n = 4, red; n = 5, violet red; n = 6, blue; n = 7, blue green and n = 8, blue green.

The Carr-Price colour reaction is the reaction in which a solution of antimony trichloride in chloroform produces a deep blue colour when mixed with vitamin A. It was recommended for the quantitative and qualitative estimation of this vitamin, but unfortunately it has since been found to give the same test with any carotenoid. Another disadvantage of the reaction is that the colour produced is short lived (about 10-20 second) and spectroscopic ex-

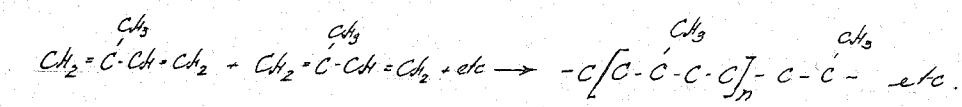
amination of the blue colour, which could make the test specific, is therefore very difficult. Various modifications of the test have been proposed but none of them have met with any general approval.

The foregoing summary of methods of carotenoid investigation is very brief. If further information is desired the reader would be well advised to read the section on carotenoids in Gilman's "Advanced Organic Chemistry" or some of the papers of Paul Karrer of the chemical properties and methods of investigation of carotenoids. It is interesting to note that the chromatographic method which today is deemed so important was discovered and reported by Tswett in 1906. He used it in his own investigations, but it was then forgotten and only recently reinvestigated. It is finding more and more use in the field of organic chemistry as is shown by its recent applications to the separation of sterols and of optically active isomers.

The formation in nature of carotenoid pigments is like so many other natural synthesizing processes, not understood. However, several interesting theories have been proposed to account for their formation and it would not be amiss to consider them here, provided that we keep in mind that none of them have much experimental basis.

Of these theories, the more logical ones usually consider isoprene ($CH_2 = C(CH_3)_3-CH = CH_2$) as the building unit from which the pigments are built up. The polymerization of isoprene could take place in one of the following three ways:

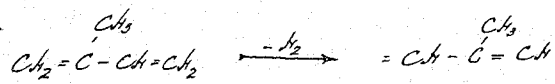
1. By direct linear head-to-tail union -



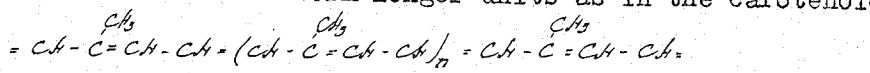
Such addition might go on indefinitely, will form either cyclic or acyclic hydrocarbons and is usually considered to be the origin of the terpenes.

2. A similar type of polymerization with the concurrent hydrogenation which would explain the formation of such hydrocarbons as that from which phytol ($C_{20}H_{40}O$) is derived.

3. Addition with subsequent dehydrogenation in the 1:4 position -



These units then unite to form longer units as in the carotenoids

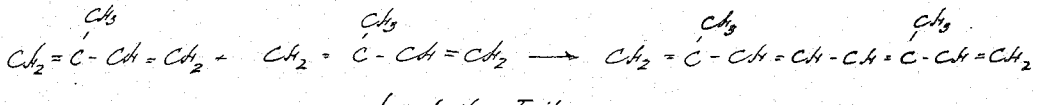


Thus n isoprene units give $2n+1$ double bonds and it is significant to note that the number of such bonds in most of the common carotenoids is uneven (7, 9, 11 or 13).

The Diels - Alder diene synthesis has strengthened these theories by showing the relatively mild conditions under which such

reactions can be accomplished.

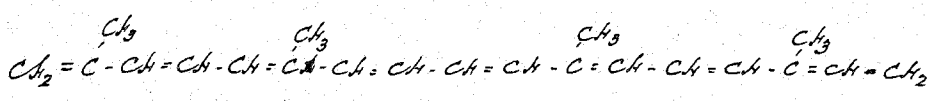
Karrer and his associates early recognized that direct head-to-tail union of isoprene molecules proceeds to a limited extent and that two of the relatively short chains then unite to a longer one in such a way that one half mirrors the other and a symmetrical molecule is formed. Considering this fact with polymerization type 3 above we could then postulate the following reactions -



Head-to-Tail

I

If now two of these new molecules were to unite tail-to-tail in accordance with Karrer's statement we would have the structure

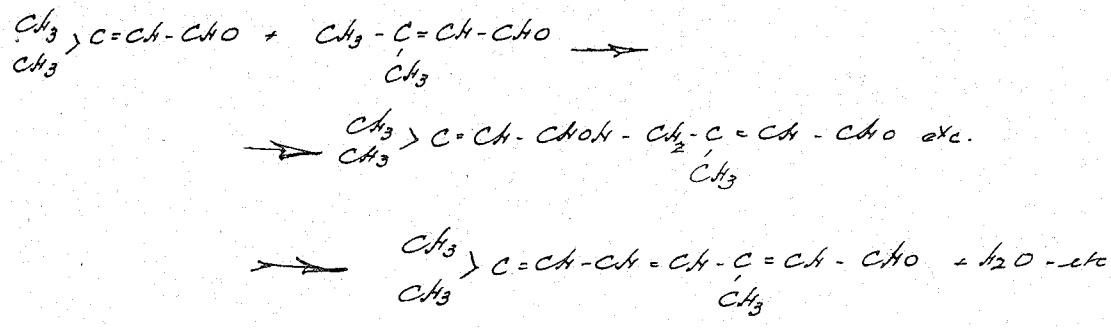


II

It is seen that whereas in formula I the methyl groups are in the 1,5 position, in the new compound (formula II), although the outer methyl groups are in the 1,5 position, the two central ones are with respect to each other, in the 1,6 position. It is rather startling to find that this same arrangment exists in the natural carotenoids as will be seen on examining the formula given on page 5,5a. Some support is also given to the idea of dehydrogenation in the process by the oxygen requirement of ripening fruit.

Karrer and others have advanced another hypothesis to explain

carotenoid formation, which is based upon the assumption of -methyl-crotonaldehyde as the primary unit in place of isoprene.

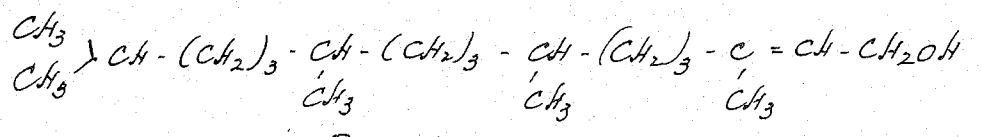


These theories have suggested methods of synthesizing carotenoids but those tried so far have been unsuccessful.

If any of the above theories are to be considered, a problem of obvious interest is the determination of the antecedent C_{20} compound from which the C_{40} compounds are derived. Willstätter and Mieg suggested that phytol might play such a role. If such were the case it would obviously account for the close association of carotenoid and chlorophyll pigments in the green parts of plants since the chlorophyll molecule owes about one third of its mass to the phytyl group. Furthermore, Karrer and his coworkers have taken completely hydrogenated phytol and then, after converting it to bromide, condensed it by the action of potassium to a perhydrolycopene of formula $C_{40}H_{82}$ which is identical with that obtained from the catalytic hydrogenation of the tomata pigment lycopene.

In considering phytol as a precursor to carotenoids, the chlorophyll-carotenoid ratio must be examined. It is well known that carotenoid content increases with the break down of chlorophyll and

this suggests that the phytol source could be the green pigments themselves. Kuhn and Brockman investigated this possibility and reported that the chlorphyll decomposed was insufficient to account for all the carotenoid formed. Their results could be taken to suggest that the phytol normally used in chlorophyll synthesis is now being used in carotenoid synthesis and the fact that carotenoid synthesis can proceed in the dark, whereas the green pigments require light for the completion of their synthesis could also be adopted to this idea. A more logical hypothesis, however, seems to be that phytol and the carotenoids have a common ancestor. Such a theory is supported by the presence in organs of plants, of large quantities of carotenoids with no associated chlorophyll. Such organs are the carrot root, and tomatoes which have been grown in the absence of light ( ).



PHYTOL

Such are the theories which have been propounded to account for the production of carotenoids in plant tissues. It seems probable that the first product formed would be a hydrocarbon from which could be derived and carotenols and other oxygenated derivatives by direct oxidation.

Following is a list of the known pigments of the carotenoid group. The first list is that given by P. Karrer (6) in 1936, the second that of M.T. Bogart (7) at about the same time. Karrer's list is somewhat more rigorous in what is included as a distinct pigment

but most of the pigments included in Bogart's list are probably distinct:

1. Karrer

1. Antheraxanthin
2. Astacin
3. Azafrin
4. Bixin
5. Capsanthin
6. Capsorubin
7. α-Carotene
8. β-Carotene
9. γ-Carotene
10. ζ-Carotene
11. Cryptoxanthin
12. Echinenon
13. Englenarhodon
14. Flavorhodin
15. Flavoxanthin
16. Fucoxanthin
17. Lycopene
18. Pectenoxanthin
19. Pentaxanthin
20. Rhodopin
21. Rhodopurpurin
22. Rhodovibrin
23. Rhodoviolascin
24. Rhodoxanthin
25. Rubixanthin
26. Sulcatoxanthin
27. Taraxanthin
28. Violaxanthin
29. Xanthophyll (Lutein)
30. Zeaxanthin

List given by H. Bogart

Name	Formula	Substituents	Occurence
1. Antheraxanthin	$C_{40}H_{56}O$		(from place discovered maybe others)
2. Astacin	$C_{40}H_{58}O_3$		Anthers of Lilium tigrinum

List given by M.J.Bogart (Cont'd.)

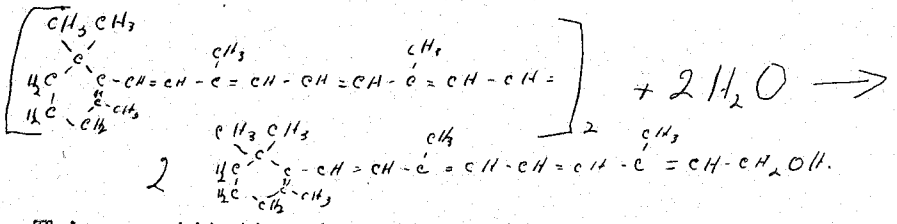
Name	Formula	Substituents	Occurance
3. Azafrin	C27 H38 04	(OH)2 COOH	Escobedia scabrifolia
4. Bixin	C25 H30 04	(COOH) (COOCH3)	Annatto (Bixa orellana)
5. Capsanthin	C40 H58 03	(OH)2 C = O	Capsicum annum
6. Capsorubin	C40 H60 04	(OH)2 (C=O)2	Capsicum annum
7. α-Carotene	C40 H56		Carrot - Daucus Carota
8. β-Carotene	C40 H56		Carrot - Urtica ureus
9. γ-Carotene	C40 H56		Carrot - Urtica Ureus
10. δ-Carotene	C40 H56		Gonocaryum pyriforme
11. Citraurin			Oranges
12. Crocetin	C20 H24 04	(COOH)2	Saffron - Crocus sativus
13. Cryptoxanthin	C40 H46 O	OH	Ground Cherry- Physalis alkakeugi
14. Cynthiaxanthin			Halocynthia papilosa
15. Echinenone	~~C40 H56 O~~ $C_{40}H_{54}O$		Sea Urchin
16. Eschscholtzxanthin			Eschscholtzia Californica
17. Euglenarhodon	C40 H56 03	(CO)4	Euglena hiliorubescens
18. Flavorhodin			Purple Bacteria
19. Flavoxanthin	C40 H56O$_3$		Ranunculus acer
20. Fucoxanthin	C40 H56 06	(OH)4 (CO)2	Brown Algae
21. Glycmerin			A Mussel

22. Isolûtèin			Green leaves
23. Lycopene	C40 H56		Lycopersicum esculentum
24. Lycophyll	C40 H56 O2	(OH)2	Solanum dulcamara
25. Lycoxanthin	C40 H56 O		Tomata & Bitter Night shade berries
26. Myxoxanthophyll	C40 H(54-60) O5		Myxophyceae
27. Myxoxanthin	C40 H54 O	C=O	do
28. Pectenoxanthin	C46 H(52-56) O7		A Mussel
29. Pentaxanthin	C40 H(54-60) O5		Sea Urchin
30. Retinene			Animal Eyes
31. Rhodopin			Purple Bacteria
32. Rhodopurpurin	C40 H56-58		Do
33. Rhodevibrin			Do
34. Rhodoviolascin	C42 H60 O2	(OCH3)2	Do
35. Rhodoxanthin	C40 H50 O2	(CO)2	Taxus baccata berries
36. Rubixanthin	C40 H56 O	OH	Rosa rubiginosa
37. Salmic acid		COOH	Salmon
38. Sarcinene			Sarcina lutea

39. Spirilloxanthin			Sulphur Bacteria
40. Sulcatoxanthin	C40 H52 O8 ?		Sea Anemone
41. Taraxanthin	C40 H56 O4	(OH)4?	Dandelion
42. Violaxanthin	C40 H56 O4	(OH)4?	Viola tricolor
43. Violaerythrin			Sea Anemone
44. Xanthophyll (lutein	C40 H56 O2	(OH)2	Green Parts of Plants
45. Zeaxanthin	C40 H56 O2	(OH)2	Yellow corn

Investigations of recent years have made it clear that carotene is a provitamin A, and that the vitamin A effect of foods runs essentially parallel with their carotene content. Carotene is transformed in the animal body into Vitamin A, which like carotene itself tends to accumulate in the liver.

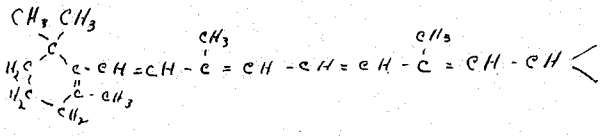
The most obvious explantion of this formation of Vitamin A from carotene is that it consists in a simple hydolysis, which cuts the carotene moecule in two, exactly in the middle.



This constitution for Vitamin A was proposed and established by Karrer and his coworkers (8). A convincing proof of the structure of the carbon skeleton was provided by the synthesis of perhydrovitamin A from B-ionone and the identity of this synthetic product

with a perhydrovitamin A obtained by the catalytic reduction of a carefully purified Vitamin A from liver oils.

According to the present state of our knowledge of the connection between structure and vitamin A activity, only those compounds which contain the following complex exhibit provitamin A activity, this complex apparently being easily transformed to Vitamin A in the living organism.



A change in the location of the double bond in the ionine cycle ⟨ or the insertion of an OH therein, destroys the Vitamin A effect. Thus α, β and γ carotenes are all active, but B-isomer with two such complexes, is approximately twice as potent as the other two. Similarly, the carotenol cryptoxanthin (3 - Hydroxy - B- carotene) functions as a provitamin A, but rubixanthin (3 - Hydroxy - γ - carotene) and zeaxanthin (3, 3' - dihydroxy - B- carotene) neither of which contains an unhydroxylated B- ionone, are devoid of such properties.

In addition to the three carotenes and cryptoxanthin, the only other naturally occurring carotenoid, and the sole zoocarotenoid reported to show vitamin A activity is the echinenone of the sea urchin. Although its constitution is still unknown, it would follow from this property that enchinenone contains the Vitamin A complex.

The following synthetic products also show the provitamin A properties:

B - Carotene diiodide	C40 H56 I2
α - Carotene "	"
Di- Hydro - B - carotene	C40 H58
Di-hydro β - carotene	C40 H58
B - carotene oxide	C40 H56 O
Oxy - β - carotene	C40 H56 O2
Semi - β - carotenone	C40 H56 O2

It is surprising that B - ionone, which is itself devoid of any provitamin A action, should appear to be so essential a part of those polyenes possessing this property; and that a product herto-fore of interest chiefly because of its violet perfume should suddenly be discovered to be playing a leading role in the field of carotenoids and vitamins.

Human vision seems to depend upon the bleaching of the eye's sensitive "visual purple" by the light with the formation of an orange "visual yellow" whose color is said to be due to a yellow pigment related to the carotenoids, termed "retinene" by Wald(9)

This retinene is liberated from visual purple not only by light but also by the action of chloroform. It disappears from the retina either by reversion to "visual purple" or by transformation into vitamin A and other colorless products. In this degredation of visual purple to retinene and Vitamin A and its rebuilding from them again, it is not clear just how much is due to the retinane and how much to the Vitamin A. Some of the latter is lost in the process, so that the organism requires a constant outside source

of supply, for this automatic regulation of the eye's sensitivity does not function properly without it. At present however, there is no definite evidence that the retinene is the sole, or even the main, source of Vitamin A in light adapted retinas or that its structure is distinct from that of B - carotene.

The use of pilchard and other fish oils in the feeding of chickens has raised several interesting problems which are related to the carotenoid pigment content of the oils. The pigments of egg yolks have been studied by Heilbron et al (10), Brown ("), and others. Although the characteristic pigments are lutein, zeaxanthin and cryptoxanthin ( ), Brown has shown that the pigment content of the diet can influence the pigmentation of the yolk to a considerable extent.

After feeding chickens on a carotenoid-free diet until the yolks were free from these pigments, he added pigments pericarp to the feed as a carotenoid source. The shells contain zeaxanthin, capsanthin, cryptoxanthin and carotene. He added also taraxanthin and violaxanthin. The The yolks of the eggs were again analysed and the pigments determined. Based upon feed consumption and the egg production, 65% of the ingested xanthophylls are deposited in the egg yolk, but the absorbtion of individual pigments varies within wide limits.

Lutein and zeaxanthin are deposited to a much greater extent than capsanthin and cryptoxanthin. Lycopene and carotenes are apparently unavailable as are taraxanthin

and violaxanthin. It would appear that the structure of the pigment has considerable influence upon its deposition in the yolk. The hydrocarbons and the highly oxygenated compounds above show almost no deposition while cryptoxanthin containing a single hydroxyl group is but little better. It seems signifigant that zeaxanthin and lutein, which are respectively, 3,3'- dihydroxy -β- carotene and 3.3'- dihydroxy α- carotene, and which therefore have very similar structure, should be almost equally well deposited.

A problem closely related to the above is that of the pigment content of the fat deposits of various animals. These deposits may represent a possible Vitamin - A storage, and are therefore of considerable physiological interest. Zechmeister and his co-workers (12) have given the problem some attention in an attempt to clarify the picture, but their observations have so far, served only to complicate the problem. They found that the fat deposits of horse and cow contain only carotene, whereas those of fowls and humans contain lutein but no carotene. Swine on the other hand, judging from the extreme whiteness of the fat probably cantain no carotenoids.

Chalmers has made the interesting observation (unpublished) that chickens fed upon a diet containing pilchard oil, store up in the liver, a dark brown pigment. The position of the liver in the vitamin - A picture suggests a relation between this pigment and members of the carotenoid group, and its storage in the liver is obviously of

considerable interest.

As a preliminary investigation to the study of the problems mentioned above, the determination of the carotenoids of commercial pilchard oil was undertaken. This oil is obtained from the whole fish after a short steam cooking, by pressure. It is then "cold cleared" by cooling to remove stearins. The oil thus obtained is of a dark olive brown color but may vary condiderably with the season in which the fish are caught. The fact that the viscera of the fish are not removed prior to extraction means that the stomach contents will probably influence the pigmentation of the oil, since the lower animal forms and algae are very high in carotenoids, many of them peculiar to the classification group to which the organism belongs.

The most common feed of pilchards is said to be the "red feed" which is composed of small crustaceae, the cocopods. Since in the lower animals forms the most common pigment is astacin, it seems probable that the cocopods might introduce this carotenoid into the oil. However, if the feed contained diatoms or other algae, it might be expected that numerous other pigments such as fucoxanthin, characteristic of the brown algae and myxoxanthin or myxoxanthophyll characteristic of the blue green algae, might be present. Both of these groups contain small forms and so could conceivably form part of the feed.

Experimental Procedure.

The separation of the pigments of pilchard oil presented a problem of unusual interest. Examinations of this oil have been made by Tompkins (13) and by Bailey (14). Tompkins reported the presence in the oil of chlorophyll which may give a distinct greenish tint and which together with the red and yellow pigments undoubtedly accounts for the usual olive brown color.

Bailey examined the carotenoid content of pilchard oil and reported the presence of carotene, xanthophyll and fucoxanthin, He used small samples of oil (50cc), dissolved in CS_2, which he passed over a column of alumina. The pigments obtained were identified by their solubility, color reactions and positions on the column. Bailey also examined canned pilchards ie. fish from which the viscera had been removed and found that fucoxanthin was absent from the oil. When commercial oil was treated in a manner similar to the canning process, fucoxanthin was not distroyed. Bailey therefore concluded that the source of the fucoxanthin was the viscera. He gives the following pigment concentrations for a dark colored oil:

Carotene--	0.06 - 0.25 mg per 100 gr. oil
Xanthophyll--	0.49 - 0.84 mg
Fucoxanthin--	0.16 - 0.84 mg

It is well known from the work of Heilbron (15) and Karrer (16) that fucoxanthin present in fish brown algae goes over to zeaxanthin in drying of the alga. Heilbron ( ) has reported that this same change takes place on the adsorbtion

column, while Zechmeister ( ) has given evidence that it takes place in solution. It seems possible therefore that Zeaxanthin may also be present in pilchard oil, although Bailey failed to observe any sign of it.

Zechmeister (24) in his examination of the pigments of horse and hen fat reported an attempt to separate the pigments by shaking up the melted fat with the adsorbent. We have tried to apply this method to the pilchard oil, but obtained poor separation.

It is usual in removing oil from carotenoid extracts to saponify the mixture with alcoholic KOH, and then to extract the mixture with ether or petrol ether, after the addition of water. This method is satisfactory when the quantity of oil is small, but in this case presented considerable difficulties. First, the amount of soap formed was extremely large and precipitated upon addition of only small amounts of water. This precipitation interfered seriously with any attempts at extraction of the pigments from the saponified mixture probably partly due to adsorbtion of pigment upon the soap but more because it tended to destroy the interface thus preventing separation. If just sufficient water was added not to cause precipitation a better separation was obtained but large amounts of the extracting solvent dissolved in the alcohol layer. Furthermore certain of the pigments were found to be exceedingly soluble in alcohol and therefore tended to remain in the alcoholic layer. An attempt was made to remove the soaps

by salting out or by precipitation as calcium salts of the acids. The removal and washing of the precipitates however was very difficult and because of the fear of losing pigments in the precipitate, either through adsorbtion, or failure to wash thoroughly, this method was abandoned.

The possibility of extracting the pigments from the oil was then examined. Fats solvents were of course, of no use. Of the others, alcohols were the only ones which showed any promise, methanol was least soluble in the oil. By comparison with a chromatograph, the effect of an alcohol extraction was determined. The extraction was made by emulsifying oil and alcohol in the ratio of two volumes of oil to one of methanol, at 50 degrees. The emulsion was allowed to break and the layers separated. Three such extractions served to remove by far the greater part of the extractable pigment, but the chromatogram showed that only one of the pigments was removed.

The alcohol extracted was evaporated down to a dark oily residue, which was dissolved in ether and petrolether. On cooling, some amorphous, white material precipitated and was filtered off. The chromatograph of this extract showed predominantly one orange-red band which was later identified with fucoxanthin, and traces of one or two other yellow bands and some green material. Since the yellow pigments were present in traces only, it was concluded that they were probably carried over mechanically in oil drops or were distributed very slightly in the alcohol.

Besides fucoxanthin, the extract contains large amounts of sterol like material together with some oil, probably rather highly unsaturated, judging from the intensity of the red color produced upon saponification. These waxy materials are readily adsorbed on the chrom atograph and have the effect of cementing together the adsorbent material to make it almost impermeable to the pigment solution. It was thought that perhaps the addition of petrol ether to the oil might reduce the solubility of the xanthophyll fraction sufficiently to allow extraction with alcohol. It was unsuccessful however, because the presence of small amounts of oil in benzine makes it a fairly good solvent for carotenoids. Another difficulty was caused by the change in specific gravities brought about. Methanol is intermediate in solubility between oil and petrol ether but a mixture of oil and petrol ether has a specific gravity very close to that of the alcohol and separation is very slow.

The method finally adopted for separating the pigments was the chromatographing of the oil itself. Preliminary experiments indicated that activated alumina was more satisfactory than activated magnesia for the purpose. Magnesia is not sufficiently strong and allows the pigments to pass through the column too rapidly but has the further disadvantage of becoming a dirty brown color in the presence of oil which tends to screen the lighter bands. In early experiments the oil was dissolved in light petroleum to give more rapid filtration but it was later found that the pure oil was equally satisfactory and was thereafter used.

Glass tubes five feet long and five cm. in diameter were packed to a height of 45 cm. with a mixture of activated alumina (Alorco--100 mesh) and a filter-aid (Johns-Manville Hyflo Super-cel) in equal parts by weight. The capacity of such a column was about one litre of oil. When that volume had been passed through, the lower bands had reached the bottom of the column and washing was usually impossible.

Three columns were set up and the oil passed over them. The following zones developed: (1) A broad green band covering the top four or five cms. of the column. (2) Three bands close together, the upper two orange red and the third one orange. (3) Considerably below these, two bands close together, the upper one green, the lower one yellow. The filtrate from the columns was light yellow. The lower yellow and green bands were allowed to pass through the column in order to obtain better separation of the upper bands.

The adsorbent was pushed out and the columns, still containing considerable quantities of oil, were divided into three parts corresponding to the green upper zone, the central zone containing the three pigments, and a lower zone containing no coloring matter.

The green pigment at the extreme top was very tightly adsorbed. An alcohol extract of the oil removes some of the green but on shaking the alcohol extract with petrol ether this pigment goes over into the upper layer. The position of the bands on the column, together with the solubility and

absorbtion maximum (686 mμ 610 mμ and 537 mμ in oil) indicate that the green materials are chlorophylls and derivatives.

The adsorbed carotenoids of the central zone were eluted with methanol without any attempt to separate them, and were then transferred to a mixture of chloroform and carbon disulphide and after concentrating and drying over sodium sulphate, were reghromatographed on alumina. Two deep yellow and one light yellow band appeared. Of these, one of the deep yellow bands remained tightly adsorbed upon the column but the other two passed through into the filtrate upon washing with more of the solvent.

The adsorbed pigment was eluted with methanol and then cromatagraphed from CS_2, and washed well with 5% alcoholic petrol ether. After elution, it was evaporated twice to dryness from CS_2, redissolved in 1-2 cc. of CS_2 and allowed to stand overnight in the ice box. After filtering, the solution, petrol ether (B.P. 35-60) was added and the mixture cooled. An estimated one mg. of pigment was obtained and redissolved in CS_2. The amount of petrol ether added for crystallization appears important because if it is not correct a resinous precipitate is obtained.

This pigment showed absorbtion maxima of 506, 476, 444 mμ in CS_2; remained completely in the alcohol layer upon distribution between petrol ether and 70% alcohol; and gave a green color with sulphuric acid. It was concluded to be fucoxanthin.

The filtrate from the column on which the fucoxanthin was adsorbed, was evaporated almost to dryness and redissolved in petrol ether, and chromatographed on a column of alumina. A deep yellow band remained on the column and the filtrate came through deep yellow.

The original oil filtrate containing the lower green and yellow bands with some of the light yellow oil which came through first was then saponified and extracted with petrol ether after careful addition of water. This solution was concentrated and adsorbed on a column of two parts magnesia and one part of filter aid. A diffuse yellow band formed on the column and was eluted with methanol. The filtrate from the column, which was light yellow in color, was not adsorbed upon alumina or magnesia from petrol ether, was soluble in petrol ether and not in alcohol, and so corresponded to the pigment of the oil filtrate first obtained. This pigment which is probably a hydrocarbon, must be the carotene of Bailey. Its concentration in the original filtrate, as determined by W. Chalmers with the tintometer, corresponds to approximately .06 mgs. carotene per 100 cc. of oil.

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