STUDIES ON THE METABOLISM OF β-CAROTENE AND RELATED SUBSTANCES.

A Thesis

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by

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SUMMARY

The subject matter of this thesis has been presented in three parts, each of which forms a complete and separate investigation, as follows:-

1. The Biosynthesis of ^{14}C-β-carotene.

The aim in this investigation was to establish a method for the production of radioactive β-carotene and vitamin A which could then be used in elucidating various problems connected with the metabolism of β-carotene and vitamin A and the mode of action of vitamin A in the animal body.

A satisfactory method was developed by allowing cultures of detached tomato leaves to photosynthesize $^{14}CO_2$ over periods of several days. The β-carotene was then isolated from the leaf tissue and the radioactivity determined. After making various modifications in the technique it was possible by this method to produce ^{14}C-β-carotene with a specific activity of 0.05 - 0.06 μc./mg.C. starting from $^{14}CO_2$ with a specific activity of 7 - 8 μc./mg.C. Although this activity represents

a very low count rate when measured by the end-window G-M counter, it was possible, using a gas-counter, to achieve high efficiency counting (27 c.p.m./μg. β-carotene) which would extend considerably its application in animal experiments.

It was discovered that the β-carotene was always associated with a colourless impurity which possessed radioactivity. The two substances could not be separated except by converting the β-carotene into a pure derivative (dehydro-β-carotene).

Other biological methods for the production of ^{14}C-β-carotene were investigated but these were unsatisfactory compared with the method described.

2. The Chemical Oxidation of β-carotene.

The aims were (i) to devise a method for the oxidation of ^{14}C-β-carotene to give radioactive retinene in good yield, and (ii) to study the possible analogy between the chemical oxidation and the biological conversion of β-carotene into vitamin A.

Satisfactory yields (10 - 15%) of retinene were produced by the H_2O_2/OsO_4 oxidation of β-carotene and the method was fairly reproducible.

The number of oxidation products detected was far greater than that obtained by previous workers. In addition to retinene,

β-apo-8'-, 10'- and 12'-carotenals were found to be major products. These were identified by spectroscopic evidence (by application of empirical relationship between absorption spectra and degree of conjugation) and by chemical tests.

A possible mechanism of the oxidation reaction is put forward on the basis of the results obtained. This is confirmed by theoretical considerations.

3. The Conversion of β-carotene into Vitamin A in vivo.

The hypothesis which is suggested is that the conversion of β-carotene in vivo involves first the fission of a terminal double bond, followed by a progressive oxidation from the open chain end until vitamin A aldehyde is formed. This would then be reduced to the alcohol and absorbed. This concept is favoured more than the central double bond hydrolytic fission hypothesis since (i) it explains a large body of previously established results, and (ii) it is in agreement with facts regarding the stability of the molecule obtained from the chemical oxidation results.

In testing the hypothesis, it was found, firstly, that the β-apo-carotenals are vitamin A precursors. Secondly, in

studying the intermediate stages of the metabolism of the β-apo-carotenals it was found that these can undergo β-oxidation. Thus it appeared that once the β-carotene molecule has been split, further oxidation probably proceeds progressively by a β-oxidation process. It was shown that the β-oxidation is probably inhibited when retinene is reached because this possesses a terminal methyl group in the β position with respect to the aldehyde group. The β-apo-carotenals possess methyl groups in the α and γ positions and thus are not sterically hindered towards β-oxidation. Confirmatory evidence for this mechanism is discussed.

An attempt to isolate β-apo-carotenals as intermediates in the conversion process was unsuccessful.

A mechanism of the conversion reaction is suggested.

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FOREWORD.

This thesis is a record of work carried out in the Department of Biochemistry, University of Liverpool, from September, 1952 to October, 1954. The primary aim of this work was to study methods for the production of isotopically-labelled β-carotene and its application in elucidating various problems connected with the metabolism of β-carotene and vitamin A in the animal body. This aim has been fulfilled at least to a partial extent and at the same time the work has yielded some interesting observations in related fields of study, such as carotenoid metabolism in the higher plants. Thus, although the investigations have demanded a presentation in three separate and distinct parts, it is felt that these are all unified by the common theme of the problem of carotenoid metabolism in living organisms.

I should like to express my gratitude to Professor R. A. Morton, F.R.S., for making this research possible by

placing the necessary facilities at my disposal and arranging generous financial assistance for which I thank the Agricultural Research Council.

I wish also to record my indebtedness to Dr. John Glover who has not only given me the benefit of his valuable experience but has been a constant source of help and encouragement.

Finally, I should like to thank Dr. T. W. Goodwin for much helpful advice and criticism, Mr. R. A. Creed for preparing the photographs, and Mrs. J. S. Lowe for typing the manuscript.

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CONTENTS

GENERAL INTRODUCTION

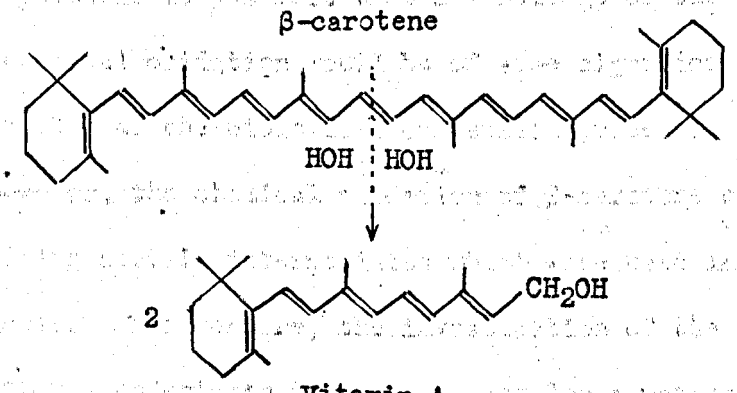
There are many problems connected with the biochemistry of the carotenoids and vitamin A which are still largely unsolved. Although the purely chemical problems of their structure and synthesis have been clearly elucidated, knowledge concerning their metabolism and the mode of action of vitamin A in the animal body is far from complete. Apart from the function of the latter in the visual process, which has been the subject of much elucidation in recent years, the other aspects of the vitamin A deficiency syndrome, viz., retarded growth, hyperkeratosis and xerophthalmia, have not yet been related to any precise biochemical mechanism in which vitamin A, or perhaps some derivative of vitamin A, participates. Similarly, comparatively little is known concerning the mechanism by which the carotenoid vitamin A precursors are transformed into vitamin in the animal body.

In view of these outstanding problems it was considered that if isotopically-labelled β-carotene and vitamin A were

available, these would provide new and valuable research tools which, in addition to augmenting existing research, would perhaps open up new methods of approach to the problems.

A study has, therefore, been made of possible methods by which isotopically labelled β-carotene or vitamin A could be synthesized.

The other major problem which has been the subject of detailed investigation is the question of the mechanism of the transformation of the carotenoid provitamins A into vitamin A in the animal body. This conversion is usually represented as a hydrolytic fission of the central double bond of the β-carotene molecule to form two molecules of vitamin A, thus:



Vitamin A.

Although there is no conclusive evidence for the occurrence of such a reaction mechanism <u>in vivo</u>, it is still favoured by many

workers. A large amount of the experimental evidence does, however, suggest that an alternative mechanism should be given serious consideration. This is that the provitamin A molecule may be split oxidatively and not hydrolytically and that the initial attack may occur, not at the central double bond, but at one of the terminal double bonds, but again there is no direct evidence for such a mechanism. At present, therefore, the problem of the conversion of provitamins A into vitamin A *in vivo* is confused and controversial and for this reason the present investigation was undertaken.

The problem of the biological degradation of β-carotene was approached initially by a study of its chemical oxidation. Since many chemical and biological reactions have basically similar patterns it was felt that a knowledge of the mechanism of the chemical oxidation would be of some significance in the consideration of the biological conversion process. At the same time, however, the chemical oxidation of β-carotene was the means of obtaining certain intermediates which were used in the animal experiments. Furthermore, the investigation of the chemical oxidation was undertaken in order to develop a method for obtaining isotopically labelled vitamin A aldehydes from labelled β-carotene in good yield, and in this way, establish a complete and convenient method for the production of isotopically labelled vitamin A.

In view of these important applications of the chemical oxidation of β-carotene, apart from its own intrinsic interest, it was considered justified in presenting it as a complete and separate study.

The subject matter of this thesis has been presented, therefore, in three separate parts, as follows:-

(1) The biosynthesis of ^{14}C -β-carotene.

(2) The chemical oxidation of β-carotene.

(3) The conversion of β-carotene into vitamin A *in vivo*.

CHAPTER I.

GENERAL EXPERIMENTAL METHODS

A. THE ISOLATION AND DETERMINATION OF CAROTENOIDS AND VITAMIN A.

The usual course of the procedure for the isolation and estimation of the carotenoids and vitamin A involves the following stages:-

(1) The preparation of the materials to be examined and the extraction of the lipid.

(2) Separation of the carotenoids and/or vitamin A by chromatographic resolution of the lipid.

(3) Estimation by spectrophotometric methods.

(4) The preparation of pure derivatives for radioactive assay.

1. The extraction of carotenoids and vitamin A.

(i) Extraction of lipid from plant leaves.

The literature abounds with references to the extraction and estimation of carotenoids in green leaf material and a comprehensive bibliography has been compiled by Gridgeman (1950). The following method for the extraction was, however, found to be rapid and efficient and was used throughout the investigation with little modification.

When a leaf sample was removed for analysis, excess moisture was removed by pressing the leaves gently between sheets of filter paper. The leaves (3 - 10 g. fresh weight) were then cut into small pieces, transferred to a beaker and boiling ethanol added; this kills the leaves by stopping enzymic process and also dehydrates the tissue. After about half-an-hour, the ethanol, now containing some dissolved lipid and other material, was decanted off. The residual leaf tissue was then transferred to a glass mortar (20 oz.) and extracted with peroxide-free ether (i.e., freshly distilled over reduced iron) containing 5 - 10% ethanol, the extraction being facilitated by breaking up the leaves by grinding with a glass pestle. The ethereal solution was then decanted off and the extraction repeated with a further quantity of ether-ethanol. Finally, the residue, now in particulate form, was moistened with acetone, ground with the pestle and extracted with ether.

The residual leaf powder was then transferred to a tared sintered (G.4) crucible, washed with ether, dried in an oven at 120° for 2 - 3 hours and then weighed.

The alcohol and ether extracts were combined and placed in a one-litre separating funnel. The extract was washed several times with distilled water to remove water-soluble substances and ethanol. The ether extract was then dried over anhydrous Na_2SO_4

in the cold. The Na_2SO_4 was filtered off on a sintered (G.4) Buchner funnel and the filtrate collected in a 500 ml. round-bottomed flask ("Quickfit"). The ether was removed by distillation under reduced pressure employing all glass "Quickfit" apparatus. The green lipid residue was then transferred to a tared 50 ml. conical flask and the remaining solvent evaporated off under nitrogen on a water-bath. The weight of the lipid was then determined.

(ii) Extraction of lipid from animal tissues.

The tissue (mainly intestine, liver or kidney of rats) was cut into small pieces and ground in a mortar with 4 - 5 times its weight of anhydrous Na_2SO_4. The mixture was then extracted with peroxide-free ether in a 400 ml. beaker with gentle heating on a water-bath. The ether solution was decanted off and the extraction repeated 3 - 4 times. The ether extracts were combined and filtered on a sintered (G.4) Buchner funnel. The extract was then reduced to dryness under reduced pressure and the residual lipid transferred to a tared 25 ml. conical flask. Residual solvent was then evaporated and the lipid weighed.

Intestinal contents, obtained by flushing out the intestine with water, were extracted by adding approximately 0.5 vol. of ethanol and the whole triturated in a mortar to break up any solid material. The mixture was then transferred to a separating funnel

and extracted with ether. The ether extract was then washed with water to remove the ethanol, dried over Na_2SO_4 and filtered. The ether extract was then treated as described for intestine, etc.

2. The separation of carotenoids and vitamin A by adsorption chromatography.

The development of the technique of adsorption chromatography has provided the biochemist with an extremely useful method for the separation of mixtures of compounds and in many cases it has completely superseded other less efficient and more tedious physical and chemical methods. In this connection, mention may be made of the great advances which occurred in carotenoid research due to the application of the technique. The method is therefore of considerable importance and it has been dealt with, in great detail, in numerous monographs (e.g., Strain, 1942; Zechmeister and Cholnoky, 1943). It is proposed, therefore, to discuss only briefly the principles of the method before describing the general methods used in the present work.

(i) Principles of method.

Essentially the method of adsorption chromatography consists in allowing a solution of the mixture of substances to be separated to pass through a vertical column of a suitable adsorbent.

The mixture will then separate into a series of bands, the relative position of each band on the column depending upon the adsorption affinity of the adsorbent for each constituent compound of the mixture. It is found that the degree of resolution can be increased by allowing quantities of the solvent to percolate through the column, or if necessary, by increasing the polarity of the solvent. This is termed the development of the chromatogram. Each separate zone can then be removed from the column (process of elution) by two main methods:-

(a) by extruding the column of absorbent and cutting it into the appropriate sections, or

(b) by development of the chromatogram with solvents of increasing polarity so that the bands are washed separately from the column ("running" chromatogram).

The latter method is usually adopted for quantitative work.

The chromatographic behaviour of the carotenoids and related substances is closely related to their chemical structures. In general it can be said that the more polar the molecule the more tightly will it be held on the column so that the number of polar and unsaturated groups in the molecule will determine to a large extent the position at which it is adsorbed, e.g., the

xanthophylls (containing -OH groups) are much more strongly adsorbed than carotenes. This fact is of importance when selecting an absorbent suitable for a particular separation. Also it is a useful guide when used in conjunction with spectroscopic properties, in trying to decide the structure of an unknown compound.

(ii) Experimental methods.

Materials and apparatus.

The absorbent which was used almost exclusively was Grade 0 activated alumina (Spence). It was found, however, that in many cases it was necessary to weaken the alumina slightly in order to achieve optimal separation. This was done by mixing the activated alumina with a certain quantity of deactivated alumina. The latter was prepared by stirring activated alumina with an excess of methanol, allowing it to stand for 1 - 2 hours, removing the solvent by vacuum filtration and drying overnight at 30 - 40° (Goodwin, 1951).

The solvent used was A.R. light petroleum (40 - 60°). As far as possible a stock of light petroleum from single batch was used for each set of experiments because of the possible variation in the small amounts of polar constituents present in different consignments.

The "polar" solvent used for chromatographic development and elution was diethyl ether. This was always dried with sodium and distilled over reduced iron immediately prior to use to remove peroxides.

The apparatus employed consisted simply of a long glass tube constricted at its lower end and fused to a short narrow tube which served as the outlet. The size of the column used depends on the amount of material to be separated. In the present work two main sizes were used - (a) internal diameter 1.5 cm. for weights of alumina up to 30 g., and (b) internal diameter 2.5 cm. for 50 - 100 g. alumina. When using the larger type of column it was often found advantageous to place centrally within the column a solid glass rod of diameter 0.9 cm. (the rod was made to fit the column centrally by fusing three small glass feet to each end). This artifice, originally used by Glover (1949) enabled better resolution to be obtained by eliminating to some extent the uneven flow which causes the head of an eluting bond to assume a paraboloid shape, an effect which becomes more pronounced with larger diameter tubes.

Procedure.

The constricted end of the chromatography tube was plugged lightly with a small piece of non-absorbent cotton-wool which was

then moistened with the solvent to remove trapped air. Suitable proportions of activated and deactivated alumina were weighed out and well mixed. Excess light petroleum was then added and the mixture stirred to remove air bubbles. The slurry of alumina was then carefully poured into the tube. If any cracks or air bubbles developed in the column of absorbent then it was extruded and the preparation repeated. When the column had been prepared, approximately 100 ml. of the solvent was allowed to run through to remove any foreign fat-soluble material present in the alumina. At no time during the preparation of the column or in the subsequent separation was the column allowed to run dry of solvent.

The material under examination was dissolved in a minimum volume (usually 5 - 10 ml.) of light petroleum and the solution carefully poured on to the column. When all the mixture had been absorbed, development was commenced by allowing light petroleum to percolate through the column until the mixture began to separate into well-defined bands. Further development was then continued by progressively increasing the polarity of the solvent by the addition of small quantities of ether. When optimal resolution was attained the bands were eluted by running through suitable volumes of solvent of the necessary degree of polarity. In some cases a certain degree of overlapping and intermingling of the

zones occurred and it was necessary then to collect intermediate fractions separately and to subject these, and sometimes the main fractions, to rechromatography in order to obtain the pure substance. For the elution of very strongly adsorbed substances, e.g., the xanthophylls, it is found that light petroleum containing more polar solvents, such as ethanol or acetone, is required.

The polyene hydrocarbons are normally susceptible to the action of light when they readily undergo isomerization and oxidation. These effects are greatly enhanced when the substance in its adsorbed state is exposed to light. It was necessary, therefore, to protect the chromatogram from strong sources of light. This was usually accomplished by wrapping a piece of black paper round the chromatography tube.

When all the zones had been eluted from the chromatogram, each fraction was distilled in vacuo to remove the solvents and the residue made up to a suitable volume with light petroleum and stored in the dark at 0° until examined spectrophotometrically.

3. The spectrophotometric estimation of carotenoids and vitamin A.

The yellow to red colours characteristic of the carotenoids are due to a selective absorption of visible radiation, mainly in the 400 - 500 mμ region. This selective absorption of light is

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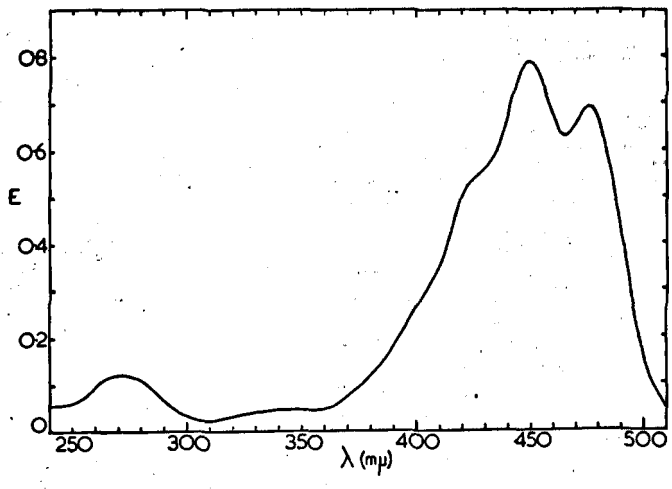


Fig. 1.

Absorption Spectrum of all-trans-β-carotene (in light petroleum).

is due to the presence within the molecules of the conjugated double bond system and of certain other groups (e.g., >CO, -CHO) which together constitute the chromophoric system. Each carotenoid will therefore possess a characteristic absorption spectrum according to its constitution. It is possible from theoretical considerations to derive certain empirical rules and relationships between the spectral properties of a substance and its chemical structure which are of great value in the identification of unknown substances. This aspect, however, will be dealt with more fully later.

The absorption spectra of the carotenoids usually consist of two or three maxima in the visible region (see Fig. 1. for absorption spectrum of all-trans β-carotene) while vitamin A, because of its smaller conjugated double bond system, exhibits single selective absorption in the near U.V. region. The technique of absorption spectrophotometry for the identification and assay of these substances consists in the determination of the positions and intensities of the maxima.

(i) General principles.

When monochromatic light passes through a homogenous medium the light transmitted by each succeeding layer of given thickness (dl) is a constant fraction of the intensity of light (I) entering

that layer. This is Lambert's law which can be expressed more fundamentally as the differential equation,

$$\pm \frac{dI}{dl} = \alpha I$$

Integrating between the limits $l = o$ and $l = x$, the expressions,

$$\log_e \frac{Io}{Ix} = \alpha x \quad \text{or} \quad \log_{10} \frac{Io}{Ix} = Kx$$

are obtained.

The absorption due to a solution varies with the concentration as well as with the thickness of the solution and Beer showed that the absorption is proportional to the number of molecules in the light path, i.e.:-

$$\alpha = \beta c$$

where β is the absorption coefficient per unit concentration c. Lambert's and Beer's laws can therefore be combined in the single equation:-

$$\log_e \frac{Io}{Ix} = \beta cx \quad \text{or} \quad \log_{10} \frac{Io}{Ix} = Kcx = E$$

Where β is the absorption coefficient and K the extinction coefficient. When the concentration is expressed in g. mol./l and x in cm. the "extinction" or "density" E is given by

$$E = \epsilon cx$$

where ϵ is the molecular extinction coefficient. When the

natural logarithmic base is employed the analogous constant is the molecular absorption coefficient ϵ^1. In practical absorption spectrophotometry the usual constant employed is the extinction of a 1% (w/v) solution of the substance measured in a 1 cm. cell ($E^{1\%}_{1cm.}$).

$$E^{1\%}_{1cm.} = \frac{1}{cx} \log 10 \frac{Io}{Ix}$$

where c is the concentration expressed as a percentage.

The following proportionality factors are useful for the interconversion of constants:-

$$\epsilon = E^{1\%}_{1cm.} \times M.W/10; \qquad \epsilon^1 = 2.3\,\epsilon \quad ; \qquad E = 0.4343\,\beta cx.$$

(ii) Apparatus and methods.

The instruments used were the photoelectric spectrophotometers, Beckman Model D.U. and Unicam, Model S.P. 500. Both these instruments are of fundamentally the same design and consist essentially of a light source, monochromater system and photoelectric apparatus. Photoelectric instruments of this type are extremely accurate and sensitive and determinations can be made with rapidity. For these reasons they have completely replaced the older types of intrument which depend on the more laborious and less accurate photographic or visual measurements.

The substance for spectrophotometric assay was prepared by dissolving it in the solvent and making up to a suitable volume, a procedure which often necessitated a further dilution of a portion of the original solution in order to obtain a suitable extinction reading. For accurate assay extinction values in the range 0.2 - 0.8 were aimed at and it was found that with practice it was possible to assess fairly accurately the degree of dilution required to give a reading within this range. In the case of U.V. absorbing substances, however, a trial and error method had to be used.

When a large number of analyses were carried out (e.g., carotenoid estimation in green leaves) it was not always practical to measure the complete spectrum of each fraction but to measure only the extinction at the main maximum. This was true particularly for β-carotene which can be identified readily by its characteristic chromatographic adsorption properties and it was only necessary to make a few extinction measurements at wavelengths in the region of the main maximum in order to locate the highest extinction value. This was sometimes found to occur at a wavelength slightly below the absorption maximum for the pure all-trans substance because of the presence of small amounts of stereoisomers. The error introduced by basing the calculation on the $E^{1\%}_{1cm.}$ value for the

all-trans substance is not, however, of great significance in this type of determination.

For the assay of vitamin A, certain correction procedures were employed since the U.V. spectrum of vitamin A in all natural extracts is overlaid with absorption due to non-vitamin A substances. Correction for this irrelevant absorption was made by employing the procedure described by Cama et al. (1951).

The data employed for the calculation of the results from the spectral measurements were as follows:-

$E^{1\%}_{1cm.}$ 328 mμ. of vitamin A alcohol in 40-60° light petroleum = 1830 (Cama et al. 1951)

$E^{1\%}_{1cm.}$ 369.5 mμ. of vitamin A aldehyde " " " " = 1685 (Ball et al. 1948)

$E^{1\%}_{1cm.}$ 449.5 mμ. of β-carotene " " " " = 2550 (Rosen, 1948)

(iii) Estimation by the Carr-Price reaction.

In addition to estimation by the U.V. absorption spectrum, vitamin A (and retinene) was also estimated by means of the blue colour it gives with $SbCl_3$. The reagent consists of a saturated solution of anhydrous $SbCl_3$ in $CHCl_3$ (ethanol-free). Two drops of acetic anhydride were added to 0.4 ml. - 0.5 ml. of a $CHCl_3$ solution of vitamin A. 4 ml. of the $SbCl_3$ reagent were then added quickly, mixed and the mixture transferred rapidly to the cell of a

Hilger-Nutting spectrophotometer and the extinction at the absorption maximum determined visually. The blue colour produced with vitamin A fades rather quickly so that it was usually necessary to carry out two or three determinations to obtain an accurate result.

For the determination of the complete absorption spectrum or the accurate location of the absorption maximum of a blue colour (e.g., in investigating the properties of the β-apo-carotenals) it was necessary to employ the following technique. An approximate location of the maximum was obtained, if possible, by a visual examination of the spectrum, using the Hilger-Nutting instrument. Extinction values were then obtained at various wavelengths within the region of the maximum by means of the Beckman spectrophotometer. The spectrophotometer was adjusted (wavelength, dark current, slit width) after placing the solution in the cell. 3 ml. of the reagent was then added quickly, the cell holder placed in position and the extinction read, at an average time of 15 seconds after mixing. The whole procedure was then repeated at different wavelengths, using a fresh solution each time.

Data used for the calculation of the results from the spectral measurements were as follows:-

$E^{1\%}_{1cm.}$ 620 mμ. for vitamin A alcohol = 5070 (Cama et al. 1951)

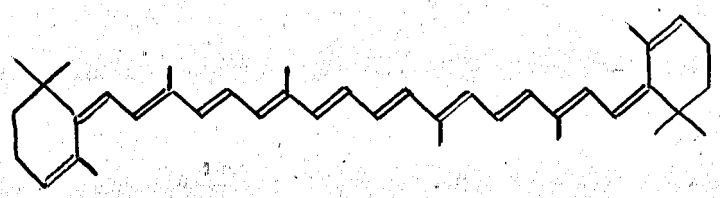
$E^{1\%}_{1cm.}$ 664 mμ. for vitamin A aldehyde = 3400 (Ball et al. 1948)

4. The preparation of pure derivatives of β-carotene and vitamin A aldehyde.

For the accurate determination of the radioactivity of a substance it is essential that it should be free of any impurities possessing activity. It was found (vide infra) that β-carotene derived from plant sources was always associated with a colourless substance which could not be detected by spectroscopic means. This contaminating substance possessed radioactivity and it could not be separated from the β-carotene by means of chromatography. It was necessary, therefore, to devise methods for preparing derivatives of β-carotene or its degradation products which could be readily separated from the impurity by means of filtration or chromatography. Two main methods were used: (i) the formation of the insoluble β-carotene iodide which could be filtered and washed free of impurity, followed by its conversion into dehydro-β-carotene for spectroscopic estimation and radioactive assay, and (ii) the preparation of retinene 2,4 dinitrophenylhydrazone followed by its isolation by chromatography.

(i) The formation of dehydro-β-carotene.

Dehydro-β-carotene (isocarotene) is formed by the decomposition of the iodine addition products of β-carotene with thiosulphate, acetone, mercury, or finely divided silver (Kuhn and Lederer, 1932). According to Karrer and Schwab (1940) it has the constitution:-



It was prepared by a modified method of Kuhn and Lederer (1932) as follows:-

500 mg. β-carotene was dissolved in 500 ml. light petroleum and the solution cooled to -10° in a freezing mixture (ice-salt). 400 mg. iodine in 250 ml. light petroleum at -10° was then run in over one minute with vigorous agitation and the shaking or stirring was continued for 2 minutes. The dark coloured iodine adduct which had formed was then filtered rapidly on sintered glass (G.4) funnel and the precipitate washed several times with light petroleum, previously cooled to -10°. The β-carotene-iodide was then dissolved in 1.5 l. acetone and the solution left to stand

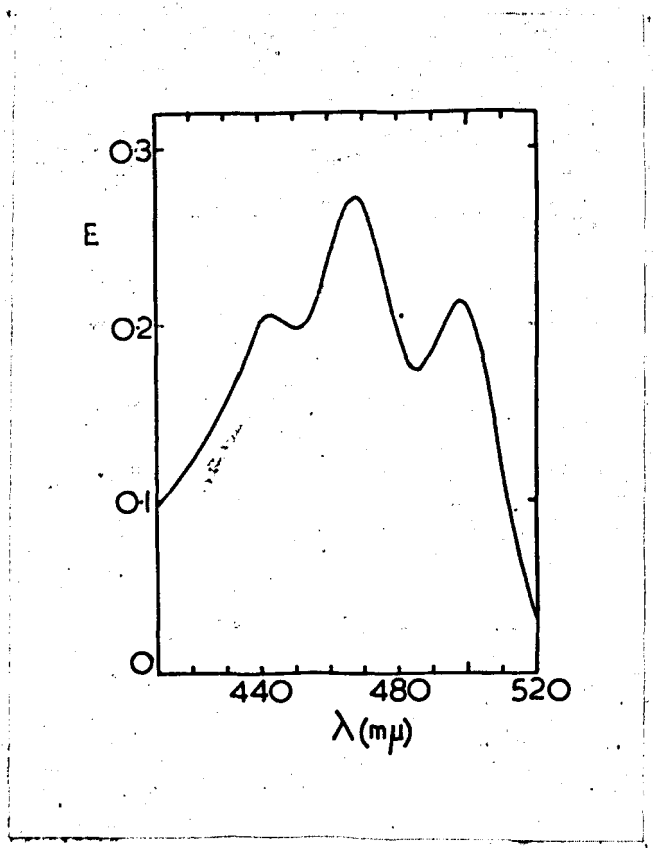


Fig. 2.

Absorption Spectrum of dehydro-β-carotene (in light petroleum).

at room temperature for about 15 minutes. 200 ml. of a 2.5% solution of sodium thiosulphate was then added and the orange-coloured solution lightened, indicating complete decomposition of the iodide. The pigment was extracted with light petroleum and washed with water. The extract was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo. The dehydro-β-carotene was then purified by means of chromatography on alumina (20 - 25% deactivated). The resulting chromatogram is described in Table 1.

Table 1.

Separation of Dehydro-β-carotene on a mixture of 3:1 (W/W) activated and deactivated alumina.

(Zones numbered in order of increasing absorptive power)

Zone No.	Description	Eluant	Absorption maxima (mμ.) (in light petroleum)
5.	narrow orange band.	-	-
4.	pale diffuse pink.	-	-
3.	pale diffuse pink.	-	-
2.	intense red band.	20% ether.	443, 468, 498
1.	diffuse pale reddish orange.	10-12%	442, 464, 492

The main red band was eluted and its spectrum in light petroleum determined (Fig. 2.) Absorption peaks occurred at 443 and 498 mμ. with the main maximum at 468 mμ. These values

agree well with those obtained in hexane solution by Zechmeister and Wallcave (1953) but are hypsochromically displaced by about 8 mμ. from those given by Karrer and Jucker (1950) for a solution in petroleum.

Quantitative extinction measurements of dehydro-β-carotene were made by Hausser and Smakula (1934). Their data, however, refer to solutions in carbon disulphide but by interpolation with data given for β-carotene in hexane solution the $E^{1\%}_{1cm.}$ at the main maximum was computed as 2950. This is in agreement with values calculated from molecular extinction coefficients in hexane solution given by Zechmeister and Wallcave (1953) for dehydro-β-carotene and its isomers, as follows:-

All-trans compound	$E^{1\%}_{1cm.}$	at	471	=	3108.
Neo A	"	"	"	467	=	2977.
Neo D	"	"	"	464	=	2921

As a reasonable approximation, therefore, the $E^{1\%}_{1cm.}$ of dehydro-β-carotene in light petroleum has been taken as 3000.

(ii) The preparation of retinene 2,4 dinitrophenylhydrazone.

An ethanolic solution of 2,4 dinitrophenylhydrazine was added to an equivalent amount of retinene in ethanol. A few drops of concentrated HCl were then added to the mixture, the colour of

which darkened immediately, indicating the formation of retinene 2,4 dinitrophenylhydrazone. The mixture was diluted with water, extracted with light petroleum and the extract washed with water. Pure retinene 2,4 dinitrophenylhydrazone was then obtained by adsorption chromatography on paper, using n-heptane as the developing solvent (vide infra). The spectrum of the pure derivative (in ethanol) shows bands at 442 and 260 mμ. with $E^{1\%}_{1cm.}$ 1166 and 346, respectively (Ball et al. 1948).

B. RADIOACTIVE ISOTOPE TECHNIQUES.

1. Fundamental Principles of Radioactivity.

The nuclei of atoms are built up by combinations of protons and neutrons, the latter being the uncharged analogue of the proton. Since the nuclear charge determines the number of extranuclear electrons which in turn determines the chemistry of the atom, no change occurs in the chemical behaviour of the atom when neutrons are added to the atomic nucleus. Consequently, there are nuclei and hence atoms, which vary in nuclear mass but not in chemical nature. These are called isotopes. Non-radioactive isotopes represent stable combinations of neutrons and protons; e.g., nucleus of carbon must contain in addition to six protons no more than six or seven neutrons, corresponding to the two stable carbon nuclei, $^{12}_{6}C$ and $^{13}_{6}C$. The combination of eight neutrons with six protons causes the unstable configuration $^{14}_{6}C$ existing in carbon-14. The corresponding stable combination is one consisting of seven protons and seven neutrons, viz., $^{14}_{7}N$. $^{14}_{6}C$ is, therefore, transformed into $^{14}_{7}N$ when a neutron is changed to a proton. This process involves the emission of a negative β-particle.

$$n \longrightarrow p + \beta^-$$

In some isotopes the redistribution of particles for stability may occur by the transformation of a proton into a neutron with the

concomitant emission of a positive β particle (positron).

$$p \longrightarrow n + \beta^+$$

This occurs when $^{11}_{6}C$ is converted to the non-radioactive isotope of boron, $^{11}_{5}B$. In some cases the nucleus may not be able to supply the necessary energy for the ejection of a positron in which case the nucleus reduces its positive charge by capturing an electron from the inner electron shell (K-shell). This process, called K-electron capture, involves also X-radiation because of the necessary redistribution of outer electrons.

In the case of certain heavy atoms (nuclear charge $Z > 82$) the particle redistribution of the radioisotopes may involve α-particle emission. In all these forms of particle emission by radioactive atoms, viz., β^- emission, β^+ emission or K-electron capture, and α-particle emission, there may also be an emission of electromagnetic radiation as γ-rays or X-rays.

Radioactivity is a chance phenomenon to which statistical principles have to be applied. Experimentally it can be shown that the rate of decay of a radioactive isotope obeys an exponential law, the rate of decay at any instant being proportional to the number (N) of unstable nuclei present, as follows:-

$$dN/dt = -\lambda N \text{----------} (1)$$

where λ is the distintegration constant.

Integration of this equation between the limits N = o and N = t

gives:-

$$\log_e N/No = -\lambda t \quad \text{----------} \quad (2)$$

which can be written:-

$$Nt = No\, e^{-\lambda t} \quad \text{----------} \quad (3)$$

The time required for the radioactivity of a sample to fall to one-half its original intensity is known as the "half-life". Substituting $Nt/No = \frac{1}{2}$ in equation (2)

$$\log_e \tfrac{1}{2} = -\lambda T_{\frac{1}{2}} \quad \text{----------} \quad (4)$$

or

$$\log_e 2 = \lambda T_{\frac{1}{2}} \quad \text{----------} \quad (5)$$

Substituting $2.303 \log_{10} 2$ for $\log_e 2$, equation (5) becomes:

$$T_{\frac{1}{2}} = 0.693/\lambda$$

For certain short-lived isotopes it is essential that the half-life should be known accurately since the experiment may be performed over a period which is commensurate with this time.

2. Production and properties of Carbon-14.

The radioisotope which has been used in the present investigation is carbon-14 (^{14}C). The production of this isotope will be outlined briefly, followed by a description of its chief properties. For more detailed information reference should be made to the standard texts (e.g., Calvin et al., 1949; Kamen, 1951; Siri, 1949).

^{14}C was first discovered by Ruben and Kamen (1940, 1941),

when carbon, enriched with ^{13}C, was bombarded with deuterons in the cyclotron

$$^{13}C + {}^{2}H \longrightarrow {}^{14}C + {}^{1}H.$$

The development of nuclear reactors in connection with atomic energy research has now made ^{14}C production possible on a large-scale by the neutron bombardment of nitrogenous substances such as ammonium nitrate, beryllium nitride or calcium nitrate.

$$^{14}N + n \longrightarrow {}^{14}C + {}^{1}H.$$

Numerous determinations of the half-life of ^{14}C have been made; values reported are 5,300 ± 800, 5,100 ± 200 and 4,700 ± 500 years (see Calvin et al., 1949). Because of its long life the value of ^{14}C as a tracer was recognised immediately, but because of the low energy of the β-emission its effective use was dependent on the development of sensitive and quantitative measurement techniques. ^{14}C emits β-particles with a maximum energy of about 0.15 Mev. This corresponds to a range in aluminium of 27.9 ± 0.3 mg./cm.^{2} (see Calvin et al., loc. cit.)

3. The measurement of Radioactivity.

The quantitative determination of radioactivity is based on the ionization effects produced when the radiation emitted passes through a gaseous medium. These effects are due to the interaction

between the moving charged particle and the atoms of the gas molecules which results in the formation of ion pairs; i.e., a heavy positive ion and an electron. If a potential difference is applied to the system the ion clouds will move towards the electrodes, and depending on the applied voltage, will be collected before recombination can occur. At higher potentials avalanche processes occur which result in a multiplication of the ionization produced. When this multiplication factor is constant, the ionization collected is a direct function of that produced and the counter is then operating in the "proportional region". At higher potentials the multiplication approaches a saturation where all ionization pulses, regardless of initial strength, are multiplied to a constant final size. This is known as the Geiger region.

The most commonly used instruments for the radioactive assay of β-emitting isotopes (e.g., ^{14}C) are the ionization chamber and the Geiger-Müller counter, although proportional counters are now coming into prominence since they have many advantages compared with the former types. The counters used in the present investigation were of the Geiger-Müller type.

The Geiger-Müller Counter.

(a) Construction.

The Geiger-Müller counter consists essentially of a cylindrical

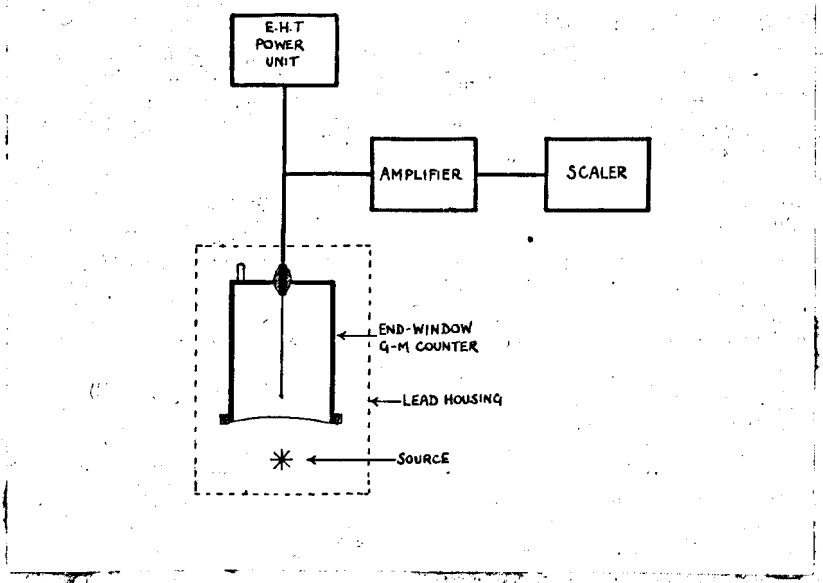


Fig. 3.

End-window G.M. Counter and Component Equipment.

Fig. 4.

Gas Counter; A: glass thimble; B: graphite cathode; C: connector to vacuum line.

cathode and a coaxial wire anode, usually made of iron or tungsten. The inter electrode space is filled with a pure gas or a mixture of gases and the whole apparatus is enclosed in a suitable envelope. The two main types of counter which have been used in the present work are (a) the end-window G-M counter, in which one end of the counter tube is closed by a thin mica window, through which the radiations enter (Fig. 3.) and (b) the gas counter, in which the sample for assay in a gaseous form is introduced directly into the counter (Fig. 4.) The end-window counter used was a self-quenching type in which the gaseous medium consists of a mixture of a simple gas with small percentages of "quenching" admixtures which are usually organic substances or halogens. The gas counter, on the other hand, with a CO_2 filling, is not self-quenching and requires an external quench circuit (vide infra).

(b) Operation.

When an ionizing particle enters the G-M tube a few ion pairs are produced in the gas space. Under the action of the applied voltage, electrons are accelerated towards the wire anode while the positive ions move towards the cylindrical cathode, but the movement of the latter is less rapid because of their heavier mass. The movement of the positive ions can, therefore, be neglected. As the electrons approach the anode their acceleration

is increased because of the increased potential gradient and they can thus gain sufficient energy to cause further ionization by collision with the gas molecules. This process continues so that eventually a great number of electrons are accelerated towards the anode (electron avalanche) and thus giving rise to a drop in potential at the anode (negative pulse). In the meantime a stationary positive space charge is created near the cathode by the slower moving positive ions. This causes the discharge to cease (usual duration = 10^{-7} sec.) by decreasing the effective voltage gradient at the anode which usually results in the formation of a small positive pulse at the anode immediately following the negative pulse. Before the counter can initiate another pulse the positive ions must diffuse sufficiently towards the cathode so that the original potential gradient is restored. The time taken for this to occur is known as the "dead" time of the counter (10^{-3} - 10^{-4} sec.).

The dead time can be further prolonged by spurious discharges produced by protons and electrons which are formed when positive ions strike the cathode. This effect is obviated by mixing the gas (e.g., argon) in the space with small quantities of an organic vapour (e.g., ethanol), or a halogen, which acts as quenching gases. The positive argon ions moving towards the cathode collide with

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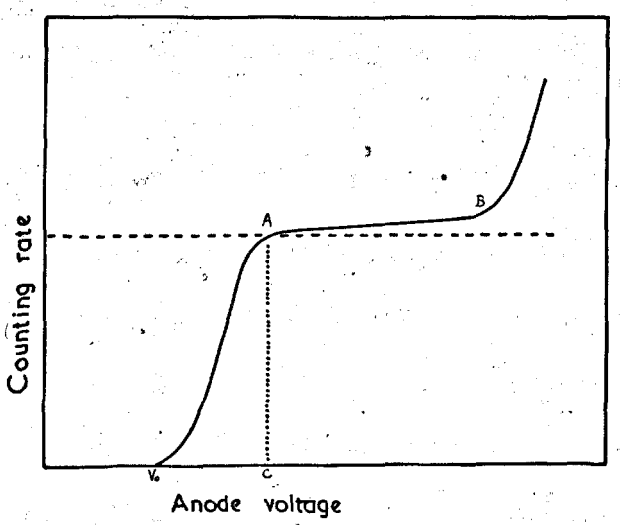


Fig. 5.

Counting rate/voltage characteristic of G-M counter.

alcohol molecules and the charge is transferred so that the positive ion cloud which arrives at the cathode is almost entirely composed of positive alcohol ions. These draw electrons from the cathode to form neutral excited molecules which predissociate (a property characteristic of excited polyatomic molecular or halogen atoms) before they can strike the cathode, and thus preventing the liberation of secondary electrons.

(c) Characteristics.

The performance of a G-M counter is determined by its threshold voltage, the length and slope of its "plateau", its efficiency, pulse characteristics, maximum counting rate, temperature coefficient and useful life.

The ionization-voltage characteristics of the G-M tube are shown in Fig. 5. At low voltages the charge collected during each pulse is too small to actuate the recording apparatus. As the voltage is increased a value is reached at which the charge collected is just enough to be detected. This is the starting or threshold potential. Further voltage increases cause larger pulses to be formed until a voltage (A) is reached when practically all the pulses are detected. The counting rate then remains constant even when the voltage is further increased. This voltage region (AB) is called the plateau and the G-M tube is always

operated in this region (about 50 - 100 v. beyond the threshold) since any slight variation of the voltage supply will then have a negligible effect on the recorded counting rate. At higher voltages the count rate increases rapidly and eventually a continuous discharge occurs. Most end-window counters have a plateau slope of 0.05% per volt and operate at working voltages between 300 and 1200 volts. A variation of 1% in voltage at an operating voltage of 1000 volts will therefore cause a change in counting rate of only 0.5%.

For high accuracy a knowledge of the temperature coefficient of the tube is important. This usually varies from a 0.2 to 5 volts/°C. shift in threshold voltage.

The maximum count rate of a G-M tube varies from 20,000 c.p.m. for alcohol-quenched tubes to 60,000 c.p.m. for halogen-quenched tubes.

The effective life of the counter also depends on the type of quench substance used; 10^8 - 10^9 counts for organic vapour-quenched tubes and unlimited for the halogen type.

(d) Component Equipment.

The component parts of the Geiger-Müller counting system are shown schematically in Fig. 3. They consist of:-

(a) the G-M counter itself, which produces a pulse for each particle which enters its sensitive volume;

(b) a stabilized power unit to supply the E.H.T. required to operate the counter;

(c) an amplifier to amplify the pulses produced by the counter to the necessary level required to operate the scaler;

(d) the scaler and register which registers and totalizes the pulses received;

(e) a source-mounting to ensure that the radioactive sample preserves a standard geometry with respect to the counter; and

(f) a lead housing for the counter, source mounting and the source to be assayed to ensure that the background counts are reduced to a minimum by screening extraneous radiation (e.g., cosmic rays).

4. Method of Radioactive Assay using the End-window Counter.

(i) Preparation of samples for assay.

(a) ^{14}C -β-carotene.

30 - 50 μg. of the ^{14}C β-carotene or dehydro-β-carotene contained in a 25 ml. conical flask were transferred quantitatively to the counting dish (area = 2.8 sq. cm.) using chloroform as the solvent. The $CHCl_3$ was then evaporated slowly under the heat

from an infra-red lamp while the planchet was carefully manipulated to ensure the formation of an evenly plated film. When dry, the sample was ready for counting. Because of the comparatively low specific activity of the ^{14}C -β-carotene, it was counted at the position of maximum geometry (i.e., shelf 1. of the source mounting).

(b) $\underline{^{14}CO_2}$.

$^{14}CO_2$ was assayed with the end-window counter by converting it to barium carbonate which can then be plated on a planchet and counted.

The $^{14}CO_2$ was absorbed in 5% NaOH (vide infra) and barium carbonate precipitated by a method similar to that used by Dauben et al. (1947) as follows:

An amount of ammonium chloride (20% solution) equivalent to the alkali used for the absorption of the $^{14}CO_2$ was added to the solution in the absorbing vessel. The mixture was then transferred to three 25 ml. bottles and 3 ml. of a10% solution of barium chloride added to each. The bottles were stoppered with rubber bungs and the precipitate centrifuged down. The supernatant was then tested with a drop of the $BaCl_2$ solution to ensure that precipitation was complete. After the precipitates had been washed three times with CO_2-free water and bulked together in one bottle, ethanol was added to form a slurry of $BaCO_3$. This was allowed to stand for a few

seconds to allow coarse particles to settle and then a small portion was pipetted on to a tared counting dish. The dish was placed under an infra-red lamp and the alcohol allowed to evaporate slowly to form a uniform film. When dry the dish was weighed and the radioactivity determined, correction for self-absorption being calculated by the method to be described. The remainder of the $BaCO_3$ precipitate was transferred to a tared sintered glass (G.4) crucible, washed with ether, dried at 120° and weighed.

(ii) Counting method.

The counting apparatus used consisted of a thin end-window (less than 2.5 mg./cm.^{2}) halogen-quenched G-M tube operating at 700 volts (20th Century Electronics Ltd.), a preamplifier, and a Type 200 scaling unit.

After setting up the equipment (see Taylor, 1951) the counter efficiency was checked daily by placing a standard radioactive source (U_3O_8 and $Ba^{14}CO_3$) in the lead-castle mounting. The background count was then determined by counting for a period of not less than half-an-hour. The background counting rate is variable and should, therefore, be determined immediately prior to the assay. The count rate of the radioactive sample is then determined over a suitable period, depending on the degree of activity of the substance. For weak samples, the activity was determined at the position of

maximum geometry (i.e., shelf 1, geometry 1.6%), while samples of higher activity were counted on shelf 2. (geometry 1%). Whenever possible duplicate assays were made.

Before the absolute specific activity of the sample can be calculated from the count rate certain corrections have to be applied.

(iii) Correction procedures.

(a) Coincidence correction.

If the sample has a very high activity, two or more β-particles may enter the G-M tube while it is in the conducting state and hence would not be recorded. To determine this factor, the sample must be diluted before assay and it was assumed that at low counting rates, coincidence is negligible.

(b) Background correction.

The net activity of the sample was calculated by subtracting the background count from the total count rate of the sample.

The statistical error of a given number of counts is given by the relation:

$$E = \sqrt{(N_s + N_b) + N_b}$$

where N_s and N_b are the counts over the same period of the sample and background, respectively. The count rate of the sample will then be given by:

$$N = N_s \pm E.$$

(c) Geometry Factor.

The relative position of the sample with respect to the window has to be taken into account in comparing the activity with a standard preparation and in calculating the absolute specific activity.

(d) Self-absorption correction.

Due to the low energy of the β-radiation of ^{14}C, an absorption of the radiation occurs in the substance of the source. For very thin layers of the sample (e.g., in the assay of ^{14}C -β-carotene) the self-absorption is negligible but for thick-layered samples the self-absorption may be appreciable and the suitable correction has to be made. This was the case in the assay of $Ba^{14}CO_3$ in which the percentage of carbon is small which necessitates the assay of comparatively large amounts of the material and thus producing thick layers. To determine the self-absorption, the sample thickness (mg./cm.^{2}) was calculated and the necessary correction factor found by interpolation on the standard self-absorption correction curve. (Yankwich et al., 1947).

5. Method of Radioactive assay using the Gas Counter.

(i) Principle.

In this method the material to be assayed in the form of $^{14}CO_2$ is introduced directly into the sensitive volume of the G-M counter. By means of this technique of using the sample as an integral part of the counter, geometric efficiencies greater than 50% can be obtained. Consequently the method is of great utility for the assay of weak radioactive samples.

CO_2 alone is a poor counting gas but it was found (Brown and Miller, 1947) that the addition of small quantities of carbon disulphide (2 cm. pressure with CO_2 pressures between 10 and 60 cm.) produced a good plateau. CS_2 acts as a charge transfer gas and tends to eliminate secondary electron emission caused by CO_2 ions striking the cathode. There is not, however, an efficient molecular dissociation of CS_2 ions at the cathode surface and consequently there is no quenching action. It is necessary, therefore, to employ an external electronic quenching circuit. The types of circuit most commonly employed are those devised by Neher and Harper (1936) and Neher and Pickering (1938). These function by terminating the discharge at the end of each pulse by lowering the potential gradient across the tube. Recently Broda and Sverak (1954) have claimed that good counting characteristics can be obtained by employing $^{14}CO_2$ gas pressures up to 40 cm. without

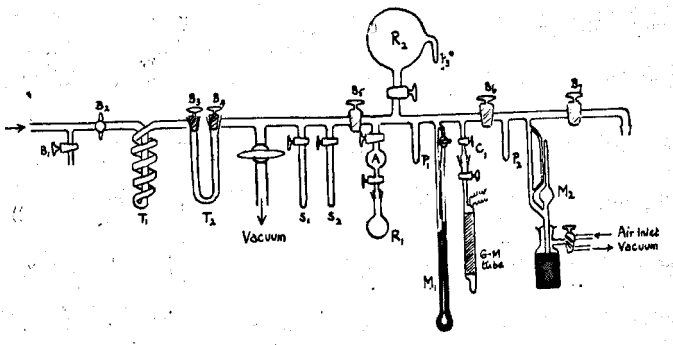


Fig. 6.

High-vacuum System for filling Gas-counter.

the admixture of CS_2. Also, Bradley et al. (1954) have assayed pure $^{14}CO_2$ at 20 cm. pressure by counting in the proportional region.

In the present work low CO_2 pressures (1 - 4 cm.) with CS_2 pressures of 1 - 2 cm. were used mainly. In this way good counting characteristics were obtained at lower operating voltages (see Eidenoff, 1950).

(ii) Method.

The radioactive sample was combusted by the wet combustion method (Evans and Huston, 1952). The CO_2 evolved was then absorbed directly in CO_2-free 0.1 N. NaOH. (In order to obtain an adequate CO_2 pressure in the G-M tube it was sometimes necessary to add a certain amount of inactive carbonate as $NaHCO_3$). The tube containing the alkali was then fitted with a dropping funnel and connected to the high-vacuum system (Fig. 6.) and the apparatus evacuated while the tube was frozen in liquid nitrogen. The tap B2 was then closed and CO_2 generated by rapid dropwise addition of 75%lactic acid. The moist CO_2 was then transferred to trap T_1 with liquid nitrogen and then to trap T_2 while the trap T_1 was surrounded with ethanol-solid CO_2 to retain water. The CO_2 was then transferred to the pip P_1, taps B_5 and B_6 closed and the pressure of the gas measured by means of the manometer M_1.

Meanwhile the counter tube (30 ml. capacity) had been evacuated and filled with 1 - 2 cm. CS_2 from R1. The G-M tube was then filled with CO_2 to a pressure of 1 - 4 cm.

The count rate was then determined by connecting the G-M tube (screened with lead bricks) to a Neher-Harper quench circuit, amplifier and scaling unit. A 6C6 valve was used in the Neher-Harper circuit with a grid resistance of 6 megohms. 100 - 200 volt. plateaux were usually obtained with the operating voltage in the region 3000 - 3200 volts.

The background was determined by filling the tube with equivalent pressures of inert CO_2 and CS_2.

The efficiency of the gas counter was calculated by comparison with the end-window counter; it was found to be 60%.

P A R T I.

THE BIOSYNTHESIS OF ^{14}C -β-CAROTENE

CHAPTER II.

I N T R O D U C T I O N

Up till now, there have been no published reports of methods for the production of isotopically labelled β-carotene on a sufficiently large scale and with the necessary degree of specific activity to make it suitable for use in animal experiments for the investigation of carotene and vitamin A metabolism. There is available, however, a commercial preparation of ^{14}C -β-carotene derived from algae of specific activity 2 μc/mg. dry weight, which had been allowed to photosynthesize $^{14}CO_2$ (Nuclear Instrument and Chemical Corporation, Chicago, U.S.A.).

Small amounts of ^{14}C -β-carotene have been produced in experiments designed to investigate carotenogenesis in micro-organisms. Grob et al. (1951) isolated ^{14}C -β-carotene from

Phycomyces blakesleeanus which had been grown on a medium containing ^{14}C -acetate. Using 2- ^{14}C -acetate there was a 25% incorporation in the β-carotene but when 1- ^{14}C -acetate was used, the incorporation was 50%. In some similar experiments by Glover et al. (1951) ^{14}C -β-carotene with a specific activity of 0.31 μc/mg. carbon was obtained. The incorporation of 2- ^{14}C acetate was, however, only 13%, and the results suggested that acetate is not a specific carotene precursor since most of the labelled acetate was dissimilated and ^{14}C was found in the lipids, protein, carbohydrate and CO_2. Furthermore, when acetate is the sole carbon source, the amount of carotene synthesized by Phycomyces is very small. For these reasons, and because of the comparatively high expense of ^{14}C -acetate, the use of this organism for the biosynthesis of ^{14}C -β-carotene is not a practical proposition, unless the specific carotene precursor, isotopically-labelled, could be prepared.

It was necessary, therefore, to explore the possibility of using other methods which would enable isotopically-labelled β-carotene (or vitamin A) to be produced efficiently and inexpensively in sufficiently large amounts and possessing the necessary specific activity for use in animal experiments.

The synthesis of Isotopically-labelled Compounds.

The use of isotopes in biochemical research is now a well-established technique and there is little need to emphasize their importance in the study of biological systems. Ever since the classical work of Schoenheimer and Rittenberg which established entirely new concepts of metabolic processes, isotopes have been applied with considerable success to problems of metabolism in animals and plants.

Two types of isotope are available; these are (a) the stable ("heavy") isotopes and (b) the radioactive isotopes. In making a choice of a suitable isotope for a particular problem, however, a long-lived radioactive isotope if available, is usually given first place because of the comparative ease and rapidity of its detection and assay using inexpensive apparatus; the assay of stable isotopes is more laborious and requires costly equipment. The stable isotopes, however, are used in cases where no suitable radioactive isotope exists (e.g., nitrogen) or when it is required to doubly label a compound (e.g., as in $^{14}CH_3{}^{13}COOH$ in which the metabolism of both carbon atoms can be studied in relation to each other). In the case of β-carotene or vitamin A a radioactive isotope can be used since radioactive isotopes of both carbon and hydrogen are available.

Essentially the production of a radioactively labelled

compound consists in introducing the radioactive atoms into the substance which is to be studied. The labelled substance will then emit a radiation which is characteristic of the isotope or "tracer" used so that it is possible, by detecting this radiation, to follow the course of metabolism of the substance after it has been administered to the living organism. This depends upon the fact that living organisms distinguish only very slightly or not at all between the differences in mass of the various isotopes of an element when these are small. In fact, the elements in nature exist in constant isotopic proportions (e.g., natural carbon contains detectable quantities of the radioisotope carbon-14 which results from a transmutation of atmospheric nitrogen under the action of cosmic rays).

Of the radioactive carbon isotopes, carbon-14 is the most important. It is a long-lived (half life = 5300 ± 800 years) and weak β-emitting (max. energy = 0.15 mev.) isotope. Because of the low energy of the β-radiation its use is dependent on sensitive and quantitative measurement techniques, but nevertheless carbon-14 is probably the most important single radioactive isotope available to the biochemist. Since its discovery by Ruben and Kamen (1940, 1941), carbon-14 has completely replaced the short-lived (half life = 21 mins.) carbon-11 which was used previously. Carbon-14, therefore, appears to be the obvious choice for the preparation of

radioactive vitamin A or β-carotene, although the possibility of using tritium might also be considered.

For the production of isotopically-labelled compounds, two main methods can be used. These are: (a) chemical synthesis and (b) biosynthesis. The former method is used widely for the preparation of relatively simple labelled compounds. It has the advantage that there is little dilution and loss of the isotope and that the positions of the labelled atoms are known. In the case of more complex molecules, such as carbohydrates and steroids, however, chemical syntheses are often long and tedious, resulting often in low yields. This is where biosynthetic methods have the obvious advantage in being able to utilize the versatile synthetic abilities of plants and micro-organisms.

^{14}C-labelled β-carotene could be obtained by either method but the biosynthetic method is preferable for the reasons stated. ^{14}C -vitamin A could then be obtained by the chemical oxidation of the β-carotene (vide infra).

The direct chemical synthesis of radioactive vitamin A should, however, be regarded as an alternative, since the intermediates used in the industrial synthesis can be obtained and it should not be difficult to modify the technique in order to introduce atoms of carbon-14.

In the present investigation biosynthetic methods for the production of ^{14}C -β-carotene have been studied. Before discussing the choice of a suitable biological system it should be stated that the principal factor which determines the usefulness of any radioactive compound is the specific ~~radioactivity~~ labelled content. This can be defined as the ratio of the number of radioactive atoms to the total number of atoms of the same element. This should be as large as possible because (a) it may be necessary to dilute the labelled compound with non-radioactive carrier in certain experiments, and (b) the substance must be able to withstand dilution during metabolism. Its resultant activity must, therefore, be of an order which is detectable and measurable, so that the higher the initial specific activity, the better. It should be mentioned here that the detection and assay of a radioactive isotope is dependent upon the efficiency of the counting system which is being used. The properties of isotopes and their measurement will, however, be dealt with more fully in a later chapter.

The Distribution of β-carotene in Nature.

Detailed summaries of the occurrence of carotenoids in nature have been given in two recent monographs by Karrer and Jucker (1950) and Goodwin (1952), so it is necessary to give only a brief

survey here. β-carotene is widely distributed in the plant kingdom; it occurs in all the green parts (ie.; chlorophyllous tissues) of the higher plants (Phanerogams) together with other carotenoids which are mainly xanthophylls. The β-carotene content of the higher plants varies according to the species but it is usually within the range 200 - 700 μg./g. dry weight, and it can form up to 50% of the total carotenoid content. In fruit, β-carotene is usually present only as a minor constituent.

In the cryptogams, β-carotene occurs widely but not so uniformly and extensively as in the higher plants. It is present in a number of fungi from which Phycomyces blakesleeanus may be singled out for attention since it has been the subject of intensive research on carotenogenesis. In this organism the β-carotene content can rise to a maximum of 1.4 mg./g. dry weight (Garton, Goodwin and Lijinsky, 1951).

Of the algae, the chlorophyceae (green algae) have been the subject of a great deal of investigation in respect of carotenoid content. β-carotene (0.05% of dry wt.) and lutein are usually found to be the predominating carotenoids. The alga Trentepohlia aurea, however, is probably unique in that it produces only β-carotene, which is present in a high concentration, viz. 2 mg./g. dry weight (Heilbron, 1942; Tischer, 1936). This organism appears

to present an interesting possibility for the production of ^{14}C -β-carotene but preliminary experiments, to be described later, proved disappointing.

The only other sub-group which will be considered is the bacteria. In general, the major bacterial carotenoids appear to be xanthophylls or their derivatives, while β-carotene is either absent or present only as a minor constituent. The bacteria can therefore be ruled out straight away as being possible sources of radioactive β-carotene.

From this brief survey it can be seen that most of the higher plants and certain fungi and algae can be considered as possible systems for the biosynthesis of ^{14}C -β-carotene.

The selection of a suitable biological system for the biosynthesis of ^{14}C -β-carotene.

The final choice of a suitable system for the biosynthesis of ^{14}C -β-carotene will depend on a number of factors, which may be enumerated as follows:-

(i) The product must have a high specific activity. To achieve this the organism must be able to incorporate the carbon-14 source fairly rapidly and extensively into the β-carotene which is being synthesized. In other words, the carbon-14 substrate must be a specific precursor of the β-carotene and the organism must either

be actively engaged in synthesizing β-carotene or one in which there is a rapid turnover of the substance.

(ii) The organism should have a high β-carotene content. This will enable the labelled-β-carotene to be obtained in good yield and also allows for an optimal utilization of the isotope.

(iii) In the case of a micro-organism (e.g., an alga or fungus) this should be one which is capable of rapid reproduction and be suitable for fairly large scale cultivation on a synthetic medium.

If tissues of higher plants are used, these should be able to withstand the rigours of in vitro culture conditions for moderately long periods.

(iv) Finally, the choice of a suitable system will be governed somewhat by the technical considerations involved in the design and setting-up of the apparatus. For a convenient and rapid method a fairly simple experimental set-up is to be preferred to an elaborately designed apparatus.

The organisms which appear to be best suited to fulfill these conditions are the higher plants. The main advantage is that their

use avoids the necessity of the lengthy and tedious procedure of culturing large colonies of micro-organisms, which would utilize the ^{14}C label in the form of the acetate which is expensive compared with $^{14}CO_2$. In this connection, mention has already been made of the disadvantages of using Phycomyces for the biosynthesis of ^{14}C - β-carotene. The other advantages are that the β-carotene content of plant leaves is comparatively high (200 - 700 μg./g. dry weight) and that ^{14}C can be administered by allowing the plants to photosynthesize in the presence of $^{14}CO_2$ which can be generated quite conveniently from radioactive barium carbonate, $Ba^{14}CO_3$.

The detached leaf culture method.

During recent years many biosynthetic techniques using higher plants have been developed. R.H. Burris et al. (1949) have described equipment for the biosynthesis of isotopically labelled compounds by growing plants in closed systems. The use of excised leaves from large plants is, however, a more general technique, particularly for the biosynthesis of compounds requiring relatively short photosynthetic exposures to isotopically labelled CO_2. This method has been used successfully, for example, in the preparation of the ^{14}C-labelled sugars and starch (Putman et al., 1948; Noggle and Bolomey, 1951; Porter and Martin, 1952).

For the biosynthesis of radioactive β-carotene the use of the whole plant is inadvisable, partly because of the greater technical difficulties involved, but mainly because of the greater metabolic requirements of the whole plant which would result in a dilution of the isotope at the centres of synthesis.

In some preliminary experiments by Dr. J. Glover (1952) the relative incorporation of ^{14}C into β-carotene obtained from whole plants (tomato) and from excised leaves maintained in closed systems, was compared. The specific activity of the β-carotene from whole plants varied within the range 0.03 - 0.19 μc./mg. carbon, while that of the β-carotene from detached leaves was 0.59 μc./mg. Carbon. Although these values probably do not represent the true values of specific activity because of the possible presence of radioactive impurities, they do serve to illustrate the greater efficiency of the detached leaves in maintaining a higher isotope concentration because of the absence of translocation. The detached-leaf culture method is, therefore, to be preferred.

The detached leaf method has been applied in many investigations in plant physiology and in a critical review Yarwood (1946) has examined the technique in detail. The main conclusions regarding the physiological and biochemical factors affected by leaf-detachment are briefly as follows:-

(i) Respiration continues at first at a rate comparable to that of attached leaves, but falls off with increasing time from detachment to a general decline in rate associated with exhaustion of carbohydrates. Respiration can resume, however, when carbohydrate is restored by allowing the leaves to photosynthesize CO_2.

(ii) The rate of photosynthesis of detached leaves decreases as the carbohydrates increase. The carbohydrate level of the leaves can reach a higher value than that attained by attached leaves and photosynthesis may entirely cease in leaves with such high accumulations of carbohydrate, but is resumed after the leaves have been kept in the dark for a period.

(iii) Protein synthesis can proceed in the dark if the leaves are supplied with carbohydrates and a nitrogen source.

(iv) Translocation in detached leaves is reduced and almost entirely eliminated.

Basically the principle of the method for the biosynthesis of ^{14}C -β-carotene is to exhaust young growing leaves of their stores of carbohydrates and then to allow them to fix new CO_2

containing the ^{14}C label. However, a number of preliminary experiments had to be carried out in order to obtain some information regarding the variations in concentrations of the carotenoids in detached leaves and thus establish the optimal conditions for the biosynthesis of radioactive β-carotene with high specific activity. Furthermore, the experiments were necessary to enable an apparatus to be designed suitable for the experiments on the incorporation of the tracer.

CHAPTER III.

THE METABOLISM OF β-CAROTENE IN DETACHED LEAVES.

Our knowledge concerning carotenoid metabolism in detached leaves is almost entirely due to Bandurski (1949) and his work is an important contribution to an understanding of the mechanism of carotenogenesis in higher plants. Considered in relation to the present investigation, however, the most important result of Bandurski's work is that detached leaves synthesize considerable amounts of carotenoids when the leaves are exposed to light and CO_2. The amount synthesized is greater than that synthesized by attached leaves, the difference being explained by assuming that precursors are not translocated in the excised leaves. Furthermore, Bandurski found that carotenoid synthesis can proceed in the dark provided the carbohydrate-starved leaves were supplied externally with a source of glucose or sucrose.

The dark synthesis of carotene in the presence of glucose occurs, however, only one-fifteenth as rapidly as the corresponding synthesis in light. It seems, therefore, that although the synthesis can proceed in the dark from precursors derived from

carbohydrate, light has an accelerating action on the process. In the absence of light and a supply of carbohydrate there is a slow destruction of carotene. Thus it appears that the concentration of carotene in the leaf may be maintained by the simultaneous processes of synthesis and destruction. In other words, the carotene may be in a state of active turnover.

This concept is in agreement with the results obtained by a number of workers, which have shown that the carotene of plants undergoes marked diurnal and seasonal variations (Murneek, 1932; Bernstein et al., 1945; Roberts, 1948; Kuppers et al., 1948). An interesting observation on the variation in carotene concentration in leaves was also made by the Russian workers Zafren and Tyukina (1948). They found that plant materials which had been wilted for a period of several hours regenerated carotene upon being placed in water to restore their turgidity.

The concept of a carotene turnover in green leaves has an important bearing on the problem of the biosynthesis of ^{14}C -β-carotene, since by allowing the leaves to photosynthesize in the presence of $^{14}CO_2$ there should be a considerable incorporation of the isotope in the β-carotene, resulting in a product with a high specific activity. The experiments which are to be described were aimed at investigating the variations in carotenoid concentrations

in detached leaves in order to determine the optimal conditions for the isotope experiments.

The leaves chosen for these experiments were those of the tomato plant (Lycopersicum esculentum Mill.). The reasons for this choice were as follows:-

(a) they have a relatively high β-carotene content (40 - 50 µg./g. fresh weight);

(b) they are rapidly growing tissues;

(c) they can withstand a comparatively high humidity and temperature, the type of conditions which prevail in the closed system of culture under artificial illumination;

(d) they are easily procurable over the greater part of the year (March - November).

With the exception of the first experiment, all the leaves used were from plants grown in greenhouses.

EXPERIMENTAL.

(i) Selection of leaves and apparatus.

The top immature leaves were cut from the plants at about 8.00 - 8.30 a.m. and carried to the laboratory in a moist container. Leaf samples were prepared immediately by cutting off the leaves close to the base of the petiole with a scalpel and placing them in five groups, each of approximately 5 g. fresh weight. In order to obtain uniform samples of the leaves the method of selection used was the "statistical method" described by Vickery et al. (1949). This method equalises the effects of the differences between the plants and the differences between leaf positions on individual plants and it gives more uniform samples than either the "opposite-leaf" or "leaf-size" methods.

One of the leaf-samples was taken as a control and treated for analysis. The remaining four samples were then carefully floated on distilled water (approx. 100 ml.) contained in four large desiccators, each of 7 litres' total capacity. Care was taken to ensure that the leaf-laminae were not wetted unduly by using a minimum amount of water and by as little handling or moving of the vessels as possible to prevent undue agitation. It was important, however, to ensure that the cut ends of all the leaf

petioles were immersed in the water.

The covers of the desiccators were then fitted after greasing the ground-glass joint. Each desiccator was fitted with a rubber bung carrying one inlet and one outlet tube, the former being allowed to project within a few centimetres of the surface of the leaf culture, while the latter was allowed to project only a short distance below the bung. This arrangement facilitated an efficient circulation of the gaseous phase in the culture vessels. The vessels were connected together in series by means of rubber tubing fitted to the inlet and outlet tubes. At the inlet side a wash-bottle containing 60% KOH and a solid-KOH tower were connected and a slow stream of air was drawn through the vessels from the outlet side for about one hour. The vessels, now containing air substantially free from CO_2, were sealed off, disconnected and placed in the dark at ambient room temperature for 24 hours. During this period the leaves use up their remaining carbohydrate reserves.

Immediately after the dark period one of the leaf samples was removed for analysis. The remaining three vessels were placed in a water-bath of constant temperature (25° C.) and inlet and outlet tubes connected to form a closed circuit with a small bellows pump and a 150 ml. conical flask containing 63 ml. CO_2 over dilute lactic acid. This volume of CO_2 resulted in an initial CO_2 percentage of 0.3 in the atmosphere of the culture vessels

(total capacity 21 litres). The pump, which was operated mechanically by an electric motor, was then started in order to maintain a thorough mixing and circulation of the atmosphere of the apparatus. The pumping rate was approximately one litre per minute so that a complete circulation of the atmosphere was effected approximately every 20 minutes.

Circulation of the atmosphere by pumping and illumination of the leaf surfaces was maintained continuously throughout the experiment. Leaf samples were removed for analysis after 24, 48 and 72 hours.

(ii) Illumination.

The leaf surfaces were illuminated by four frosted 100 watt tungsten lamps fitted laterally in a holder and provided with an aluminium reflector. The lamps and reflector were fixed horizontally by means of retort stands at a distance of 18 inches above the surface of the leaves, producing a luminous intensity of 400 foot-candles (measured by a Sangamo Weston Photronic Cell, Model S.123) at the surfaces of the leaves. This light intensity was used since Bandurski (1949) found that the optimal light intensity for carotene synthesis lies between 400 and 600 foot-candles.

(iii) Separation and estimation of the carotenoids.

When a leaf sample was removed for analysis, excess moisture was removed by pressing the leaves gently between sheets of filter paper. The lipid was then extracted according to the method previously described.

The lipid was then dissolved in 5 ml. light petroleum and chromatographed on 15 g. alumina contained in a column of internal diameter 1.5 cm. The alumina used was a mixture of activated alumina (3 parts) and deactivated alumina (1 part). The column was developed with light petroleum and the resulting chromatogram is described in Table 1. The zones were eluted by gradually increasing the polarity of the eluant with ether (peroxide-free). α-Carotene and neo-β-carotene B, a pale yellow band, were eluted with 1 - 2% v/v ether in light petroleum. This zone was followed closely by β-carotene, a broad orange zone, which was eluted with 4 - 8% ether.

Above the β-carotene zone a number of fainter coloured bands could be seen. These began to separate into well-defined bands with increasing polarity of the eluant, the first bands being eluted with 12% ether and the last with 60% ether. A good separation was difficult at this stage owing to a considerable overlapping of many of the bands. All these bands were designated collectively as

the "post-β-carotene fractions" and they were retained for re-chromatography in an attempt to separate and identify the constituents.

Finally, the xanthophylls, which are normally obscured by the chlorophyll which is strongly absorbed near the top of the column, were eluted with 20% (v/v) ethanol in light petroleum. This also carried down a part of the chlorophyll so that the resulting eluate was a xanthophyll-chlorophyll mixture. To separate them, therefore, it was necessary to saponify. The fraction was reduced to dryness under nitrogen, 0.5 ml. 60% KOH and 5 ml. ethanol added, and the mixture heated under nitrogen for about 15 minutes. The mixture was then extracted with ether and the ether extract, containing the xanthophylls, washed with water and dried over anhydrous Na_2SO_4.

The carotenoids were then estimated spectrophotometrically. The fractions obtained from the chromatography were distilled under reduced pressure to remove the solvent and the residues made up to a known volume with light petroleum, and if necessary, a further dilution made in order to obtain a suitable reading on the spectrophotometer. In the case of the xanthophylls, which are only sparingly soluble in light petroleum, the reading was made either in ether solution or in ethanol solution, the latter being preferable due to the greater tendency towards oxidation of carotenoids by ether peroxides.

Table 1.

Separation of carotenoids from Tomato-leaf Lipid on a mixture of 3:1 (W/W) mixture of activated and deactivated alumina, using light petroleum containing ether as developer.

(zones numbered in order of increasing adsorptive power.)

Zone No.	Description.	Name of pigment.
4.	Green band.	Chlorophylls and xanthophylls.
3.	Number of minor pink and yellow bands.	"Post-β-carotene" fractions.
2.	Broad orange zone.	β-carotene.
1.	Yellow band.	α-carotene and neo-β-carotene B.

In all cases the carotenoid solutions were protected from the possibility of photoxidation by storage in the dark at 0° C.

The extinction values for α- and β-carotene were measured at the main maxima (446 mμ and 448 - 449 mμ, respectively, in 40-60° light petroleum), and the concentration calculated assuming the $E^{1\%}_{1cm.}$ value to be 2500 in each case.

Similarly, the highest extinction value of the total post-β-carotene fraction was measured (446 - 448 mμ). Since the composition of this fraction was unknown, the $E^{1\%}_{1cm.}$ was assumed as 2500, a value which probably represents a reasonable approximation. For the xanthophyll mixture the main maximum occurred in ether and ethanol solution at 444 - 446 mμ and again an $E^{1\%}_{1cm.}$ of 2500 was assumed, since lutein, which is the major xanthophyll occurring in the leaves of higher plants, has an $E^{1\%}_{1cm.}$ at 446.5 mμ of 2550 in ethanol solution. (Zscheile et al., 1942).

Table 2.

The variations in carotenoid concentrations of detached tomato leaves.

Carotenoid	Amount in µg./g. dry tissue. Control Sample	After 24 hr. respiration minus CO_2 in dark.	Period of continuous photosynthesis with 0.3% CO_2 following on the dark period. 24 hr.	48 hr.	72 hr.
I.					
α- & β-carotene	330	90	45	209	151
post-β-carotene fractions	46	124	63	116	131
xanthophylls	-	563	643	582	663
total carotenoid	-	777	751	907	945
II.					
α- & β-carotene	509	212	204	370	462
post-β-carotene fractions	109	190	75	148	111
xanthophylls	1112	1283	1275	1311	1336
total carotenoid	1730	1685	1554	1729	1909
III.					
α- & β-carotene	573	334	-	589	529
post-β-carotene fractions	64	119	-	34	39
xanthophylls	1374	1036	-	1013	977
total carotenoid	2011	1489	-	1636	1545

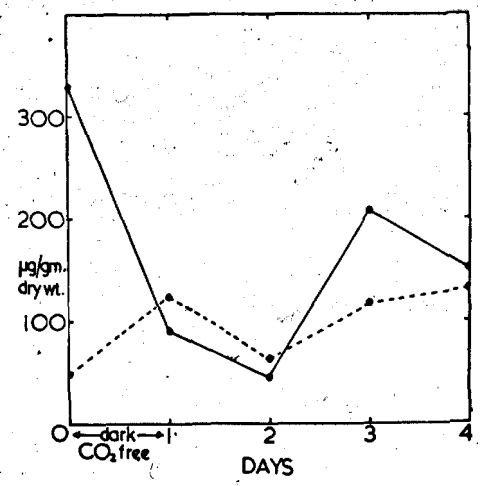


Fig. 1.

Variation in carotenoid concentrations of detached tomato leaves. (Experiment I.)

——— α- and β-carotene.

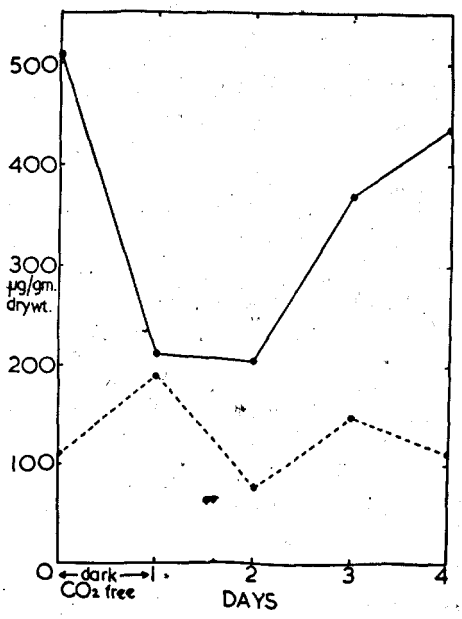
..... post-β-carotene.

Fig. 2.

Variation in carotenoid concentrations of detached tomato leaves. (Experiment II.)

——— α- and β-carotene.

..... post-β-carotene.



RESULTS.

The results of the first three experiments are summarized in Table 2. These experiments show that the α- and β-carotene content of detached tomato leaves had fallen considerably (by 50 - 75%) from its original level after the leaves had been maintained in the dark for 24 hours. After 24 hours' exposure to constant illumination in an atmosphere containing 0.3% CO_2 there was a further slight decrease in α- and β-carotene in both experiments. At the end of the 48-hour period of constant illumination, however, the α- and β-carotene had risen again and at the end of the 72-hour period α- and β-carotene had in the second experiment risen to a value approaching that of the original level, while in the first experiment the concentration had shown a fall (see Figs. 1 and 2.)

These results indicate that a destruction of β-carotene occurs when detached tomato leaves are kept in the dark in an atmosphere which is initially CO_2-free. When the leaves are again exposed to light in an atmosphere of CO_2, photosynthesis is resumed and the carotene concentration begins to rise towards its initial level after an induction period of 24 hours, during which the concentration shows comparatively little change.

Examination of the "Post-β-Carotene"Fractions.

These experiments gave results (See Table 2 and Figs. 1 and 2) showing that the post-β-carotene fractions underwent a variation in concentration according to the conditions to which the leaf-culture was subjected. In the first experiment the total post-β-carotene fraction was initially 46 μg./g. dry weight, but after the end of the 24-hour dark period the concentration had risen to 124 μg./g. dry weight; the concentration dropped again (to 63 μg.) after the first 24-hour light period but had risen to 131 μg. at the end of the 72-hour light period. Similarly, in the second experiment the concentration of the total fraction increased from 109 μg. to 190 μg./g. dry weight during the 24-hour dark period but dropped to 75 μg. in the 24-hour light period, increased again (to 148 μg.) in the next 24-hours and then fell again (to 111 μg.) at the end of the 72-hour light period (See Figs. 1 & 2.).

It can be seen that these changes can be related to the variations which occur in the α- and β-carotene concentrations. The decrease in carotene in the dark period coincides with the increase in the post-β-carotene fractions. At the end of the 24-hour light period, however, the post-β-carotene fell again to a level approaching its initial value, while the carotene concentration remained at its low level, but as the carotene increased

again in the next 24-hour light period, the post-β-carotene also increased.

One is tempted at first to regard the differences in the pattern of the results obtained at one period from that at a previous period as reflecting absolute changes in the amounts of the various components present, but this would be strictly incorrect for a dynamic system since the figures really only show the change in the equilibrium proportions of a reaction sequence. However, the total quantity of carotenoid present tends to be fairly constant, suggesting that if the reaction rates of these lipid soluble substances are slow then there may be a correlation between the metabolism of the carotenes and the post-β-carotene fractions. The significance of this relationship, however, can only be appreciated if we have some knowledge of the nature of these post-β-carotene fractions. These pigments were therefore investigated to try to establish their constitutions.

All the post-β-carotene fractions from Experiment 2. were combined and the solvent removed in vacuo. The residue was dissolved in 5 ml. light petroleum and chromatographed on a column (1.8 x 6 cm.) containing 100% deactivated alumina. The resulting chromatogram is described in Table 3.

Table 3.

Separation of Post-β-Carotene Fractions on Deactivated Alumina, using Light Petroleum containing Ether as Developer.

Zone No.*	Description	Eluant	Absorption maxima, mμ.ø (in light petroleum).
8.	Yellow	20% ether	-
7.	Pink-yellow	20% "	428, 451, inflexion at 475.
6.	Pink	6% "	425, 448.
5.	Pink	6% "	
4.	Yellow	3% "	426, 452.
3.	Yellow	2% "	447, shoulders at 430, 470.
2.	Orange-pink.	2% "	445, 469, inflexion at 420.
1.	Yellow	100% petrol	-

*The zones are numbered in order of increasing adsorptive power.

øThe main maxima are underlined.

The zones were eluted and their spectra in petrol solution determined. (See Figs. 3 - 7).

Results and Discussion.

The spectrum of fraction 4 (Fig. 5) bears a close resemblance to that of β-carotene 5,8 monofuranoid (mutatochrome or citroxanthin). According to Karrer and Jucker (1950) the absorption maxima of this compound in petroleum ether are at 427 and 456 mμ and the absorption spectrum (in ethanol; abs. max. 423, 452 mμ) obtained by Karrer and Jucker (1944) is reproduced in Fig. 5. for comparison. Incidentally, this compound may also be identical with an unidentified pigment obtained from the carotenoids of corn seedlings by Moster et al. (1952). This pigment (No. 6.) showed maxima at 430 and 456 mμ in hexane, the ratio of the peaks λ 430/λ 456 being 1.13. This agrees well with fraction 4 with λ max. at 426 and 452 mμ in petrol (the difference being explained by the spectral shift caused by the different solvents), and the ratio λ max. 426/λ 452 equal to 1.10. Also, fraction 4 may correspond to a pigment with λλ max. 403, 428, 452 mμ (in Skellysolve B) found adsorbed above β-carotene by chromatography of commercial β-carotene (Bauerfeind et al., 1944). Pigments with similar chromatographic and spectroscopic properties were also found in yellow corn

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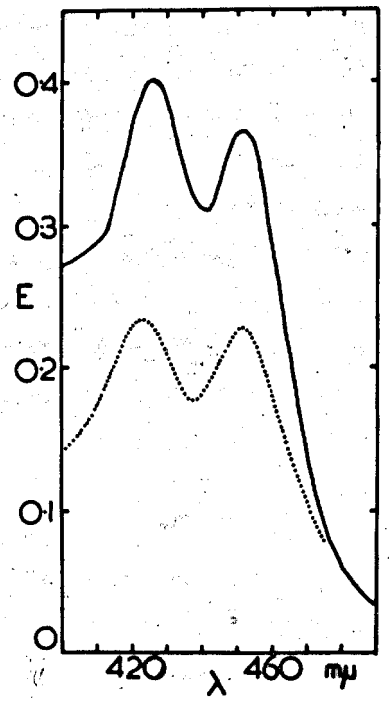
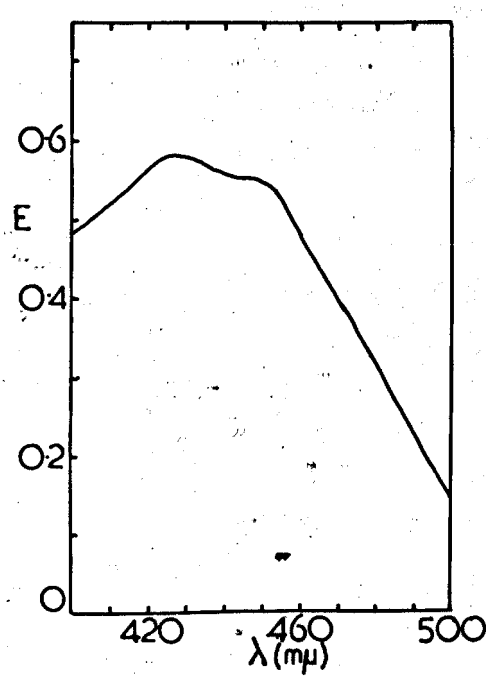


Fig. 5.

Post-β-carotene fraction 4 ——— ; mutatochrome. (in ethanol) .....

Fig. 6.

Post-β-carotene, fraction 5.



(Baumgarten *et al.* 1944), e.g., pigment D4a, λλ max. 402, 426, 451.

If it is accepted that the fraction 4 pigment is in fact the furanoid oxide of β-carotene (although this has yet to be established with certainty) it raises the interesting possibility that the other post-β-carotene fractions may also be oxygenated derivatives of β-carotene and, perhaps, of α-carotene. On this basis, therefore, it is possible to assign the following tentative structures to the post-β-carotene fractions (Table 4.)

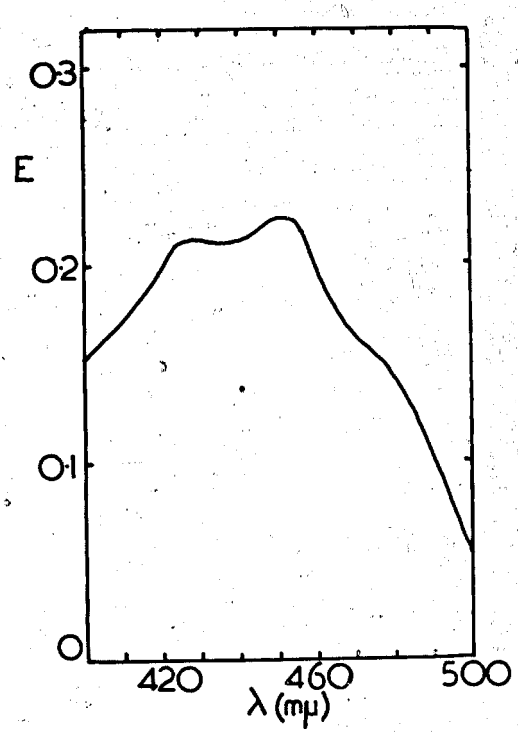


Fig. 7.

Post-β-carotene, fraction 7.

Table 4.

Spectroscopic Properties of the Post-β-Carotene Fractions and their possible identity with compounds of known structure.

Fraction	Observed maxima (in light petroleum.)	Possible Structure.	Actual maxima (in light petroleum) Ref. Karrer & Jucker (1950).
2.	445, 469.	β-carotene 5,6, 5^1, 6^1, diepoxide.	443, 470.5
4.	426, 452	β-carotene 5,8 monoepoxide (mutatochrome)	427, 456.
5.	425, 448.	β-carotene 5,8; 5^1, 8^1 diepoxide (aurochrome)	428.
7.	428, 451.	β-carotene 5,6, 5^1,8^1 diepoxide (luteochrome)	451, 482 (in CS_2)

Of all these possible structures, probably the only one which can be accepted with any degree of certainty is that of fraction 4. One obvious criticism which can be directed against the above scheme is the anomalous position of fraction 2, identified as a β-carotene diepoxide; it would be expected that a diepoxide, because of its greater degree of polarity, would be adsorbed on the column above the monoepoxides, yet it is shown in a position

below that of the mono-furanoid oxide (fraction 4.).

However, until further work can be done to establish unequivocally the nature of these pigments, all that can be said at the moment is that spectroscopically, they appear to resemble closely the carotene epoxides. On this assumption it is possible to formulate a hypothesis which implicates the epoxides in the general metabolism of β-carotene.

The increase in concentration of the post-β-carotene fraction and the decrease in the β-carotene concentration which occur in the dark period may be interpreted as indicating that the production of epoxides is the first step in the oxidative destruction of β-carotene. It is unlikely, however, that the renewed synthesis of β-carotene in the light, merely involves regeneration or reduction of the epoxides for the following reasons. Firstly, the increase in β-carotene epoxide and furanoid concentrations at the end of the dark period amounted to only a small fraction (27 - 32%) of the decrease in the "carotenes". Secondly, illumination of the leaves brought the β-carotene oxides back quickly to their normal level, while the carotene remained at a low concentration. Thus it appears that once the oxides are formed they are probably oxidatively degraded further, while new β-carotene is synthesized from the smaller carbon compounds of the

metabolic pool. This concept is in agreement with the work of Bandurski (1949) who observed different temperature coefficients for the synthesis (2.9) and destruction (1.6) of carotene in detached bean leaves.

Discussion of the significance of the variations in carotene concentration in detached leaves.

Bandurski (1949) has established that carotene destruction in detached leaves may occur in two ways:-

(1) By an enzymic destruction which occurs in the dark. At higher temperatures (30 - 35° C.) in the presence of glucose a net increase of carotene was observed, so that it appears that this destruction is being counteracted by a simultaneous synthesis.

(2) Destruction by photoxidation. This was shown by the smaller net increase in young leaves exposed to light intensities higher than 600 ft.-c.

A third possible way in which carotene destruction might occur was suggested. This was that the carotene may participate in some metabolic reaction in which it is used up. As will be seen later, this may not be different from the first.

In the present experiments the decrease in carotene concentration which occurs during the 24-hour dark period indicates that

the rate of destruction exceeds considerably the dark synthesis, if it can be assumed that the latter is taking place at all. The transformation of some of the β-carotene into another carotenoid, xanthophyll or epoxide, might also account for some of the loss. This is discussed more fully in the next section. The leaves used in the experiments were taken from the plant in the early morning when the carbohydrate reserves were probably at a very low level. Consequently, during the 24-hour dark period there would be very little carbohydrate or none at all available for carotene synthesis, since the whole of the low carbohydrate reserve probably would be required for the essential metabolic requirements of the plant. The further slight fall in carotene content which occurs in the 24-hour light period immediately following the dark period suggests that the rate of destruction (now by photoxidation as well as by enzymic destruction) is slightly greater than the rate of light synthesis. This can be interpreted as being due to the leaves building up their reserves of carbohydrate which become available for the immediate metabolic needs of the leaves, but leaving little available for carotene synthesis. Furthermore, photodestruction of carotene may be greater in the absence of an oxidizable substrate, such as carbohydrate, which might normally protect the pigment from

photoxidation. This is supported by the fact that the photoantoxidation of chlorophyll is prevented by an external supply of oxidizable substrate (Franck and French, 1941).

The increases in the carotene content which occur at the end of the 48 and 72-hour periods indicate that synthesis is now proceeding at a greater rate than the destruction, suggesting that the leaves are gradually restoring their carbohydrate reserves. The fall in carotene content which occurred in the first experiment at the end of the 72-hour period may be attributed either to a necrosis of the leaves (some of the leaves were showing signs of chlorosis) or to the presence of narcotic substances which may have infiltrated into the atmosphere of the apparatus from the air in the laboratory.

The significance of the results of these experiments in relation to the metabolism of the carotenoids in the intact plant must be viewed with caution. Bandurski (1949), however, has discussed the validity of the detached leaf culture technique and concludes that differences which do exist between the detached leaf and the intact plant, are differences of degree rather than kind. One can say, therefore, that the results represent the general pattern of carotene metabolism in higher plants, but probably on a much exaggerated scale. The results of these

experiments confirm the earlier work of Bandurski (1949) and the pattern of carotene metabolism which emerges is that the carotene content of leaves is normally maintained by the simultaneous processes of synthesis and destruction. In other words, it appears that the carotene in the leaf is in a state of constant turnover.

This idea is in agreement with the results (previously discussed) obtained by other workers, which have shown that the carotene concentration of plants can undergo marked variations, according to the environmental conditions.

The concept of an active turnover of leaf carotene has an important bearing upon the production of ^{14}C -β-carotene since a substance which is being synthesized constantly should incorporate extensively the labelled precursor, resulting in a product of high specific activity. On this basis, therefore, the detached leaf culture method appears to be a suitable system for the biosynthesis of radioactive β-carotene. The question remains, however, is the β-carotene synthesized de novo or is it merely regenerated from breakdown products formed in its destruction? An examination of the results for the concentrations of the "post-β-carotene" fractions and the determination of their probable identities has thrown some light on this question.

The significance of the Results in relation to the Problem of the function of Carotenoids in Higher Plants.

The role of the carotenoids in the metabolism of the higher plants is far from clear. The widespread distribution of these pigments in relatively high concentrations and their intimate association with chlorophyll in the chloroplasts, implies that they have some specific function in plants, but the precise nature of this function is still far from being understood.

Most of the theories of the function of carotenoids in plants have been based on two characteristic properties: (a) the ability to absorb oxygen and (b) the ability to absorb light energy.

Carotene when exposed to air absorbs oxygen fairly rapidly but in the leaf it appears to be stable. This observation led Arnaud (1889) to suggest that carotene probably participates in some oxidation-reduction system. This idea was elaborated by Willstätter and Stoll (1913) into a hypothesis in which a carotene to xanthophyll oxidation was coupled to a chlorophyll b. to chlorophyll a. reduction. These workers could not, however, produce any experimental evidence for such a system, although they found that the xanthophyll/carotene ratio can undergo considerable variation. More recently, Sapozhnikov and Lopatkin (1950) have studied the effect of different environmental conditions on this ratio. The xanthophyll/carotene ratio increased in the presence

of narcotics, the increase being attributed to an inhibition of the dark (enzymatic) raction in photosynthesis, which the authors suggested is involved in the enzymatic conversion of xanthophylls to carotene. A further claim for a carotene-xanthophyll inter-conversion has been put forward by Moster and Quackenbush (1952). They suggested that β-carotene and zeaxanthin are interconvertible, zeaxanthin formation being favoured by either a high light intensity or decreased temperature (photochemical reaction), while β-carotene formation was temperature-dependent (enzymatic reaction). A similar inter-relationship was suggested for lutein and violaxanthin. (It should be noted that the interpretation of the results of these workers is open to criticism since variations of a particular carotenoid were expressed as percentages of the total carotenoid rather than as actual concentrations.)

The results of the present experiments do not suggest that there is an interconversion between carotene and xanthophylls; the total xanthophyll concentration remained at a fairly constant level so that any variation in the xanthophyll/carotene ratio could be related to a change in carotene concentration rather than to a change in xanthophyll concentration. In the cases where there were definite changes in the xanthophyll concentrations, these could not be correlated significantly with changes in the carotene

concentrations. On the other hand, there did appear to be a significant correlation between carotene and carotene epoxides. It has already been suggested that the carotene epoxides may be the first stages in the oxidative destruction of carotene in the plant, a process which is probably irreversible. It would be interesting to speculate now whether carotene epoxide formation is part of some fundamental biochemical system of the plant.

The capacity of the carotenoids to act as oxygen acceptors has caused many authors (see Rabinowitch, 1945) to suggest that they may have a catalytic function in the binding of oxygen in respiration. This idea was strengthened when Karrer and his co-workers (see Karrer and Jucker, 1950) found that a number of carotenoid-epoxides and furanoids existed in nature. The carotenoid epoxides are sensitive to traces of dilute mineral acids which rupture the epoxide ring to form the isomeric furanoid oxide together with the original carotenoid, which is formed as a by-product by the loss of oxygen. The ease with which these inter-conversions proceed has led Karrer et al. (1945) to suggest that similar processes operating in the plant may play a part in biological oxidations.

The possibility that the carotenoids, instead of being involved in respiration, may alternatively participate in the

reverse process, viz., the liberation of oxygen in photosynthesis, has been discussed by Rabinowitch (1945). It is believed that the intermediate between the photolysis of water and liberation of molecular oxygen is a peroxide, probably an organic peroxide, since its formation would be reversible. Double bond peroxides (moloxides) appear to be a possible type of reversible peroxide and these might be formed from carotenoids. Dorough and Calvin (1951) have put forward a hypothesis which involves carotenoid epoxides as intermediates in the formation of double bond peroxides. The carotenoid is initially oxidized to an epoxide which, under the influence of light or enzymes, is converted to the furanoid derivative. The furanoid ring can then be envisaged as rupturing in such a way that the effect is to move the oxygen along the polyene chain. This effect could possibly take place from both ends of the molecule so that the two oxygen atoms would eventually meet at the centre to form a peroxide, which would then decompose to yield the original carotenoid and molecular oxygen.

This hypothesis, however, is questionable on theoretical grounds since it is doubtful if such a mechanism is thermodynamically possible. Dorough and Calvin (loc. cit.) attempted to prove that the oxygen of carotenoid epoxides and furanoids is derived from the photolysis of water, but their experiments were inconclusive.

CHAPTER IV.

THE BIOSYNTHESIS OF ^{14}C -β-CAROTENE USING THE DETACHED LEAF-CULTURE METHOD.

Having established the pattern of metabolism of the carotenoids in detached tomato leaves the next stage in the investigation was concerned with the production of ^{14}C-labelled β-carotene. The possibility of obtaining the compound with a relatively high specific activity (1 - 5 μc./mg.) appeared promising, since by allowing carbohydrate-depleted leaves to photosynthesize $^{14}CO_2$ the isotope should be incorporated extensively in the β-carotene during its resynthesis. The results obtained, however, did not confirm these expectations, the resulting product having a much more moderate specific activity. (0.01 μc./mg.C.). After making various modifications in the apparatus and improvements in technique, however, it was possible to raise the specific activity to a higher level (0.05 - 0.06 μc./mg.C.) starting with $^{14}CO_2$ at 7 - 8 μc./mg.C. This level of activity is suitable for many of the research requirements and the effectiveness of its utilization has been further increased

by using the most efficient method of radioactive assay, known as gas-counting.

The preliminary experiment for the production of radioactive β-carotene will be described. Later sections will deal with the modifications and improvements in technique made in subsequent experiments, and the use of alternative carbon precursors, such as acetate. Finally, the possibility of obtaining more active preparations by using other organisms, such as algae, will be discussed.

EXPERIMENTAL PROCEDURE.

The procedure adopted in these experiments for the preparation of the leaf-cultures was essentially the same as that described in the previous chapter. The leaves were collected in the morning of the commencement of the experiment and uniform leaf samples were selected. One sample, the control, was taken for immediate analysis and the remaining four samples carefully floated on distilled water contained in four 7-litre capacity desiccators. These were filled with CO_2-free air and as an extra precaution the air was also passed through wash-bottles containing fresh oxalated blood and lead acetate solution to remove traces of carbon monoxide and hydrogen sulphide, respectively.

After the 24-hour dark period and the removal of a sample for analysis, the remaining three desiccators (each containing 9 - 10 g. fresh wt. of leaves) were connected together in a closed circuit which also contained the circulating pump, a "bubbler" containing lactic acid to observe the pumping rate and efficiency, and a CO_2 generator which consisted of a 50-ml. round-bottomed flask, with side arm (the outlet) and fitted with a small dropping funnel to which the inlet tube was connected.

Radioactive barium carbonate ($Ba^{14}CO_3$) equivalent to 232 micro-curies was weighed out and this was diluted with inactive $BaCO_3$ to form a total weight of 485 mg. This is equivalent to 108 mg. CO_2 so, therefore, the specific activity of the $^{14}CO_2$ was 2.15 μc./mg. or in terms of carbon, 7.9 μc./mg.C. Since the total capacity of the three desiccators was approximately 20 litres the initial concentration of CO_2 would be 0.27%.

The $Ba^{14}CO_3$ was placed in the flask of the CO_2 generator and the dropping funnel filled with 75% lactic acid. The flask was evacuated and then connected into the closed circuit. The lactic acid was allowed to run into the flask slowly to generate the $^{14}CO_2$, all the taps of the apparatus were opened and the pump started to circulate the gas mixture, and after a short while the lamps, giving an intensity of 400 ft.c. at the leaf surfaces, were switched on.

The leaf samples were not subjected to constant illumination as in the previous experiments, but to a photoperiod of 16 hours light and 8 hours dark. The reason for this was that there would probably be a lower degree of pigment destruction by photoxidation in photoperiod than in constant illumination. Withrow and Withrow (1949) have shown that tomato leaves soon develop interveinal chlorosis when exposed to continuous artifical radiation but that the chlorosis is less severe under a photoperiod, particularly at lower temperatures.

During the dark period the leaf-culture vessels were covered completely with a black cloth and pumping was maintained.

After 48 hours and 72 hours photoperiod samples were removed for analysis. The residual $^{14}CO_2$ in the atmosphere of each vessel was first absorbed in NaOH by the method to be described later.

The third desiccator was also removed from the circuit at the end of the 72 hours' photoperiod and the residual $^{14}CO_2$ absorbed in NaOH. The desiccator was then connected to a pump and slow stream of air was drawn through, while the photoperiod of 16 hours light, 8 hours dark was maintained. Groups of leaves were then removed for analysis after 24, 48 and 72 hours. The object of this part of the experiment was to obtain some information regarding the turnover rate of β-carotene. The latter will be related to the

change in the specific activities of the β-carotene of the samples removed at the different time intervals.

Radioactivity Determinations.

(i) The specific activity of the ^{14}C -β-carotene.

After the spectrophotometric assay of the β-carotene obtained from the leaf samples a suitable portion, containing 30 - 50 μg., was removed for the radioactive assay. This was placed in a 25 ml. conical flask and the solvent removed under nitrogen. The residue was then transferred to a counting dish for radioactive assay as previously described.

(ii) The specific activities of the oxidation products of ^{14}C -β-carotene.

All the ^{14}C -β-carotene fractions were combined and total amount found to be 0.64 mg. This was diluted with unlabelled β-carotene to a total weight of 1.925 mg. (i.e., a 3-fold dilution) and the mixture purified by chromatography. A small portion (36 μg.) of the purified β-carotene was taken for radioactive assay while the remainder (1.7 mg.) was oxidized by the method of Wendler et al.(1950), (vide infra). The β-carotene was dissolved in 10 ml. dry ether in a 50 ml. round-bottomed flask fitted with a mechanical stirrer and the oxidation was carried out at 25° C. The reaction was controlled to achieve an optimal yield of vitamin A aldehyde (retinene). The oxidation products were separated by chromatography

on 15 g. alumina (containing 25% deactivated alumina). The products were eluted from the column and assayed spectrophotometrically. 0.25 mg. retinene was obtained, which represents a yield of 14.3%.

Radioactivity measurements were made on the oxidation products and the values obtained are shown in Table 1. Values for the original ^{14}C -β-carotene are included for comparison.

Results.

The results strongly suggest that the original β-carotene must have been associated with another, presumably colourless, substance possessing radioactivity. The count rate of the β-carotene isolated from the leaf samples after the various intervals of photoperiod showed an extremely wide and scattered variation (6 - 86 cpm./μg.). In addition, the specific activity of the retinene obtained by the oxidation of the ^{14}C -β-carotene was only half that of the original β-carotene, whereas if the latter had been pure, both substances should have had approximately the same activity. Thus it appears that the radioactive impurity has similar adsorption properties to β-carotene. Retinene, however, is slightly more strongly adsorbed than β-carotene and, therefore, presumably less of the impurity will be associated with the retinene fraction, thus accounting for its lower activity. It is difficult, however,

to explain the comparatively high activity of the 2,7 dimethyl-octatriendial, since this substance is strongly adsorbed and would, therefore, be expected to be free from the active impurity. It can only be assumed that the impurity itself is oxidized, giving rise to a more strongly adsorbing derivative.

Saponification of the β-carotene, followed by chromatography, produced no change in specific activity and it was concluded, therefore, that the contamination was not due to the presence of a glyceride or other esters, since these would give rise to strongly adsorbing alcohols after saponification and these could be separated from the β-carotene by chromatography. The problem was then to separate the impurity from the β-carotene or its oxidation products in order to obtain true values of specific activity. This was achieved by the preparation of pure derivatives of β-carotene and retinene.

Table 1.

Radioactivity determinations on the oxidation products of ^{14}C -β-Carotene.

Substance	Activity (allowing for original dilution) (cpm./µg.)	Total Activity (cpm.)
β-carotene	32	18,688
β-carotene (after purification by chromatography)	28	
Retinene [1.]	15	1,250
Retinene (after re-chromatography)	14.7	
β-apo-12'-carotenal. [2.]	3.3	165
2,7 dimethyloctatriendial [3.]	27	130
First fraction (colourless)		147

1. $E^{1\%}_{1cm.}$ at 367 mµ in light petroleum = 1685 (Ball et al.,1948)

2. $E^{1\%}_{1cm.}$ at 408 mµ. " " " = 2000 (value assumed from theoretical considerations).

3. $E^{1\%}_{1cm.}$ at 320 mµ. in iso octane = 2620 (Wendler et al., 1950)

(iii) Determination of the specific activity of ^{14}C -β-carotene by the formation of dehydro-β-carotene.

Essentially the method consists in the formation of the insoluble iodine adduct of β-carotene which can be washed free of impurity, followed by its conversion to dehydro-β-carotene for spectrophotometric estimation and radioactive assay as previously described.

16.7 μg. ^{14}C -β-carotene were diluted with inactive material to a total weight of 184 μg. (i.e., approx. 11 x dilution). This was dissolved in 0.5 ml. light petroleum and dehydro-β-carotene formed. The product was chromatographed on 5 g. alumina (25% de-activated). The dehydro-β-carotene band was eluted and determined spectroscopically using an $E^{1\%}_{1cm.}$ value of 3000 at 468 mμ. The radioactivity was then determined.

It was found that the specific activity was then only 0.01 μc./mg.C., which is approximately one-sixteenth of that obtained for the original β-carotene.

(iv) Determination of the specific activity of ^{14}C -Retinene by the formation of Retinene 2,4,dinitrophenylhydrazone.

146 μg. Retinene obtained from the oxidation of the ^{14}C -β-carotene was dissolved in 2 ml. ethanol, and retinene 2,4,dinitrophenylhydrazone prepared as previously described.

An attempt was then made to resolve the retinene 2,4,DNPH by

partition chromatography on paper (ascending technique) using the n-heptane-methanol system. The mixture was dissolved in 5 ml. petrol and a 2 ml. portion of the solution pipetted on to an 8-inch line, 2 inches from the bottom edge of a rectangular sheet of No. 1. Whatman paper. The paper was then folded into a cylinder and placed in the chromatography tank with the bottom edge immersed in the developer (n-heptane saturated with methanol). After a few hours it was found that a single orange band had travelled with the solvent front. This was eluted with light petroleum, estimated spectroscopically and the radioactivity determined. A relatively high specific activity (0.19 μc./mg.C.) was obtained indicating that the retinene 2,4 DNPH had not been resolved from the impurity.

It was decided, therefore, to try to obtain better resolution by utilizing the technique of adsorption chromatography on paper. A 2 ml. portion of the retinene 2,4 DNPH solution was pipetted on to an 8-inch line 2.5 inches from the base of a rectangular sheet (13.5 x 10 inches) of No. 1. Whatman paper. The paper was folded into a cylinder and placed in the tank with the base immersed in n-heptane. A rapid development occurred and after 4 hours five distinct bands were observed (see Table 2.). The Rf's were measured and the bands were cut from the paper and the pigments eluted with ethanol.

Table 2.

Adsorption Chromatography on Paper of Retinene 2,4 dinitrophenylhydrazone, using n-heptane as developer.

Band No.	Description.	Rf.	Absorption Maxima (mμ.) (in ethanol)
1.	Yellow.	0.84	360
2.	Narrow, pale orange.	0.42	420
3.	Broad orange	0.32	435
4.	Trace of faint red.	0.13	-
5.	Narrow orange.	0	-

The main band (No. 3.) exhibited a maximum in ethanol at 435 mμ. This was probably an isomer of retinene 2,4 DNPH since Ball et al. (1948) give the main maxima of the pure derivative as 442 mμ. It was assayed using the $E^{1\%}_{1cm.}$ of 1166 (loc. cit.) and the radioactivity determined. The pale orange band (No. 2.) absorbed immediately above No. 3. was probably also an isomer of retinene 2,4 DNPH since this gave a broad peak at 420 mμ. in ethanol. This may correspond with the isomer (λ max. 422 mμ. in $CHCl_3$) discussed by Julia and Weedon (1951). The yellow band (No. 1.) which travelled close to the solvent front exhibited a maximum at 360 mμ; this compound was probably a 2,4 DNPH with no double bonds conjugated with the imino linkage (Roberts and Green, 1946). It may have been, therefore, a saturated aldehydic or ketonic derivative of 2,4 DNPH. The compound was radioactive.

The results of the radioactive assays of the retinene 2,4 DNPH's showed a low level of specific activity (0.01 - 0.02 μc./mg.C.) of the same order as that obtained by the dehydro-β-carotene method.

(v) Determination of radioactivity of the $^{14}CO_2$ in the atmosphere of the culture vessels.

The CO_2 in the atmosphere of the leaf-culture vessels at the end of the experiment represents an equilibrium between the

simultaneous processes of photosynthesis and respiration. The level of radioactivity of the $^{14}CO_2$ is therefore an indication of the level of radioactivity in the fuel reserves (mainly carbohydrates) which are undergoing oxidation in the respiratory processes of the leaves. Thus it is important to determine the specific activity of this CO_2 in order to assess the efficiency of the system for the biosynthesis of ^{14}C -β-carotene.

The CO_2 was absorbed by displacing the atmosphere by suction with CO_2-free air and allowing the gas mixture to pass through an alkali absorber. The absorber consisted of a 150 ml. Buchner flask containing 25 ml. of 5% sodium hydroxide (carbonate-free) fitted with a sintered glass (G.3.) disperser. The suction was adjusted to give a slow but steady flow of gas, producing a fine bubble formation at the sintered disc, thus ensuring complete absorption. In later experiments the absorption was effected more rapidly and probably more efficiently by placing a CO_2 absorber (without sintered pad) in the closed circuit and allowing the atmosphere to circulate for about an hour, while the leaves were maintained in the dark.

The "carbonate-free" sodium hydroxide was prepared by centrifuging 50% sodium hydroxide. This removes sodium carbonate which is relatively insoluble at this concentration, and the

carbonate-free 50% sodium hydroxide was then diluted with CO_2-free water (boiled distilled water) immediately before use.

The method used for the precipitation of barium carbonate for radioactive assay has already been described.

Discussion of the Results.

The results of this experiment (Table 3.) showed that the ^{14}C -β-carotene synthesized was of very low specific activity. This activity, in terms of the usual 1 - 2% geometry of the end-window type counter, represents a count rate of 0.5 - 1 count per minute per microgramme of β-carotene. Although this level of activity may be suitable for some research purposes, it is too low for most, unless more efficient counting methods, such as proportional counting or gas counting, were used.

The specific activity of the β-carotene was only a small fraction (one-tenth approx.) of the activity of the $^{14}CO_2$ remaining in the atmosphere at the end of the experiment. This may mean that the turnover rate of β-carotene is relatively slow compared with that of the other cell constituents, such as carbohydrates. On the other hand, this view is difficult to reconcile with Bandurski's (1949) work and with the results of the preliminary experiments in the present investigation.

Table 3.

Preliminary experiment on the production of ^{14}C -β-Carotene

Radioactive Assay of ^{14}C-β-carotene and its pure derivatives.

Substance.	Specific Activity cpm./µg.	cpm./µg.C.	µc./mg. C.
Original $^{14}CO_2$	-	-	7.9
Residual $^{14}CO_2$ (after 48 hours)	-	-	0.58
Residual $^{14}CO_2$ (after 72 hours)	-	-	0.26
^{14}C -β-carotene (crude)	32.4	36.2	0.6
^{14}C -dehydro-β-carotene	0.58	0.64	0.01
^{14}C -retinene 2,4 DNPH (main fraction)	0.81	0.96	0.016
^{14}C -retinene 2,4 DNPH (isomer)	0.89	1.06	0.017

In a second experiment, using essentially the same technique as in the first, except that the leaves were subjected to constant illumination instead of photoperiod, it was found that the specific activity of the β-carotene was higher (0.06 μc./mg.C.), although the ratio of the specific activity of the original $^{14}CO_2$ to the total dry weight of leaf tissue were approximately the same in both cases (viz., 100 μc./g. dry wt.). The improvement was due probably to two factors; (a) that constant illumination resulted in a more efficient incorporation of the $^{14}CO_2$ coupled with a more rapid synthesis of β-carotene, and (b) the leaves, on the whole, appeared to remain longer in a healthy condition than those in the first experiment.

Nevertheless, in both these experiments the limit of effective life of the leaves was three days under constant illumination or photoperiod. After about two days some of the leaves began to show signs of wilting and at the end of three days many of them were chlorosed. The onset of necrosis of the leaf tissue will be accompanied by a general degeneration in the photosynthetic and metabolic efficiency of the organism. Thus there will be a gradual decline in the rate of turnover of β-carotene and the pigment will be destroyed by photoxidation. It appears, therefore, that the effective life of the leaf-culture may be the limiting factor in

the production of ^{14}C -β-carotene with high specific activity.

The later experiments were aimed mainly at the production of ^{14}C -β-carotene with higher specific activity by trying to prolong the life of the leaf-cultures.

THE NATURE OF THE IMPURITY ASSOCIATED WITH β-CAROTENE.

It has been shown that the apparently high specific activity of the β-carotene obtained from the tomato leaves was due to the presence of a colourless impurity which possessed radioactivity. It was impossible to separate this substance from the β-carotene by adsorption chromatography and it still persisted after saponification. The specific activity of the β-carotene could only be determined, therefore, by converting it to the insoluble iodide and thus separating it from the impurity. Since the β-carotene iodide cannot be reconverted to β-carotene but only to a new substance, dehydro-β-carotene, it is impossible to isolate pure β-carotene by this method. The presence of this impurity would tend to complicate any biological experiment in which ^{14}C -β-carotene was used because of its comparatively high specific activity and also because its metabolism in the animal body is unknown. It is important, therefore, that the chemical nature of this substance should be determined in order to devise a method for removing it

from the β-carotene without affecting the latter. From the purely biochemical point of view, a knowledge of the nature of the substance is of considerable interest since it may have an important function in plant metabolism and, possibly, in the animal body. Although it has not been possible to investigate the chemical nature of this substance in detail, some preliminary observations have been made.

The compound was isolated as follows. 116 g. Fresh weight of tomato leaves were extracted with acetone in a Waring blender. The filtered extract was evaporated to small bulk in vacuo, extracted with ether and washed with water. The ethereal extract was dried and the solvent removed. The lipid was then chromatographed on 60 g. alumina (25% deactivated) and the β-carotene fraction collected. Approximately 5 mg. β-carotene was obtained. The β-carotene was precipitated as the iodide (Kuhn and Lederer, 1932) and the mixture filtered. The filtrate, containing the colourless impurity, was collected and excess iodine removed by adding a 2.5% solution of sodium thiosulphate. The light petroleum solution was washed with water and dried. The solvent was evaporated, leaving a colourless wax-like solid.

The compound exhibited steep end-absorption in the far U.V. region but no maxima could be found. In U.V. light a green fluorescence could be seen.

A small portion of the solid was tested for the presence of an unsaturated sterol by the Liebermann-Burchard reaction. A very pale green colouration was obtained, thus indicating the presence of a trace of a sterol. It is unlikely that a sterol is the major constituent since it has been found that the main sterol component of the plant lipid is much more strongly adsorbed on the column.

All that can be said of this colourless, U.V. fluorescent substance at the moment is that it is probably a hydrocarbon. It is interesting to note that Strain (1936) reported the presence of similar fluorescent substances in leaf extracts. These occurred on the adsorption column just below the α-carotene band and above the β-carotene band; they did not show any absorption maxima or minima and saponification did not reduce the yield or alter their relative positions on the column.

FURTHER EXPERIMENTS FOR THE PRODUCTION OF ^{14}C -β-CAROTENE WITH HIGHER SPECIFIC ACTIVITY.

(i) The use of a Nutrient Salt Medium.

It was thought that by floating the excised leaves on a nutrient salt solution instead of distilled water, the life of the leaves might be prolonged. The nutrient employed was the three-salt solution described by Bandurski (1949). It consisted of KH_2POH (0.0167 M), $Ca(NO_3)_2.4H_2O$ (0.0046 M) and $MgSO_4.7H_2O$

(0.0023 M); the total osmotic pressure of the solution was one atmosphere, and its reaction was pH 4.5. The solution was made up with boiled tap water which provided micro-nutrients.

As a further modification, the whole plants were maintained in the dark for 24 hours prior to the detachment of the leaves. The reason for this was that translocation would then be possible, resulting in a more rapid depletion of the carbohydrate reserves and perhaps a greater reduction in carotene concentration. Twelve young tomato plants were placed in a large glass tank which was then placed in the dark for 24 hours. After the detachment of the leaves and placing into groups, they were carefully floated on a minimal volume (100 - 150 ml.) of the nutrient medium in the culture vessels which were contained in a water-bath at 20°.

0.3% $^{14}CO_2$ was used, with a total specific activity of 100 μc. (i.e., 23 μc. per g. dry tissue weight). During and after the generation of the CO_2, the culture vessels were kept in the dark for a time sufficient to ensure a uniform distribution of the gas throughout the apparatus. This procedure reduced the possibility of a preferential CO_2-fixation by the first culture vessel in the closed circuit.

The intensity of the illumination in this experiment was reduced to 250 foot-candles by raising the lamps to a distance of 2 feet above the surface of the leaves. This would lessen the

tendency towards photoxidation and chlorosis.

The results of this experiment showed, however, that the various modifications introduced did not improve the life of leaf-cultures since at the end of three days of constant illumination more than half of the leaves were severely chlorosed. It was decided, however, to investigate the influence of chlorosis on the concentration of β-carotene and on the specific activity of the latter.

The specific activity of the β-carotene isolated from the chlorosed leaves was less than half that of the β-carotene from the green leaves (0.0076 μc./mg.C. compared with 0.018 μc./mg.C. respectively - See Table 4.)

Table 4.

Biosynthesis of ^{14}C -β-Carotene using detached tomato leaves maintained in a nutrient salt Medium.

Substance.	Specific Activity cpm./µg.	Specific Activity µc./mg. C.	Total Activity µc.
Original $^{14}CO_2$		8.0	100
^{14}C -β-Carotene (green leaves)	0.6	0.018	0.008
^{14}C -β-Carotene (chlorosed leaves)	0.25	0.0076	0.006

Total dry weight of leaves = 4.3 g.

This is what might be expected considering the decline in photosynthetic rate which occurs with chlorosis. A rather surprising result, however, was to find that the β-carotene concentration of the chlorosed leaves (432 μg./g. dry weight) had not fallen considerably and was, in fact, higher than that in the green leaves (348 μg./g. dry weight). A possible explanation for this is that the value of total dry weight obtained for the chlorosed leaves probably does not represent the true dry weight since during necrosis there is a degradation of the high molecular weight components, such as proteins, into smaller water and alcohol-soluble compounds. These would, therefore, tend to diffuse out into the culture solution and also to be extracted during the treatment with ethanol.

(ii) Improvements in the maintenance of the leaf-cultures and the use of a heat-filter.

The next major modifications which were made were in the environmental conditions of the leaf-cultures. In the previous experiments the leaves had been floated on water or nutrient solution. This usually led to an undesirable wetting of the leaf surfaces, an effect which would inhibit a free gaseous exchange through the stomata of the leaves. A more disastrous consequence, however, would probably be due to the production of a favourable medium for the growth of bacteria and fungi, resulting

in a degradation of the leaf tissue by these organisms. It was decided, therefore, to try the effect of maintaining the leaves in a moist atmosphere rather than floating them on water. This was done by allowing the inlet tube of each culture vessel to dip below the surface of water contained in a 100 ml. beaker, so that when the pump was in operation the bubbling of the gas mixture through the water caused a humidification of the atmosphere. The bubbling also tended to produce a spray of water droplets in each container sufficient to maintain the leaf surfaces moist without undue wetting.

The second important modification was the employment of a heat-filter to absorb the infra-red component of the source of illumination. Water exhibits an appreciable absorption in the infra-red region and in the experiments where a heat-filter was not used the relatively high water content of the leaves would probably result in a considerable heat absorption. This would lead to desiccation of the leaves through water loss by heat evaporation and also to a disturbance and injury of the metabolic processes. Absorption of the infra-red radiation can be accomplished quite effectively by the use of heat absorbing glass or a layer of water. The latter method was adopted in these experiments; a large glass tank containing water at a depth of 4 inches was interposed between the light source and the culture vessels. In later experiments a

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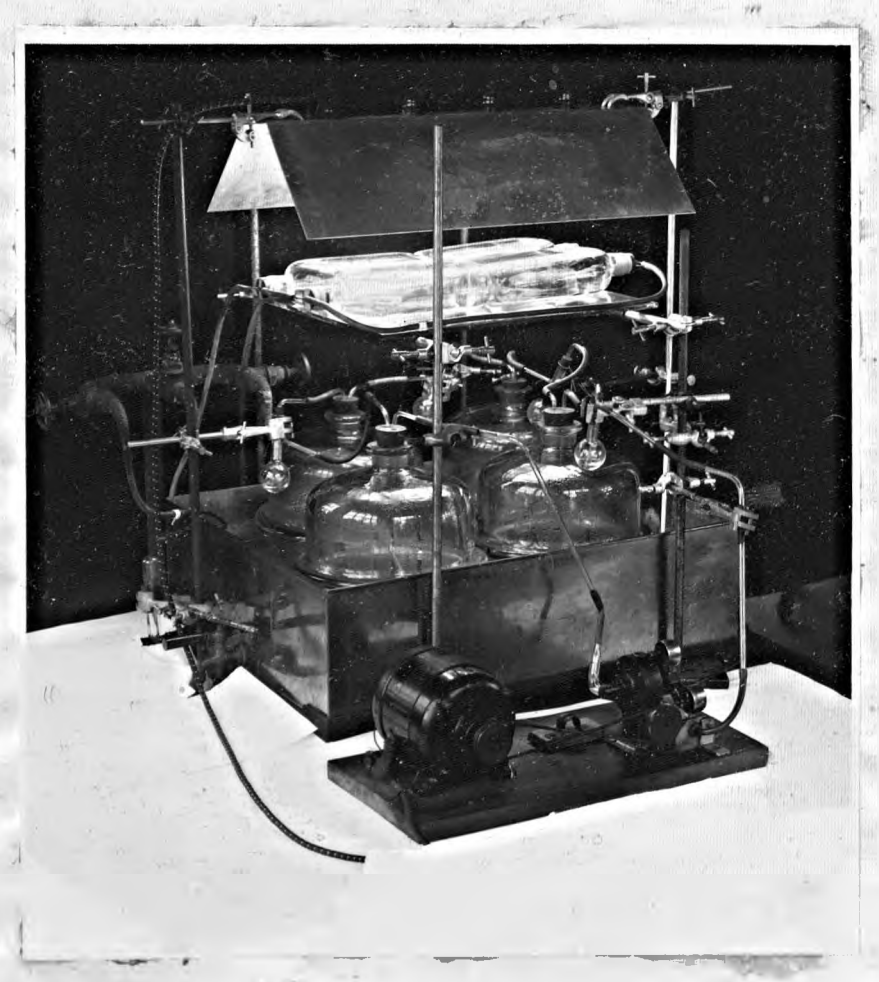


Fig. 1.

Improved Apparatus for the Biosynthesis of ^{14}C-β-carotene.

more effective heat-filter was designed by connecting six Roux bottles, supported by a glass plate, together in series and allowing a slow continuous stream of cold water to flow through them (see Fig. 1.).

A trial experiment, using inactive CO_2, was carried out on the culture of detached tomato leaves incorporating the above modifications. The leaf-cultures were maintained in a bath at 20° and subjected to constant illumination of 250 foot-candles intensity. It was found that the leaves remained in a healthy condition for a considerably longer period than in the previous experiments; it was not until the thirteenth day of culture that some of the leaves began to show signs of chlorosis.

In view of the successful result of this experiment in being able to extend considerably the life of the leaf cultures, it was decided to run experiments using $^{14}CO_2$ to see whether ^{14}C -β-carotene with higher specific activity could be obtained.

In the first of these experiments an initial CO_2 concentration of 0.1% was used instead of 0.3% as in the previous experiments. 377 μc. of $Ba^{14}CO_3$ were used, which represented 79 μc./g. dry tissue. Instead of using one CO_2 generator for the whole apparatus, each culture vessel was provided with its own generator. The reason for this was to ensure a more rapid distribution of the CO_2 in the

atmosphere and to avoid a preferential CO_2 pick-up in the dark by the leaves in the first culture vessel. In order to avoid the possibility of CO_2 diffusion through the rubber connexions, these were kept down to a minimum and as far as possible all the connexions were made glass to glass. The leaves were maintained in a moist atmosphere by the use of the "humidifiers" already described and a heat-filter was used. The culture vessels were kept in a water-bath at 20° and a light intensity of 250 foot-candles was employed. After varying periods of constant illumination some of the leaves were beginning to show signs of chlorosis. It was considered that this may have been due to an exhaustion of the CO_2 in the atmosphere resulting in a CO_2-starvation of the leaves. It was decided, therefore, to add 0.1% of inactive CO_2. At the end of the 8-day period, however, the chlorosis had become more severe so the experiment was discontinued.

It was found that the carotene concentration of the leaves at the 8-day period had fallen to almost half the initial level (viz., from 455 µg./g. to 231 µg./g. dry weight). The specific activity of the β-carotene was 0.06 µc./mg.C. (See Table 5.). It will be seen that the specific activity at the 5-day period was 0.03 µc./mg.C. while at the 7-day period it was 0.057.

Although these results are insufficient themselves to draw any valid conclusions concerning the turnover rate of β-carotene, they appear to indicate that the isotope is incorporated slowly at first and then followed by a more rapid incorporation until the seventh day when the specific activity appears to be approaching a limiting value; the latter may, of course, be due to a falling off in the metabolic activity of the leaves.

Table 5.

Improved technique for the production of ^{14}C -β-Carotene. Carotenoid Concentrations and Specific Activity Results.

	1. Control sample.	2. 24-hr. dark minus CO_2.	3. 2 days light + $^{14}CO_2$.	4. 5 days light + $^{14}CO_2$	5. 7 days light + $^{14}CO_2$	6(a) 8 days light + $^{14}CO_2$	6(b) 8 days light + $^{14}CO_2$
Dry weight (g.)	0.8152	0.7565	0.4378	0.5186	1.0966	1.0706	1.1580
Lipid weight (g.)	0.0886	0.0907	0.0556	0.0910	0.1200	0.1071	0.1149
Total dry weight (g.)	0.9038	0.8472	0.4934	0.6096	1.2166	1.1777	1.2729
Carotene (α and β) per 1 g. dry wt.(μg.)	455	203	173	497	349	231	299
"Post-β-carotene" per 1 g. dry wt. (μg.)	103	213	250	121	137	161	153
Xanthophylls per 1 g. dry wt. (μg.)	1160	1502	1581	1219	1489	1563	1485
Total carotenoids per 1 g. dry wt. (μg.)	1719	1919	2004	1838	1974	1955	1937
Specific activity of ^{14}C -β-Carotene (μc./mg. C.)	-	-	-	0.03	0.057	0.06	0.048
Total activity of ^{14}C -β-carotene (μc.)	-	-	-	0.0069	0.019	0.013	0.013

Total activity of original $^{14}CO_2$ = 377 μc. (79 μc. per g. dry wt. leaf tissue).

Specific activity of " " = 25.7 μc./mg.C.

In the next two experiments using substantially the same technique, specific activities of 0.047 μc. and 0.06 μc./mg. C. were obtained, although it was only possible to maintain the leaves under constant illumination, starting with an initial CO_2 concentration of 0.3%, for five and six days, respectively (See Table 6.)

The results of these three experiments show that it is possible, by prolonging the life of the leaf-cultures, to obtain β-carotene with consistently higher specific activities than that of the earlier experiments. The increased efficiency of the process is also indicated by the fact that the specific activity of the β-carotene was approximately one-sixth of the specific activity of the $^{14}CO_2$ remaining in the atmosphere of the culture vessels.

A specific activity of 0.06 μc./mg. C. is equivalent to a count rate of about 2 cpm./μg. β-carotene at the position of maximum geometry in the end-window counter. This is still a very low activity which necessarily limits its application, unless the more efficient method of radioactive assay by gas counting was employed.

Table 6.

Further experiments on the biosynthesis of ^{14}C -β-Carotene. Specific Activities.

Substance.	Specific Activity. (μc./mg. C.)	Total Activity. (μc.)
I.		
Original $^{14}CO_2$.	7.6	343
Residual $^{14}CO_2$.	0.27	-
Total carbon*	0.23	343
^{14}C -β-carotene.	0.047	0.094
(Total dry wt. of leaf tissue = 4.89 g.) (Total yield of β-carotene = 2.24 mg.)		
II.		
Original $^{14}CO_2$	8.2	182
Residual $^{14}CO_2$	0.37	-
Total carbon*	0.33	182
^{14}C -β-carotene	0.06	0.042
(Total dry wt. of leaf tissue = 1.86 g.) (Total yield of β-carotene = 782 μg.)		

*Computed by assuming carbon content to be 30% of total dry weight (see data by Chibnall, 1939).

Radioactivity Determination of ^{14}C -β-Carotene, using the Gas-Counter.

The ^{14}C -β-Carotene obtained from the various experiments was bulked. A small portion was taken for radioactive assay and converted to dehydro-β-carotene. 29 μg. of the latter was combusted to CO_2 which was then absorbed in NaOH by the method previously described. 7.6 mg. inert $NaHCO_3$ were added and the CO_2 generated. A G-M counter tube (30 ml. capacity) was filled with the CO_2 (pressure 17.5 cm.) and CS_2 (pressure 5.4 cm.). The net count rate was 785 c.p.m. This is equivalent to 27 c.p.m./μg. dehydro-β-carotene. Using the end-window counter the activity was recorded as 1.4 c.p.m./μg. so that the relative efficiency of the gas-counter compared with the end-window counter is 19.3.

(iii) The use of Spinach Leaves.

The possibility of using detached leaves of another type of plant which may withstand culture for longer periods, was investigated. Spinach leaves were selected for this purpose. These have a comparatively high β-carotene content (450 μg./g. dry wt.) and they appear to be hardy leaves. For a trial experiment two groups of young spinach leaves were selected and these were placed in two desiccators. The modifications adopted in the previous experiments, viz., the maintenance of a moist atmosphere

and the use of a heat-filter, were employed. An atmosphere containing 0.1% CO_2 and a light intensity of 250 ft.-candles were used. On the sixth day of constant illumination, however, most of the spinach leaves were severely chlorosed, while tomato leaves under the same conditions still appeared quite healthy and did not appear chlorosed until the thirteenth day.

This experiment, therefore, proved the unsuitability of spinach leaves for detached leaf-culture.

INVESTIGATION OF ALTERNATIVE METHODS FOR THE PRODUCTION OF ^{14}C -β-CAROTENE.

In order to try to increase the specific activity of the β-carotene it was decided to investigate the possibility of using alternative methods. Two main methods were studied. The first was really only a modification of the detached leaf-culture method, involving the use of ^{14}C-labelled acetate as the carbon-source instead of $^{14}CO_2$. The second method was concerned with the possibility of using algal cultures.

(i) The use of ^{14}C-acetate as the carbon-source in the detached leaf-culture method.

It is well known that acetic acid can be used as food to support plants in the absence of photosynthesis (Rabinowitch, 1945).

Krotkov and Barker (1948) have shown that tobacco leaves could utilize acetate in the dark after the leaves had been placed in a solution of acetate labelled with ^{14}C in the carboxyl group, since the isotope appeared in the respired CO_2. This suggested that acetate can enter the respiratory processes of the leaves. This idea receives some support from the recent work of Millerd and Bonner (1954) who have isolated an acetate activation enzyme from a number of higher plant tissues. This enzyme was found to catalyze the formation of acetyl-coenzyme A from acetate, A.T.P. and coenzyme A. Also, it has been shown by Newcomb and Stumpf (1953) that higher plants can utilize ^{14}C-labelled acetate for the synthesis of fatty acids. All this evidence seems to indicate that acetate utilization by the higher plants proceeds firstly by the formation of acetyl-coenzyme A, which is probably a normal metabolite which is concerned in the process of aerobic respiration and in biosynthetic mechanisms, functions of acetyl-coenzyme A which are now well established in animal tissues.

Comparatively little is known, however, concerning the utilization of acetate by higher plants for the biosynthesis of carotenoids. Beekmann (1953) has stated that when etiolated wheat seedlings are treated with acetate, it has no effect on carotenoid synthesis, a result which contradicts the findings of Schopfer and

Grob (1950, 1952), which suggest that acetate is the fundamental precursor of carotene in Phycomyces. It was hoped, therefore, that the present experiments might throw some light on this problem.

Experimental and Results.

In the first of these experiments, two groups (each of approx. 3 g. fresh weight) of young tomato leaves were floated on 125 ml. of acetate solution contained in two large desiccators. The acetate solution contained 7 mg. 2- ^{14}C -sodium acetate (equivalent to 100 μc.) and 5 mg. inactive acetic acid in 250 ml. water. The desiccators, containing a CO_2-free atmosphere, were connected up with a pump in a closed circuit in the usual way and to ensure a constant mixing of the culture solution, the inlet tubes were allowed to dip below the surface of the liquid. The leaves were subjected to a constant illumination of 400 ft.-candles intensity and at the end of three days the leaves were removed for analysis. The specific activity of the β-carotene was only 0.016 μc./mg. C. The atmosphere of the culture vessels at the end of the experiment was found to contain CO_2 which was ^{14}C-labelled; the specific activity was 0.03 μc./mg.C. This meant that the ^{14}C-acetate had been utilized in the metabolic processes of the leaves. This experiment, however, gave no information as to whether the ^{14}C -acetate was utilized directly for the biosynthesis

of ^{14}C -β-carotene or whether the $^{14}CO_2$ formed by the dissimilation of the acetate was photosynthesized to carbohydrate which then served as the carotene precursor. It was decided, therefore, to carry out another experiment using ^{14}C-acetate in which photosynthesis was inhibited by maintaining the leaf-cultures in the dark. This type of experiment would be useful also in determining the mechanism of carotenogenesis in the leaves of higher plants. If ^{14}C-labelled acetate is utilized directly for the synthesis of ^{14}C -β-carotene, then by oxidative degradation of the latter followed by a determination of the radioactivity of each fragment, it would be possible to obtain information concerning the mechanism of the synthesis.

This second experiment was performed using two groups of young tomato leaves, each group containing approximately 6 g. fresh weight. The leaves were placed in two 7 l. desiccators, each containing 100 ml. of the acetate solution. The composition of the acetate solution was as follows:-

2- ^{14}C -sodium acetate (100 μc.)	3.8 mg.
sodium acetate	71.4 "
acetic acid (glacial)	75.9 "
sulphanilamide	54.3 "

These were dissolved in water to make a total volume of 200 ml. The sulphanilamide (approx. 0.025%) was added to suppress bacterial

growth; Bandurski (1949) found that sulphanilamide at this concentration had no effect on carotene synthesis. Direct contact of the leaf surfaces with the culture solution was prevented by supporting the leaves on a framework of glass rods, allowing only the ends of the petioles to be immersed in the solution. The culture vessels were connected in a closed circuit with a pump and CO_2-absorber (25 ml. 10% NaOH) to remove the respired CO_2 from the atmosphere, and photosynthesis was inhibited by excluding light by covering the vessels with a black cloth. The leaf cultures were maintained at a temperature of 20° and each vessel was provided with a "water-bubbler" to provide a moist atmosphere. After four days under these conditions the leaves were removed for analysis.

The results of this experiment showed that the specific activity of the β-carotene was negligible (0.003 μc./mg. C.). The β-carotene concentration was normal (545 μg./g. dry weight) while the post-β-carotene concentration was high (157 μg./g. dry wt.). The specific activity of the respired CO_2, which was collected by the alkali absorber, was 0.18 μc./mg. C. The acetate remaining in the culture solution was estimated as follows. The solution was made alkaline with NaOH and evaporated to approximately 15 ml. volume. The solution was then made acid (pH 3) with dilute sulphuric acid and steam distilled and approximately

20 times the bulk of the original solution was collected as distillate. The distillate was titrated against 0.05 N $Ba(OH)_2$ using bromothymol blue as indicator. It was found that 22.7 mg. of acetic acid was present with a specific activity of 0.4 μc./mg. C. Since the initial concentration of acetate as acetic acid was 121 mg., it appears that approximately four-fifths of the acetate had been utilized by the leaves.

Discussion.

It is difficult to interpret these results adequately without complete data, which would involve radioactivity determinations on other components of the leaf tissue. The results do show, however, that acetate is metabolized by the leaf since a considerable amount of radioactivity appears in the respired CO_2. (The total activity as CO_2 was 33 μc; a considerable amount of CO_2 was probably lost due to a leak which developed in the apparatus, so that the actual specific activity was probably much higher). Acetate does not, however, appear to be a direct precursor of β-carotene, or at least it is not a precursor in the absence of light. This confirms the work of Beekmann (1953). Acetate, or rather acetyl-coenzyme A, cannot be ruled out as a precursor under the normal conditions of photosynthesis, since carotene synthesis has been shown to be accelerated by light (Bandurski, 1949).

The inability of acetate to act as a precursor of carotenoids in higher plants in the dark may be explained by the recent results of Krotkov et al. (1954). These workers showed that 1-^{14}C acetic acid is not utilized by tobacco leaves for carbohydrate synthesis in the dark. In the absence of light, therefore, it would seem that acetate cannot act as a precursor of carotenoids since carotenoid synthesis is dependent on carbohydrates (Bandurski, loc. cit.).

(ii) An attempt to use algae for the biosynthesis of ^{14}C -β-carotene.

The disadvantages of using algae for the production of ^{14}C-labelled β-carotene have already been pointed out. The main advantage of this type of organism is, however, that it generally undergoes rapid reproduction and therefore there is a greater possibility of obtaining ^{14}C -β-carotene with higher specific activity than in the case with detached leaves. An alga which, because of its comparatively high β-carotene content, appeared particularly suitable for this purpose, is Trentepohlia aurea. This alga is a rich source of β-carotene for it comprises 0.2% of the dry weight as compared with about 0.05% of the dry weight of leaves of higher plants (Heilbron, 1942).

An attempt was made to culture Trentepohlia aurea on a medium of the following composition:-

Agar	1%
KNO_3	0.02%
K_2HPO_4	0.002%
$MgSO_4.7H_2O$	0.002%
Soil extract (1 part soil, 2 parts water)	10%

It was found, however, that there was no perceptible growth of the organism after a period of several weeks, so it was decided to discontinue the experiments.

SUMMARY AND CONCLUSIONS.

Of the biosynthetic techniques for the production of ^{14}C - β-carotene investigated, the detached tomato leaf method using CO_2 as the ^{14}C source, was the superior method. It has been possible, using this method, to obtain labelled β-carotene with a specific activity of 0.05 - 0.06 μc./mg. carbon, commencing from $^{14}CO_2$ with specific activity 7 - 8 μc./mg. C. This level of activity represents 16 - 17% of the maximum possible incorporation of the isotope, based on the specific activity of the $^{14}CO_2$ remaining in the culture vessels at the end of the experiment (from results given in Table 6.). A similar figure (18 - 21%) is obtained if the percentage incorporation of ^{14}C is based on the maximum specific activity of the carbon (computed by assuming the carbon content of the dry leaf

tissue to be 30% from data by Chibnall (1939) assuming that all the isotope is evenly distributed.

A specific activity of 0.05 - 0.06 μc./mg. C. is very moderate and because of the low count rate (2 c.p.m./μg. C.) obtained using the end-window G-M counter, the use of the ^{14}C -β-carotene is limited. It has been shown, however, that by utilizing the greater geometric efficiency of the gas-counter the count rate can be raised to 27 c.p.m./μg. C. Thus it is possible by means of this method to detect and measure the activity of quantities of ^{14}C -β-carotene of the order of 1 μg. Effective use of the ^{14}C -β-carotene could, therefore, be made in animal experiments even in cases where a considerable dilution of the substance occurred.

The possibility that (^{14}C -β-carotene with higher specific activity might be obtained by prolonging still further the life of the leaf cultures should be considered as a problem for future research. In a recent interesting paper by Chibnall (1954) it was shown that detached bean leaves could be kept in a healthy condition for periods of several weeks by treatment with indole acetic acid. The latter was assumed to function by restoring the normal balance of protein synthesis and degeneration. In the untreated detached leaf, protein degradation proceeds at a greater rate than protein synthesis and this degradation is usually accompanied

by chloroplast degeneration which results in the chlorosis of the leaf. Incidentally, the possibility that a correlation exists between protein decomposition and the concentration of chloroplast pigments, suggested by Chibnall (1939), is of importance in the study of the general problem of carotenoid metabolism in plants.

The low specific activity of the ^{14}C -β-carotene produced by the biosynthetic method leads one to consider the possibility of obtaining radioactive vitamin A by a direct chemical synthesis. It would only be possible by this method to label one or two of the carbon atoms in the molecule and as already mentioned, the technique would tend to be long and laborious. However, it is possible to obtain a rough estimate of the degree of activity which could be obtained. Assuming that the starting material is 2- ^{14}C -sodium acetate of specific activity 1 mc., 1 g. is the least amount which could be used for the preparation of the intermediate, e.g., chloroethylacetate. If all this were used for the synthesis of vitamin A (see Baxter, 1952) the total yield of vitamin A would be 3.3 g., assuming that all reactions result in 100% yields. The specific activity of the vitamin A would then be 0.36 μc./mg.C. In comparison, starting with $Ba^{14}CO_3$ at the same level of activity, the detached leaf method would give 0.8 - 1.0 mg. β-carotene with a specific activity of 0.3 μc./mg. C. The ^{14}C-acetate is, however,

about five times as expensive as $Ba^{14}CO_3$, so that β-carotene with a specific activity of 1.5 μc./mg.C. could be produced for the same initial outlay. Hence, the purely synthetic method would not result in a product with a higher specific activity but it would have the great advantage of producing an extremely high yield of material compared with the biosynthetic method. Thus enough material could be obtained from a single synthesis to suffice for a great number of experiments. It is considered, therefore, that an investigation of the chemical synthetic method should prove profitable. However, for experiments in which ^{14}C -β-carotene is to be used directly, as in the study of the conversion of β-carotene into vitamin A, the biosynthetic method is the obvious method to use.

PART II.

THE CHEMICAL OXIDATION OF β-CAROTENE

CHAPTER V.

INTRODUCTION

This study of the chemical oxidation of β-carotene was undertaken primarily in order to devise a method for obtaining radioactive vitamin A in good yield from the labelled β-carotene; the alternative biological method would involve the tedious procedure of feeding the β-carotene to experimental animals and then isolating the vitamin A from the livers, a method which would result in very low yields (less than 5%). It was necessary, therefore, to investigate the possibility of obtaining the vitamin A by a chemical oxidation of β-carotene which would be more rapid and efficient than the biological method.

Although this was the primary aim, it was found that this study of the chemical oxidation of β-carotene had an important bearing on the question of the biological oxidation of β-carotene to vitamin A. Many chemical and biochemical processes follow

similar patterns, so by studying the mechanism of the purely chemical process it may be possible to assume that analogous reactions occur in the animal body. This possibility will be discussed in more detail when the biological conversion of β-carotene into vitamin A is considered in a later section. In addition, the chemical oxidation of β-carotene has been the means for obtaining certain intermediate compounds, the β-apo-carotenals, which were used in the investigation of the *in vivo* conversion of β-carotene into vitamin A. Thus the development of a chemical method for the oxidation of β-carotene has had important applications to the other investigations reported in this thesis. Even apart from these applications, mention should be made of the importance of the method in related studies, such as the mechanism of carotenogenesis; by the oxidative degradation into smaller fragments of β-carotene or other carotenoids which have been biosynthesized from radioactive precursors it should be possible to obtain information regarding the labelling pattern by radioactive assay of the fragments. Finally, the importance of the method in the purely chemical problems of the constitution of the carotenoids should be stated. Although the constitution of β-carotene has been fully worked out it is possible that the method could be extended to other carotenoids whose structures are unknown.

To summarize then, the chemical oxidation of β-carotene has the following applications:-

(a) It provides a method for obtaining radioactive vitamin A from radioactive β-carotene.

(b) It provides information regarding the mechanism of the reaction which is useful in studying the in vivo conversion of β-carotene into vitamin A, and incidentally provides the intermediate compounds used in this study.

(c) It can be applied to investigations on carotenogenesis to determine the labelling pattern of isotopically labelled β-carotene.

(d) The method can probably be extended to unknown carotenoids as a means of establishing chemical constitutions.

Previous Investigations on the Chemical Oxidation of β-Carotene.

The introduction of methods of oxidative degradation was an important step in the elucidation of the structure of the carotenoids. The development of these methods has been treated fully by Karrer and Jucker (1950) and will only be considered briefly here.

The oxidation of β-carotene with potassium permanganate or with ozone gave the compounds: α,α-dimethylglutaric acid, α,α dimethyl succinic acid, dimethylmalonic acid and α,α dimethyl-

δ-acetyl valeric acid (geronic acid). These products are also obtained by the oxidation of β-ionone so this proved the presence of a β-ionone ring in the β-carotene molecule. Further investigations by Karrer and his co-workers (1937) showed that by using alkaline potassium permanganate the open conjugated chain was attacked preferentially, resulting in long chain aldehydes, the β-apo-carotenals, which still possess an intact β-ionone ring. Using CrO_3, however, this reagent appears to attack mainly the double bonds in the β-ionone rings, giving rise to a series of ketonic derivatives, such as β-carotenone and semi-β-carotenone, still possessing intact conjugated chains (Kuhn and Brockmann, 1935). The oxidation of β-carotene appears, therefore, to follow two main routes, according to the reagent employed; (a) a fission of the β-ionone ring double bond, and (b) an attack on the double bonds in the open conjugated chain. In the present investigation the main interest is centred on the latter type of oxidation since the ultimate aim is the production of vitamin A and related substances possessing an intact β-ionone ring.

Ever since the conversion of β-carotene into vitamin A was shown to occur in the animal body many attempts have been made to try to reproduce the *in vivo* conversion under *in vitro* conditions or by chemical methods. Most of the *in vitro* studies have produced

negative or indefinite results (vide infra) but chemical methods have met with more success.

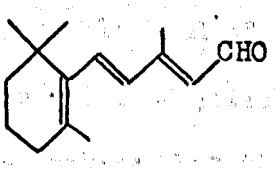
In the alkaline $KMnO_4$ oxidations reported by Karrer and his associates (loc. cit.) there was no evidence for the formation of vitamin A aldehyde (retinene) in addition to the β-apo-carotenals. This seemed rather surprising but a repetition of the method by other workers (see Hunter and Williams, 1945) also failed to reveal the presence of any vitamin A aldehyde. This naturally led to the use of other oxidizing agents. The first successful attempt to produce vitamin A aldehyde from β-carotene was reported by Hunter and Williams (1945). They used hydrogen peroxide as the oxidizing agent and the reaction was carried out in chloroform-acetic acid solution. The vitamin A aldehyde formed was reduced to vitamin A alcohol by the Pondorff method and the final yield was 0.4 - 0.5%. In order to try to improve this poor yield, Goss and McFarlane (1947) suggested the use of hydrogen peroxide together with osmium tetroxide to act as a catalyst, since OsO_4 was employed successfully by Criegee (1936) for the H_2O_2 oxidation of anethole and other ethylenic compounds.

Goss and McFarlane (loc. cit.) oxidized β-carotene (in ethereal solution) using a 2% aqueous solution of OsO_4 and a 30% aqueous solution of H_2O_2. The absorption spectrum of the product

showed maxima at 283, 325 and 370 mμ. (measured in cyclohexane). They did not attempt a chromatographic separation of the oxidation products but simply measured the intensity of the blue colour given with $SbCl_3$ at 620 mμ and calculated the yield to be 30 - 40% of the theoretical conversion of β-carotene to vitamin A. These workers assumed that vitamin A alcohol is formed directly by this reaction but as was shown later, aldehydes are the main products. Also, their method for calculating the yield can be criticized since other products of the oxidation will give blue colorations with the Carr-Price reagent. It did appear, however, from the shape of the absorption spectrum of the product, that the reaction had proceeded more effectively in the presence of OsO_4.

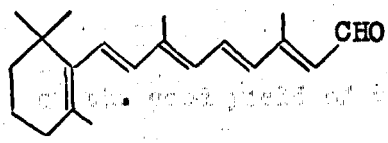
The OsO_4/H_2O_2 oxidation of β-carotene was repeated by Wendler, Rosenblum and Tishler (1950) who obtained three main products by chromatographic separation of the oxidation product. These were:-

(a) β-ionylideneacetaldehyde



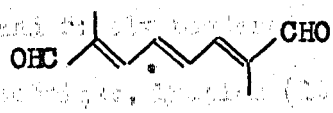
λ max. 315 mμ. (cyclohexane)

(b) Vitamin A aldehyde. (yield 30%)



λ max. 374 mμ (isooctane), 376 mμ (cyclohexane)

(c) 2,7 dimethyloctatriendial (yield 6 - 8%)



λλ max. 306, 320, 336 mμ. (isooctane)

The vitamin A aldehyde was reduced to vitamin A alcohol in good yield using lithium aluminium hydride ($LiAlH_4$). These results showed that the central double bond of the β-carotene molecule is not necessarily the main point of attack of the reagent as was assumed by the previous workers, but that fission also occurs at laterally placed double bonds. This, as suggested by Wendler et al. (1950) is more in keeping with physico-chemical theory which

predicts that the central double bond of a polyene chain possesses greater stability than the terminal double bonds (Zechmeister et al. 1943).

In view of the good yield of vitamin A aldehyde obtained by these workers it was decided to reinvestigate this reaction in more detail.

In conclusion, mention should be made of the remarkable claim of Meunier et al. (1950) that 60 - 80% yields of retinene can be obtained by agitating an ethereal solution of β-carotene in the presence of air and finely powdered electrolytic manganese dioxide. Using the same technique, Meunier (1951) also claimed that good yields of vitamin A_2 aldehyde were produced by the MnO_2 oxidation of lycopene. However, until these results are confirmed they must be accepted with some reserve. Dr. H. R. Cama (1951), formerly of this department, attempted the oxidation without success, even with MnO_2 provided by the late Dr. Meunier.

CHAPTER VI.

THE PRODUCTS OF CHEMICAL OXIDATION OF β-CAROTENE.

In this investigation the method used by Wendler *et al.* (1950) was adopted as a basis and certain modifications and refinements of the technique were developed during the course of the work, with the result that it has been possible to extend considerably information regarding the oxidation products and the mechanism of the reaction.

EXPERIMENTAL.

(i) General Procedure.

The β-carotene used was the commercial grade (Lights or B.D.H.). This was always purified immediately prior to an oxidation by chromatography on alumina (usually 25% deactivated). It was invariably found that the β-carotene contained a considerable amount of impurity which was strongly adsorbed as a series of coloured bands near the top of the column and the recovery of pure β-carotene was usually only in the 50 - 60% region. The amount of the purified β-carotene was determined spectroscopically and the

Facing Page 133.

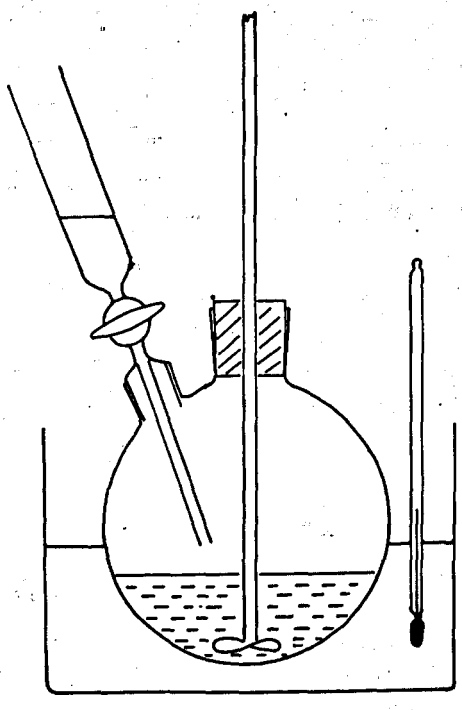


Fig. 1.

Apparatus for the H_2O_2/OsO_4 Oxidation of β-carotene.

spectral characteristic were also taken as being a good indication as to the degree of purity.

The light petroleum was then removed in vacuo in the same flask in which the reaction was to be carried out. The dry residue of β-carotene was then dissolved in an appropriate volume of dry ether, depending on the amount of β-carotene being oxidized. Up to 50 mg. β-carotene, 50 ml. ether was usually found to be the optimal volume, while for weights between 50 and 200 mg. it was convenient to dissolve the β-carotene in a volume in ml. equivalent to the weight in mg. These figures are quite arbitrary since it has not been possible to assess fully the effect of total volume on the course of the reaction, but the aim has been to try to dissolve the β-carotene in the minimum volume of solvent as far as practicable; for very small weights (less than 10 mg.) it is not possible to reduce the volume much below 10 ml. because of the practical difficulties involved, while for amounts of the order of 1 g. the reaction appears to proceed quite efficiently with the β-carotene in the form of a suspension in 500 ml. ether (Wendler et al., 1950).

The reaction was carried out in a two-necked 500 ml. "Quickfit" round-bottomed flask. One neck was fitted with an electric stirrer to agitate the reaction mixture, while in the other was placed a dropping funnel for making the reagent additions (Fig. 1.).

The flask was immersed in a water bath controlled at a fixed temperature. The preliminary trials of the reaction were then carried out according to the method of Wendler et al. (1950) except for a few modifications. The procedure was as follows (all additions are based on a weight of 1 g. β-carotene in suspension in 500 ml. ether). 100 g. Anhydrous Na_2SO_4 were added to the ethereal solution of β-carotene and the stirrer started. 5 ml. of a 2% aqueous solution of OsO_4 were added and the reaction mixture agitated until the mixture had darkened noticeably due to osmic ester formation. (According to Wendler et al. (loc. cit.) this usually occurs after about 15 minutes but with the smaller amounts of β-carotene used in the present investigation there was no detectable colour change after the OsO_4 addition and the H_2O_2 was usually added 5 minutes later). 20 ml. of 30% H_2O_2 were then added with vigorous stirring over a period of 5 minutes. The reaction mixture was then stirred for a time until it became light orange in colour (20 - 25 minutes). At this point 30 ml. of a 5% aqueous solution of $NaHCO_3$ was added slowly with cooling to 0°. After the effervescence had subsided, the ether solution was decanted into a separating funnel and the Na_2SO_4 washed by decantation with ether. The combined ether washings were washed with water several times, the first washing being retained for examination of water-soluble

oxidation products. Osmic oxides were then removed by allowing the ether solution to pass through a small column containing 5 gm. activated alumina; the osmic oxides were retained at the top of the column as a black band. This was adopted as an alternative to the method used by Wendler et al. who removed the osmic oxides by washing with 5% aqueous KOH. The ethereal solution of the oxidation product was then dried over anhydrous Na_2SO_4, filtered and the ether removed under reduced pressure. The product was then dissolved in light petroleum, examined spectroscopically and finally chromatographed to separate the various oxidation products.

In the initial experiments the primary aim was to examine the reaction in order to assess the optimal conditions for the production of vitamin A aldehyde in good yield. It was found, however, that the reaction was extremely variable and the time taken for the colour to change to that characteristic of retinene was usually considerably longer than the 25 minutes quoted by Wendler et al. and often completion of the reaction necessitated the addition of further quantities of the reagents. Wendler et al. did not appear to maintain the reaction mixture at a fixed temperature but presumably carried out the reaction at ambient room temperature. It was decided, therefore, to control this variable by maintaining the mixture at a fixed temperature throughout the

reaction. Reactions were carried out at 0°, 10° and 25° C. Although the differences in reaction rates at these temperatures did not appear to be striking it was decided to adopt a temperature of 25° arbitrarily for all oxidations. Only in this way could valid assumptions be made in comparing reaction rates when the other variables, the reagent concentrations, were varied. In spite of the controlled temperature, the reaction was still extremely erratic. This presented a severe handicap in attempting to determine the course of the reaction and in trying to standardize the procedure in order to be able to terminate the oxidation at any required stage. The variability of the reaction was also noticed by Wendler et al. (1950) who suggested that it was due to heterogeneous nature of the reaction mixture when aqueous solutions of the reagents were used. These workers mentioned that the oxidation was more effective giving greater reproducibility when carried out homogeneously by employing proportionate amounts of 90% aqueous H_2O_2 and 2% OsO_4 in t-butanol. It was decided, therefore, to adopt this procedure, but instead of using 90% H_2O_2 to prepare a solution of H_2O_2 in t-butanol and thus avoid the addition of water altogether.

(ii) Preparation of Reagents in t-Butanol Solution.

The solution of H_2O_2 in t-butanol was prepared according to the method of Milas and Sussman (1936) which is as follows:- To 100 ml. of 30% H_2O_2 were added 400 ml. of pure t-butanol (m.pt. 25.5°) and the mixture treated with small portions of anhydrous Na_2SO_4 whereby two layers separated out. The alcohol layer, which contained most of the H_2O_2 was removed and dried with anhydrous Na_2SO_4 and finally with anhydrous $CaSO_4$. This gave a solution of 7.4% H_2O_2 in t-butanol (determined by titration with standard $KMnO_4$). If necessary this solution could be concentrated by vacuum distillation of the alcohol to any desired concentration without any loss of peroxide provided all-glass apparatus is used. It was decided, however, to use the solution without further concentration since a weaker solution has an advantage in the oxidation reaction, particularly for small amounts of β-carotene, that because of the larger volume required the reagent can be added dropwise over a longer period and thus ensuring a more uniform distribution in the reaction mixture. This solution of peroxide in t-butanol kept well at 0° in a glass-stoppered bottle. The concentration of the H_2O_2 was checked at intervals and there did not appear any considerable decrease even after a period of several months.

OsO_4 dissolves readily in t-butanol and the solution is perfectly stable provided no isobutylene is present. Otherwise most

of the OsO_4 is reduced into an insoluble black colloidal oxide which is a very active catalyst for the decomposition of H_2O_2. It was found that if this black oxide does form, OsO_4 can be regenerated by adding a few drops of the H_2O_2 solution in t-butanol. A 2% (w/w) solution of OsO_4 in t-butanol was prepared. Since the density of t-butanol at 26° is 0.78 it was necessary to make the appropriate correction when calculating the volume of this reagent proportionate to 2% (w/w) aqueous OsO_4.

Using these reagents it was found that the reaction proceeded much more smoothly with greater reproducibility than when aqueous solutions were used. The oxidation was completed in a shorter time, usually less than 25 minutes, and sometimes it was necessary to reduce the H_2O_2 concentration to one-half or even one-quarter that used by Wendler et al. (loc. cit.) in order to control the rate of reaction. This was particularly important in the oxidations for the production of the β-apo-carotenals when it was only necessary to allow the reaction to proceed to the half-way stage between β-carotene and vitamin A aldehyde.

(iii) Chromatographic Separation of the Oxidation Products.

The gross product of an oxidation was chromatographed on alumina; for optimal separation a mixture of 3 parts activated and 1 part deactivated alumina was found to be best. The amount of

alumina used depended, of course, on the initial weight of β-carotene oxidized; for the smaller weights, e.g., less than 10 mg., 15 - 20 g. alumina in a column 1.5 cm. internal diameter were used, while for weights between 100 and 175 mg., 100 g. alumina were used in a 2.5 cm. diameter column. The product was poured on to the column in a minimum volume of 40 - 60° light petroleum, and after development, each zone was eluted by progressively increasing the polarity of the solvent with dry, freshly distilled ether. Each fraction was distilled under reduced pressure to remove the solvents and the spectra were then recorded in light petroleum solution.

The general chromatographic pattern of each oxidation was much the same but differing mainly in the relative amounts of each constituent product, depending upon the stage to which the reaction had been taken. The chromatographic separation which will be described is, therefore, an example of a typical oxidation, which illustrates the type of chromatogram usually obtained and comprising the complete range of the main oxidation products.

165 mg. of pure β-carotene were dissolved in 165 ml. ether in the reaction flask. 1.06 ml. 2% OsO_4 in t-butanol were added and five minutes later 11 ml. of 7.4% H_2O_2 in t-butanol were added (the OsO_4 addition was equivalent to the concentration used by Wendler *et al.*; the H_2O_2 concentration was 80% of their concentration)

and the reaction was allowed to proceed for 37 minutes at 25°. The reaction was then stopped by adding 5% $NaHCO_3$ with cooling to 0° and the further experimental procedure continued as previously described.

The gross oxidation product in light petroleum was chromatographed on 100 g. alumina (25% deactivated) in a column 2.5 cm. diameter.

The appearance of the fully developed chromatogram is shown in Fig. 2. and described in Table 1.

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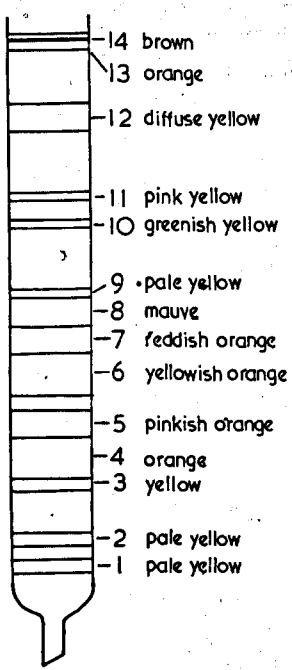

Fig. 2.

Fully developed chromatogram of oxidation products of β-carotene.

Table 1.

Separation of oxidation products of β-carotene on a mixture of 3:1 (w/w) activated and deactivated alumina using light petroleum containing ether as developer.

(Zones numbered in order of increasing adsorptive power)

Zone No.	Description	Eluant
14.	brown band.	-
13.	narrow orange.	-
12.	pale diffuse yellow.	100% ethanol.
11.	pink yellow.	100% ether.
10.	greenish-yellow.	50% ether in l. petrol.
9.	pale yellow.	24% " " " "
8.	mauve.	20-24% " " " "
7.	intense reddish-orange.	14-20% " " " "
6.	broad yellowish-orange.	10-12% " " " "
5.	pinkish-orange.	8% " " " "
4.	broad orange.	6% " " " "
3.	yellow.	6% " " " "
2.	pale yellow.	4% " " " "
1.	pale yellow.	2% " " " "

RESULTS.

(i) Spectroscopic Properties of the Oxidation Products.

The spectroscopic properties of a compound in conjunction with its chromatographic behaviour are usually useful guides to identifying the compound. Since the structure of the parent compound is known in this case the task is made simpler and certain of the products can be readily identified with compounds of known structure and properties. This is particularly true in the case of vitamin A aldehyde and other compounds such as β-ionylidene acetaldehyde, which have been characterized by previous workers using the same oxidation technique (Wendler et al., 1950). With some of the other products, however, the spectra do not appear to correspond with those of any known compounds and it has been necessary to try to characterize these by various physical and chemical methods.

The following are the spectroscopic properties (in light petroleum) of each fraction obtained from the chromatographic resolution of the gross oxidation product (see Fig. 2. and Table 1.) and where possible, a definite or provisional structure has been assigned to each compound.

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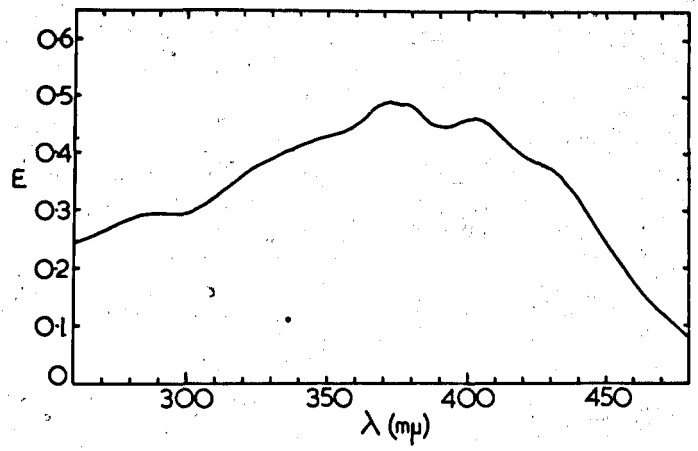


Fig. 3.

Spectrum of gross oxidation product.

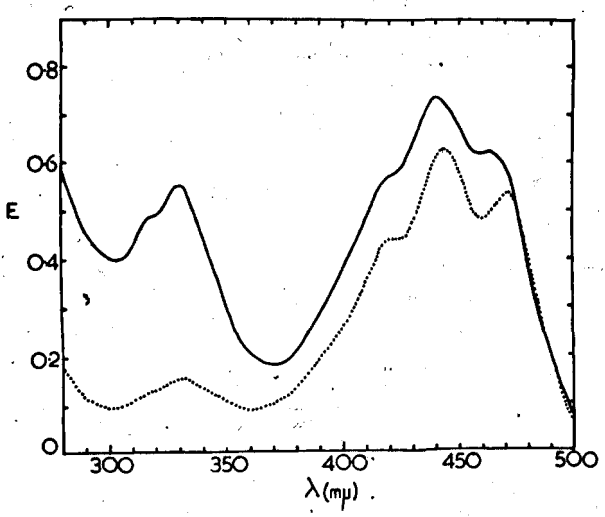


Fig. 4.

Fraction 1 ———

Fraction 2 .....

Gross Oxidation Product. (Fig. 3.)

Absorption maxima 290, 371, 379, 403, shoulder 430 mμ. It will be seen later that the absorption spectrum of the gross product is a good indication of the extent to which the oxidation has been carried. In this particular case the 371 mμ. max. is due to the presence of vitamin A aldehyde and the 403 mμ. max. and 430 mμ. shoulder indicate the presence of β-apo-carotenals.

Fraction 1. (Fig. 4.)

λ max. pale yellow zone (a) 330 mμ, shoulder 318 mμ.

" " " " (b) 440, 463 mμ, shoulder 420 mμ.

This may be a mixture of two substances, the second of which is probably an isomer of β-carotene. This fraction is only a minor constituent so that further investigation was unwarranted.

Fraction 2. (Fig. 4.)

Pale yellow zone.

λλ max. 332, 420, 443, 472 mμ.

This minor constituent appears to be a β-carotene isomer.

Fraction 3. (Fig. 5.)

Yellow zone.

λ max. (a) 280 mμ.

" " (b) 446, 476 mμ. inflexion 425 mμ.

The 280 mμ material may correspond to β-ionone (λ max. in ethanol 223, 296 mμ. according to Braude et al. 1949). The λ max. in

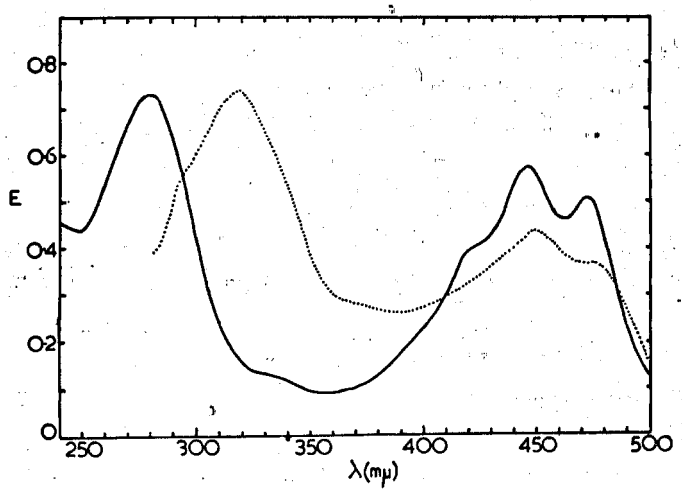


Fig. 5.

Fraction 3 ——— ; Fraction 4 .....

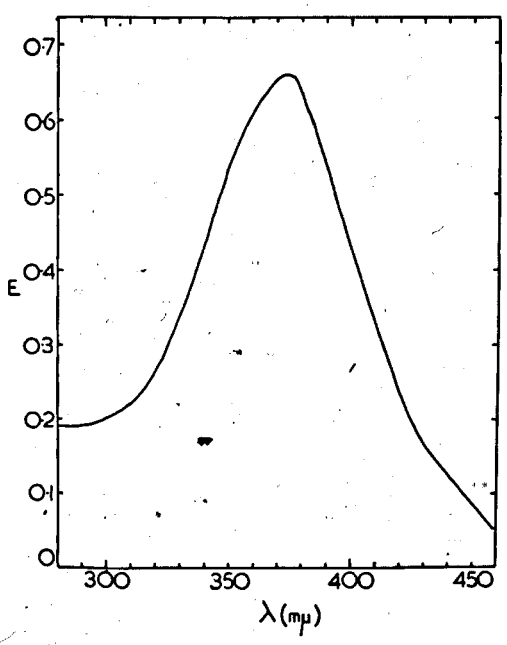


Fig. 6.

Fraction 5. (in cyclohexane)

(retinene)

alcohol would probably be in this region due to the spectral shift caused by the change of solvent. The absorbing component (b) is a β-carotene isomer.

Fraction 4. (Fig. 5.)

Orange zone.

λ max. (a) 318 mμ.

" (b) 448, 476 mμ.

The component with λ max. 318 mμ is probably identical with β-ionylidene acetaldehyde (λ max. 315 or 318 mμ according to isomeric form) which was shown to be one of the main products of this oxidation by Wendler et al. (1950). The component (b) is residual β-carotene.

Fraction 5. (Fig. 6.)

Pinkish-orange zone.

λ max. 373mμ. (in cyclohexane)

" 367 mμ. (in 40 - 60° light petroleum).

This compound is vitamin A aldehyde which was reported by Wendler et al. (loc. cit.) to be produced in 30% yield by the H_2O_2/OsO_4 oxidation of β-carotene.

Fraction 6. (Fig. 7.)

Yellowish-orange zone.

λ max. 408 mμ. Slight inflexions at 385 and 430 mμ.

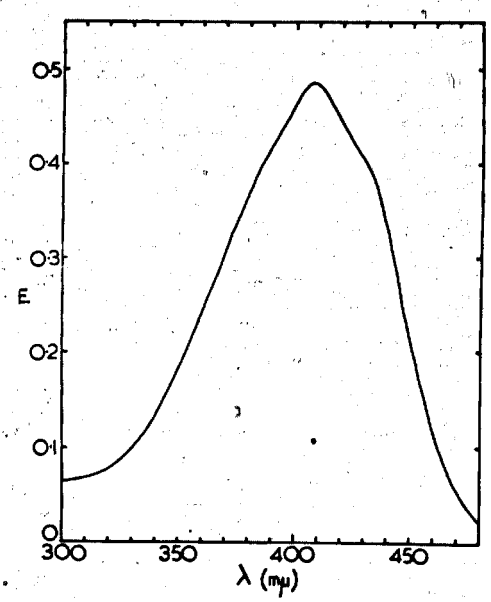
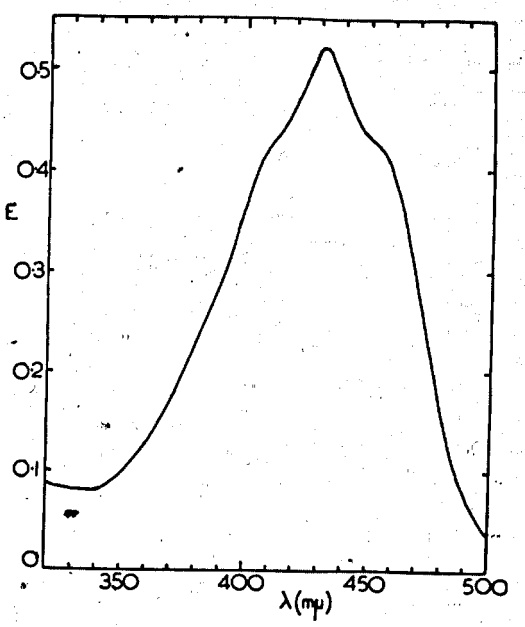


Fig. 7.

Fraction 6.
(β-apo-12'-carotenal).

Fig. 8.

Fraction 7.
(β-apo-10'-carotenal)



This substance was tentatively assigned the structure of β-apo-12'-carotenal. Yield approximately 10%.

Fraction 7. (Fig. 8.)

Reddish-orange zone.

λ max. 432 mμ. Inflexions at 410 and 454 mμ.

This substance was tentatively assigned the structure of β-apo-10'-carotenal. Yield approximately 5%.

Fraction 8. (Fig. 9.)

Mauve zone.

λ max. 452 mμ. Slight inflexions at 435 and 475 mμ.

This substance was tentatively assigned the structure of β-apo-8'-carotenal. Yield approximately 2%.

Fraction 9. (Fig. 10.)

Pale yellow band.

λλ max. 305, 319, 336 mμ. (fine structure).

This is identical with 2,7 dimethyloctatriendial reported by Wendler et al. (loc. cit.) who recorded the following maxima in isooctane: 306, 320, 336 mμ. These workers, however, described its appearance on the column as a "sharp red zone". This colour is actually due to fraction 8 (mauve band) which often masks the pale yellow of fraction 9 and in some cases the two zones may be eluted simultaneously.

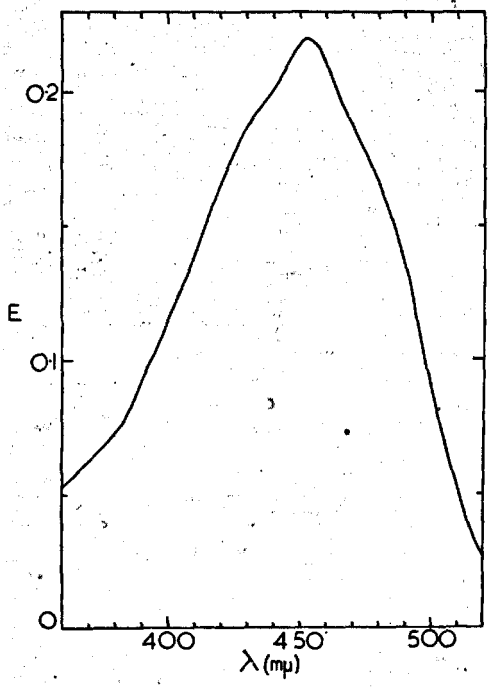


Fig. 9.

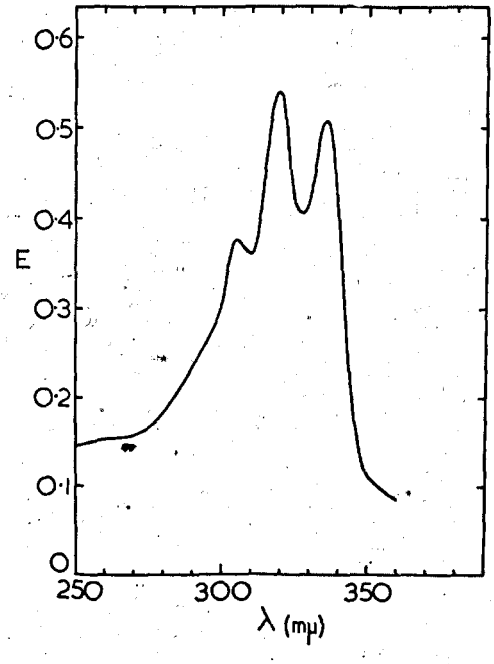
Fraction 8.

(β-apo-8'-carotenal)

Fig. 10.

Fraction 10.

(2,7-dimethylocta-triendial)



Fraction 10. (Fig. 11.)

Greenish-yellow band.

λλ max. 332, 349, 368, 401 mμ. Shoulder 378 mμ (fine structure).

This fraction was rechromatographed and a single yellow band was obtained indicating that the fraction is probably homogeneous. A redetermination of the spectrum, however, showed that the absorption maxima were now: 349, 367, 378, 402 mμ. with a shoulder at 333 mμ., i.e., the shoulder at 378 had now become a peak and the maximum previously at 332 was now an inflexion. In addition, there had been changes in the relative heights of the peaks (see Fig. 11.). These changes indicate that the substance had probably undergone isomerization. Because of its position on the column and the nature of the absorption spectrum, this substance is probably a dialdehyde, consisting possibly of five conjugated double bonds, viz., 4,9 dimethyldodecapentaendial or 2,6,11 trimethyldodecapentaendial.

Fraction 11. (Fig. 12.)

Pink-yellow band.

λλ max. 329, 350, 368, 400, 428 mμ. Inflexion 378 mμ. Probably a mixture which includes a higher dialdehyde.

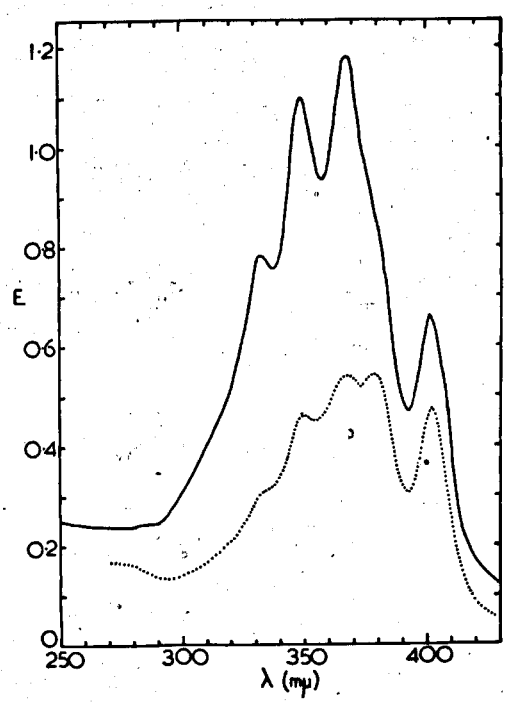


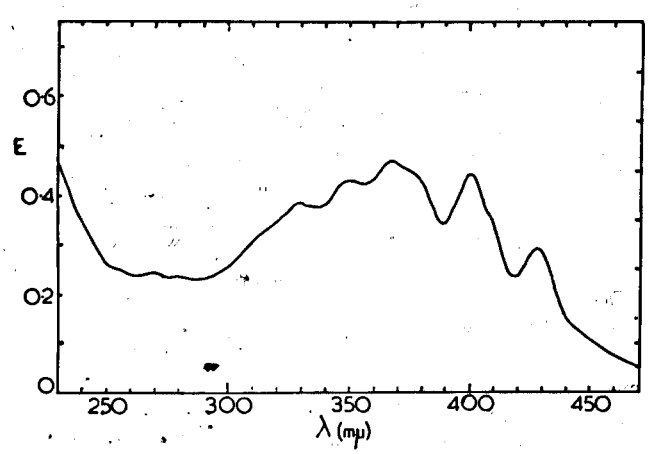
Fig. 11.

Fraction 10 ——— ;

Same after isomerization .....

Fig. 12.

Fraction 11.



Fraction 12.

Diffuse yellow zone.

Steep end absorption but no maxima in near U.V. or visible region.

(ii) Examination of the Water-soluble Products of the Oxidation.

The smaller molecular weight fragments of the oxidation would be retained in the aqueous phase after the initial washing of the ethereal solution of the gross oxidation product. These water-soluble products were examined as follows.

Excess 2,4 dinitrophenylhydrazine in HCl solution was added to the aqueous layer obtained from an oxidation in which aqueous solutions of reagents were used (since the presence of t-butanol or its oxidation products might interfere). The mixture was allowed to stand at 0° overnight and then the orange precipitate which formed was washed, dried and dissolved in ethanol. The spectrum of the 2,4 dinitrophenylhydrazone was determined. The absorption maximum was at 356 mμ. For comparison the curve for pure 2,4 dinitrophenylhydrazine in ethanol was also determined. This exhibits maxima at 260 and 351 mμ. (Fig. 13) which is in agreement with the maximum of 352 mμ obtained by Roberts and Green (1946) and the values of 258 and 350 mμ reported by Braude and Jones (1945).

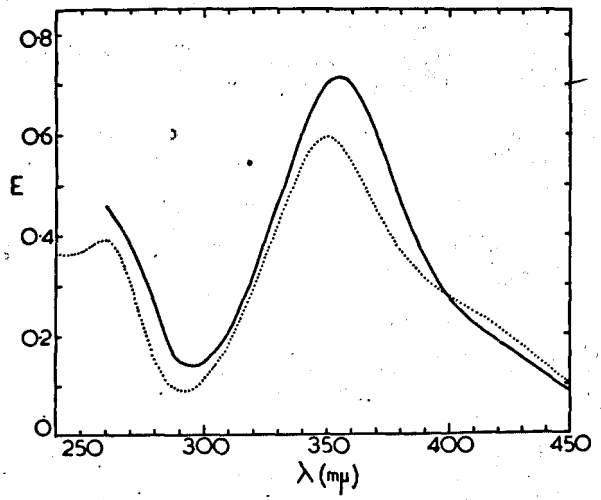


Fig. 13.

2,4 DNPH of water-soluble oxidation products ——— ;

2,4 dinitrophenylhydrazine .....

(both in ethanol)

The 2,4 dinitrophenylhydrazone(s) of the water-soluble oxidation products were then subjected to partition chromatography on paper. 17.7 mg. of the 2,4 DNPH were dissolved in 2 ml. ethanol and 2 ml. $CHCl_3$ and 0.8 ml. of this solution was pipetted on to a line 9 inches long, 1-inch from the base of a sheet (10 x 13.5 inches) of No. 1. Whatman paper. The paper was folded into a cylinder, placed in the tank and developed using the n-heptane/methanol system with ascending development. Three zones were obtained, as follows:-

	Distance from origin (cm.)			Mean Rf.
Solvent front.	27	27.8	29	
Zone 1 (large)	14.8	14.5	14.8	0.53
" 2 (very small)	22.2	22.7		0.79
" 3 (small)	23.6	25.2	26.5	0.90

Zone 1 was the major component and this was eluted with ethanol and the spectrum determined. The absorption maximum was at 355 mμ. (Fig. 13). This agrees well with the maximum of 356 mμ obtained for the 2,4 DNPH of acetaldehyde by Roberts and Green (1946). For further confirmation, the melting point and "alkali-shift" of the 2,4 DNPH would have to be determined.

(iii) Determination of the Yield of Vitamin A Aldehyde (retinene).

According to Wendler et al. (loc. cit.) the yield of retinene obtained by the H_2O_2/OsO_4 oxidation is 30%. Attempts were made to confirm this result by determining the yield of retinene obtained from a number of oxidations. The yield was found naturally to vary considerably, depending upon the stage at which the reaction was terminated. In the initial experiments, using aqueous solutions of the reagents, it was not very easy to stop the oxidation at the desired point because of the erratic nature of the reaction and because of the difficulty experienced by the operator in judging the optimal colour change. With the improved method using reagents in t-butanol and after greater practice on the part of the experimenter, it was found possible to control the reaction within broad limits.

The yields of retinene obtained from four oxidations are shown in Table 2.

Table 2.

Yields of vitamin A aldehyde obtained by the H_2O_2/OsO_4 oxidation of β-carotene.

Oxidation.	Yield of Vitamin A aldehyde (%) (i) from U.V. spectrum.*	(ii) by Carr-Price reaction. ø
(1) (Reagent in aqueous solution)	5.7	5.5
(2) " " "	8.8	-
(3) (Reagents in t-butanol solution)	11.2	-
(4) " " "	14.3	-
	10.5 (after rechromatography)	

*$E^{1\%}_{1cm.}$ in light petroleum (40 - 60°) = 1685 (Ball *et al.* 1948).

ø$E^{1\%}_{1cm.}$ at 664 mμ. = 3400 (Ball *et al.*, 1948).

It will be seen that the maximum yield obtained is considerably lower than the 30% claimed by Wendler *et al.* (*loc. cit.*) However, this figure may have been overestimated since the retinene they obtained may have been contaminated with other oxidation products such as the β-apo-carotenals. (The absorption spectrum of the gross oxidation product showed shoulders in the 410 and 450 mμ. regions, which indicated the presence of β-apo-carotenals.) The chromatographic technique they employed certainly seems to suggest this, for they attempted to separate 1 g. of gross oxidation product on an extremely small amount of alumina, viz., 100 g., and obtained only three main bands. It is inevitable, therefore, because of this gross overloading of the column, that it would not be possible to obtain a fine resolution of the oxidation products, and the fractions they obtained must have actually been mixtures.

The yields of vitamin A aldehyde obtained in the present investigation, although low, are definitely superior to those which could be obtained by the biological method. This method could, therefore, be used with advantage for the preparation of radioactive vitamin A aldehyde from ^{14}C -β-carotene.

DISCUSSION.

The results obtained confirm the earlier findings of Wendler et al. (1950) but it has also been shown that the number of major oxidation products formed is much greater than that obtained by these workers. As already pointed out, in this previous work many of the products were probably not identified due to the inadequate method of separation used.

The point of immediate interest was the nature of these other oxidation products since this knowledge would possibly throw some light on the mechanism of the reaction. An attempt was made, therefore, to try to identify the chemical constitution of these compounds. This was done by establishing provisional structures based on theoretical physico-chemical considerations and then trying to confirm these experimentally by chemical methods.

ELUCIDATION OF THE STRUCTURES OF THE OXIDATION PRODUCTS.

1. Method based on relationships between absorption spectra and chemical constitution.

Introduction.

Attempts have been made by numerous workers to formulate empirical relationships between the spectral properties of compounds and their chemical constitutions and try to apply them to the elucidation of substances of unknown structure. These empirical relationships can be roughly divided into two categories: (a) those based purely on rules derived from compounds of known constitution which are then formulated into a general equation, and (b) those which are derived from fundamental theoretical considerations based on resonance concepts or the theory of atomic or molecular orbitals. The former type is of limited application since different equations have to be formulated for different series of compounds, and in many cases the relationship breaks down when applied to a compound in the series for which it was formulated. This was found to be the case for the equations proposed by Fieser (1950) for the carotenoids and polyene systems in general. Again, the principle suggested by Frye (1951), although useful for the carotenoids, cannot be readily extended

to carotenoid derivatives. Relationships of the latter class, based on theoretical considerations, are, however, capable of a much wider application because of their more fundamental derivation.

The relationship between the degree of conjugation of the linear polyene series and the wavelength of maximum light absorption was studied by Lewis and Calvin (1939), who applied the classical theory of harmonic oscillation to electronic displacements of the mobile unsaturation electrons. The absorption of light causes the transition of the molecule from its ground state to an ionic, electronically excited state, the difference in energy of the two states determining the frequency of light absorbed. If it is assumed that the electronic displacements are harmonic oscillations, then the restoring force will be -Kx (Hooke's law), where x is the electronic displacement from the equilibrium position and K the restoring force constant. The energy required for these displacements will, therefore, be $-\frac{1}{2}Kx^2$. If the energy is quantised, then the energy levels associated with the successive energy states will be given by:

$$E_v = (v + \tfrac{1}{2})\, h\nu_o$$

where ν_o, the fundamental frequency is given by

$$\nu_o = \frac{\lambda}{c} = \frac{1}{2\pi}\sqrt{\frac{K}{m}}$$

The wavelength (λ) of the light absorbed by the unit vibrating chromophore will, therefore, be expressed by:

$$\lambda = 2\pi c \Big/ \sqrt{\frac{K}{m}}$$

If n such chromophores are joined, each with the same restoring force constant K and effective mass of vibrating electrons m, and if they vibrate in phase such that the system resembles a single oscillator, then the wavelength of the absorbed light can be expressed as:

$$\lambda = 2\pi c \Big/ \sqrt{\frac{K}{mn}}$$

By squaring both sides:

$$\lambda^2 = \frac{4\pi^2 c^2 . mn}{K}$$

the equation for a straight line is obtained,

$$\lambda^2 = An$$

where A is a constant.

It is interesting to note that the same equation was derived by Kuhn (1949) by a molecular orbital theory treatment.

The relation has been found to hold for the diphenylpolyenes (Lewis and Calvin, loc. cit.) and for the polyenals (Ferguson, 1948). Using the equation as a basis, Porter (1953) derived a relationship between the absorption maxima and degree of conjugation for the carotenes and colourless polyenes.

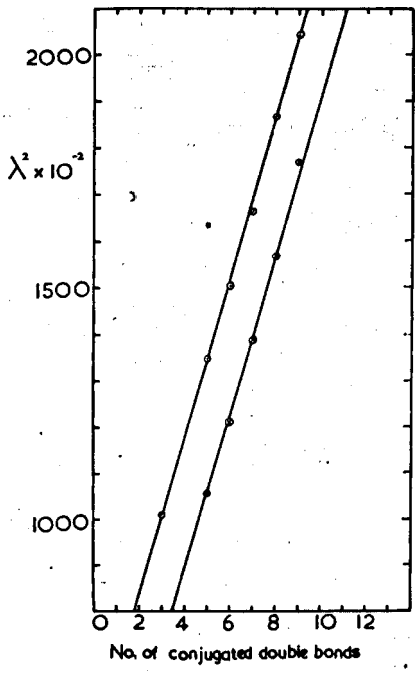


Fig. 14.

Relation between λ^2 and number of conjugated double bonds for

(i) the β-apo-carotenals —●—●—

(ii) the β-apo-carotenols —○—○—

It was decided, therefore, to try to obtain confirmatory evidence by applying the empirical relationship between absorption maxima and degree of conjugation derived by Lewis and Calvin (1939),

$$\lambda^2 = An$$

The results obtained are shown in Table 3, together with the values for known compounds belonging to the same series (β-ionylidene acetaldehyde, retinene$_1$ and retinene$_2$.) In addition, values for the alcohol series are given, the alcohols being formed by the $LiAlH_4$- reduction of the aldehydes (vide infra). It will be seen that the value of A for the different compounds is fairly constant which suggests close agreement with the equation. The values of λ^2 against n are plotted in Fig. 14. which illustrates the straight line relationship. There is a close agreement between the values of A (mean value in both cases = 16.7) for the two series of compounds, which is to be expected since the presence of the chromophoric aldehyde group in the carotenal series will merely cause a vertical shift of the curve by a constant amount.

The complete equations can be expressed in the form

$$\lambda^2 = An + B$$

as follows:-

(1) β-apo-carotenal series

$$\lambda^2 = 16.7 \times 10^3 n + 53.1 \times 10^3$$

(2) β-apo-carotenol series

$$\lambda^2 = 16.7 \times 10^3 n + 22.1 \times 10^3$$

where the values of B represent the intercepts on the λ^2-axis when n = 0.

Thus the β-apo-carotenal structures assigned to these unknown pigments formed by the oxidation of β-carotene are confirmed by the application of this empirical relationship between absorption maxima and degree of conjugation. This, however, cannot be regarded as unequivocal evidence for accepting these structures and it was necessary to obtain further confirmatory evidence by other methods.

Table 3.

Relation between the absorption maxima and number of conjugated double bonds of the β-apo-carotenal and β-apo-carotenol series.

Compound	No. of conjugated double bonds. n.	λ max. in light petroleum (mμ.)	$\lambda^2 \times 10^{-3}$	$A \times 10^{-3}$ $(A = \frac{\lambda^2 n - \lambda^2 3}{n - 3})$
(1) Aldehyde series.				
β-ionylidene acetaldehyde	3	318	100.5	
Vitamin A_1 aldehyde	5	369.5	136.6	17.75
Vitamin A_2 aldehyde	6	385	148.2	15.9
β-apo-12'-carotenal	7	408	166.5	16.5
β-apo-10'-carotenal	8	432	186.6	16.2
β-apo-8'-carotenal	9	452	204.3	17.3
(2) Alcohol series.				$(A = \frac{\lambda^2 n - \lambda^2 5}{n - 5})$
Vitamin A_1 alcohol	5	325	105.6	
Vitamin A_2 alcohol	6	348	121.1	15.5
β-apo-12'-carotenol	7	372	138.4	16.4
β-apo-10'-carotenol	8	396	156.8	17.0
β-apo-8'-carotenol	9	421	177.2	17.9

2. Chemical Methods.

(i) Formation of β-apo-carotenols.

The β-apo-carotenols were formed by the $LiAlH_4$ reduction of the aldehydes according to the method described by Brown (1951) which, briefly, is as follows:-

An excess of $LiAlH_4$ was suspended in 20 - 50 ml. dry ether in a 500 ml. two-necked flask fitted with a mechanical stirrer. The aldehyde, dissolved in dry ether, was added dropwise with stirring. The original colour was quickly dissipated and stirring was continued for about 20 minutes to ensure that complete reduction had occurred. Excess $LiAlH_4$ was then decomposed by the dropwise addition of water with cooling to 0°. The reaction mixture was washed with 10% aqueous KOH and the ether solution then washed with water until neutral, and finally dried.

The spectrum of the product (in light petroleum) was determined and the product then chromatographed on alumina to separate the alcohol from any residual aldehyde and by-products of the reaction.

(ii) Other Methods.

It was not possible, due to the pressure of other work, to subject the β-apo-carotenals to the exhaustive examination which would be required in order to characterize the compounds fully.

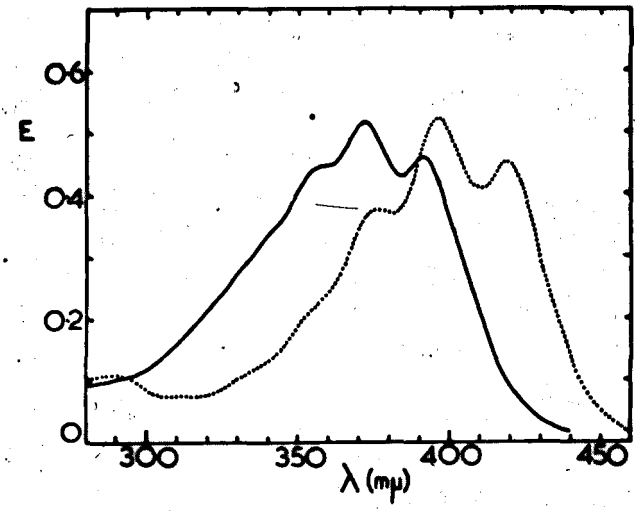


Fig. 15.

Spectra of β-apo-12'-carotenol ——

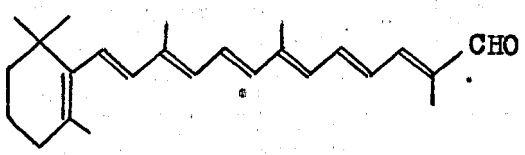
and β-apo-10'-carotenol .....

Certain properties of the compounds, however, were investigated. These included (a) the determination of the colours given by the $SbCl_3$ reagent by the method previously described, and (b) the formation of anhydro-β-carotenols by the method of Cama et al. (1952a).

Results.

(The spectral data refer to solutions in 40 - 60° light petroleum.)

(i) β-apo-12'-carotenal.



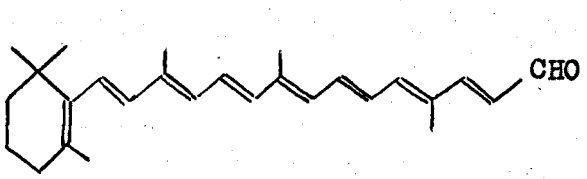
λ max. 408 mμ; slight inflexions at 385 and 430 mμ. (Fig. 7).

$SbCl_3$ colour test: greenish-blue colouration with λ max. 738 mμ.

Oxime: λ max. 392 mμ.

$LiAlH_4$ reduction gave β-apo-12'-carotenol with λλ max. 357, 372 and 391 mμ. (See Fig. 15.)

(ii) β-apo-10'-carotenal.



λ max. 432 mμ; inflexions at 410 and 454 mμ. (Fig. 8.)

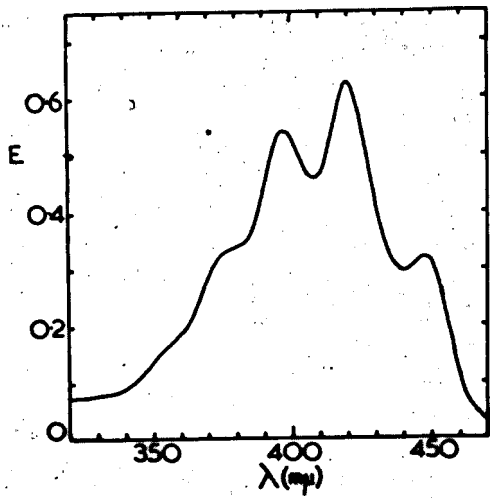
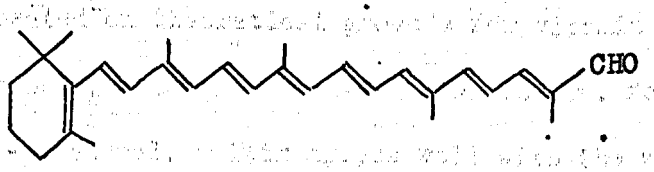


Fig. 16.

Spectrum of anhydro-β-apo-10'-carotenol.

$LiAlH_4$ reduction gave β-apo-10'-carotenol with λλ max. 374, 396, 418 mμ. (See Fig. 15.) Anhydro-β-apo-10'-carotenol: λλ max. 397, 420, 447 mμ.; shoulder at 380 mμ, infl. at 355 mμ. (Fig. 16).

(iii) β-apo-8'-carotenal.



λ max. 452 mμ; inflexions at 435 and 475 mμg. (Fig. 9.) Reduction in vivo (vide infra) gave β-apo-8'-carotenol with λλ max. 399, 421, 444 mμ.

Discussion.

The evidence for the β-apo-carotenal structures assigned to these compounds has come mainly from theoretical considerations on the relationship between absorption spectra and chemical constitution. This evidence has, however, received support from the results of various chemical tests applied to these compounds. This confirmatory evidence can be summarized as follows:-

(a) The chromatographic behaviour of the three pigments is in keeping with the structures suggested.

(b) Reduction, chemically and enzymically (vide infra) gave alcohols whose spectroscopic properties conform to those expected on theoretical grounds for vitamin A analogues.

(c) The $SbCl_3$ colour test gave λ max. 738 mμ. for β-apo-12'-carotenal. This agrees well with the value to be expected from theoretical considerations. If the compounds formed in the Carr-Price reaction are assumed to be unsymmetrical ions, λ max. should be a linear function of the number of conjugated double bonds (Ferguson, 1948). In the colour test, retinene$_1$ has a λ max. 664 mμ. (Ball et al., 1948) and retinene$_2$, λ max. 705 mμ. (Cama et al., 1952b) so that the calculated value for β-apo-12'-carotenal is 746 mμ.

β-apo-12'-carotenal and β-apo-8'-carotenal have been obtained previously by Karrer and his co-workers by the $KMnO_4$ oxidation of β-carotene and the corresponding carotenols were formed by reduction with aluminium isopropoxide and isopropyl alcohol (see Karrer and Jucker, 1950). The spectroscopic data for these compounds, however, do not agree with those obtained in the present investigation. A comparison of the two sets of results is given in Table 4.

Table 4.

Spectroscopic properties of the β-apo-carotenals and carotenols. Data obtained in present investigation compared with that obtained by Karrer et al.

Compound.	Absorption maxima (mμ.) (in light petroleum)		References.
	Present results.	Karrer et al.	
β-apo-12'-carotenal.	408	about 442 (diffuse)	Karrer and Solmssen (1937); Karrer et al. (1937).
β-apo-8'-carotenal.	452	454, 484	-ditto-
β-apo-12'-carotenol.	357, 372, 391.	No figures given.	
β-apo-8'-carotenol.	399, 421, 444.	423, 453.	von Euler et al. (1938a)

The values of the absorption maxima reported by Karrer et al. (loc. cit.) should, however, be viewed with caution since the spectroscopic measurements were probably made on the older types of instrument which depended on a photographic or visual determination of extinction values. It will be seen that Karrer et al. (1937) did not obtain a well-defined λ max. for β-apo-12'-carotenal, which leads one to suspect that they may not have actually obtained the pure substance. The main absorption maximum for β-apo-8'-carotenal shows reasonable agreement but Karrer et al. (loc. cit.) also obtained a subsidiary maximum at 484 mμ; this, however, may have actually been a shoulder or inflexion corresponding to that obtained in the present work.

Karrer and co-workers did not obtain β-apo-10'-carotenal by the $KMnO_4$ oxidation of β-carotene. β-apo-8'-carotenal appeared to be the main product of the oxidation but they did observe occasionally a very small zone on the chromatogram which they assumed to be β-apo-10'-carotenal (von Euler et al., 1938a). The amount present, however, was insufficient for isolation.

It has not been possible in the present investigation to obtain sufficient quantities of the β-apo-carotenals in crystalline form, either to make quantitative extinction measurements or for carbon and hydrogen analyses. It was necessary, therefore, to

assume extinction values in order that spectrophotometric assays could be made. It has been shown that the integrated intensity of an absorption band ("oscillator strength") can be related linearly to the number (n) of double bonds in the conjugated system (Lewis and Calvin, 1939; Braude, 1945). If, as an approximation the integrated intensity is measured by the maximum extinction coefficient it is found that the linear relation still holds (Lewis and Calvin, loc. cit.). The extinction values of the β-apo-carotenals were calculated, therefore, from the known data of retinene and β-carotene and it was found that the $E^{1\%}_{1cm.}$ values of β-apo-carotenals lie in the region 1900 - 2100. As a reasonable approximation, therefore, the $E^{1\%}_{1cm.}$ values of the β-apo-8', 10'- and 12'- carotenals were all assumed to be 2000.

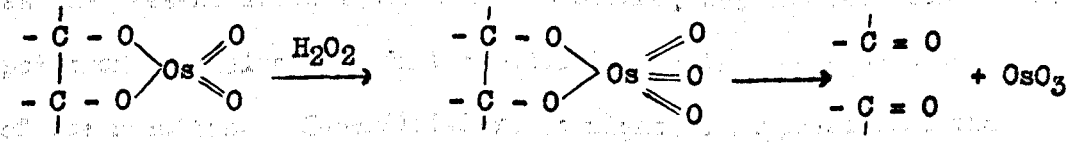
It should be noted that in the H_2O_2/OsO_4 oxidation of β-carotene, there was no evidence for the formation of β-apo-14'-carotenal, or at least if it is formed it must be so unstable that it is immediately transformed into vitamin A aldehyde. von Euler et al. (1938a), however, claimed that β-apo-14'-carotenal was formed in small quantities by the $KMnO_4$ oxidation of β-carotene.

CHAPTER VII.

THE MECHANISM OF THE H_2O_2/OsO_4 OXIDATION OF β-CAROTENE.

The H_2O_2/OsO_4 oxidation of β-carotene results in a fission of the molecule. This type of reaction is, however, very rare for most catalytic H_2O_2 oxidations do not involve fission of double bonds but merely hydroxylate the bonds with the formation of glycols (Johnson, 1951). The latter type of reaction has been widely studied and possible mechanisms suggested. So far as the oxidation of β-carotene is concerned, the same mechanism will probably apply to the initial stages of the reaction.

The H_2O_2/OsO_4 oxidation was first studied by Criegee (1936) who claimed that main products of the oxidation of olefinic substances were diketones or dialdehydes. The reaction mechanism suggested was as follows:-



in which the initial step is the formation of osmic esters.

The latter were isolated by Criegee (loc. cit.) after reacting olefins with OsO_4.

However, the work of Milas and Sussman (1936) showed that the main products of this reaction were glycols formed by hydroxylation of the double bonds. It was suggested then that the mechanism involved was a direct addition of free -OH radicals which had been formed by a catalytic split of H_2O_2 (Waters, 1945). More recent evidence, however, indicates that the more probable mechanism is the formation of a cyclic osmic ester followed by an oxidative hydrolysis (Mugdan and Young, 1949).

All the products of H_2O_2/OsO_4 oxidation of β-carotene are aldehydes, dialdehydes and ketones; there was no evidence for the formation of hydroxylated compounds. Thus the oxidation of β-carotene must either proceed by the type of mechanism suggested originally by Criegee (loc. cit.) or by the intermediate formation of glycols which are then immediately oxidized further to aldehydes.

A further and more important aspect of the oxidation as far as the present investigation was concerned, was the location of the point of initial attack on the molecule and the subsequent course of the reaction. Superficially, it might be supposed that the central double bond of the β-carotene molecule is the most vulnerable position for oxidative attack because it possesses sterically the

least hindered disposition. Previous work on the oxidation of β-carotene, however, did not confirm this supposition. The major products of the $KMnO_4$ oxidation (see Karrer and Jucker, 1950) are β-apo-carotenals which still possess the central double bond intact. Similarly, in the H_2O_2/OsO_4 oxidation of β-carotene there was evidence that fission could occur at bonds other than the central double bond (Wendler et al., 1950). A further study of this reaction in the present investigation has shown that in addition to the fragments obtained by Wendler et al. (loc. cit.) the β-apo-carotenals are also major products of the reaction. Thus the initial point of attack in the molecule may be directed mainly at a terminal double bond with the formation of a long chain β-apo-carotenal and the further oxidation may then proceed by a progressive oxidation of this compound from one end of the molecule with the production of smaller fragments. This idea receives some support from theoretical considerations regarding the stability of double bonds in extended polyene systems.

In a linear conjugated system in the normal state, the major contributing structure, A

(A) -CH=CH-CH=CH-CH=CH-

tends to resonate between the two symmetrical minor structures B and C.

(B) $^{+}$CH-CH=CH-CH=CH-CH^{-}

(C) $^{-}$CH-CH=CH-CH=CH-CH^{+}

In other words, the mobile unsaturation electrons make concerted motions from the major structure A, first in the direction of structure B and then in the direction of structure C. (Lewis and Calvin, 1939). The central double bond in such a system will, therefore, possess least double bonded character since it loses it to the neighbouring single bonds. Since the extreme forms of the minor contributing structures are probably not achieved in the normal state, the effect will become less pronounced as we move towards the ends of the molecules and the terminal double bonds will, therefore, mostly retain their double bonded character. The point of maximum stability will, therefore, be at the centre of the molecule but the stability will gradually decrease towards the ends of the molecule until points of least stability are reached. A similar theoretical argument was put forward by Zechmeister et al. (1943) to account for the fact that the central double bonds of carotenoids undergo thermal cis-trans isomerization at the relatively lowest temperature.

On the basis of the foregoing considerations the oxidation of β-carotene could be conceived as being initiated at one end of the conjugated chain (i.e., at a position of lowest stability) and

then proceeding progressively by the successive terminal oxidation of the product formed. The possibility that this type of mechanism actually occurs was investigated by carrying out a number of oxidations of β-carotene and trying to determine the course of the reaction by varying the extent to which the reactions were taken.

Experimental.

A number of H_2O_2/OsO_4 oxidations of β-carotene were carried out by the method previously described. As already stated, it was not possible to control the reaction within fine limits but a certain degree of reproducibility was achieved by employing t-butanol solutions of the reagents. The course of the reaction was studied mainly by making changes in two variables, viz., (a) the concentration of reagents employed, and (b) the time for which the reaction was allowed to proceed.

Results and Discussion.

The results of four oxidations are given in Table 1. and the spectra of the gross oxidation products are shown in Fig. 1.

Table 1.

Oxidation of β-carotene at 25° C. using different concentrations of the reagents and varying the period of the reaction.

Oxidation No.	Wt. of β-carotene oxidized (mg.)	Volume of solution (ml.)	Reagents, based on concn. used by Wendler et al. (1950)		Period of reaction (minutes)	Spectrum of oxidation product. (See Fig. 1.)
			H_2O_2	OsO_4		
(1)	21.4	100	1x	1x	75	Curve 2.
(2)	172.5	170	$\frac{3}{4}$x	1x	25	Curve 3.
(3)	85.2	90	$\frac{1}{2}$x	1x	18	Curve 4.
(4)	97.6	100	1x	1x	25	Curve 5.

N.B. Oxidation (1) was performed using aqueous solutions of the reagents.

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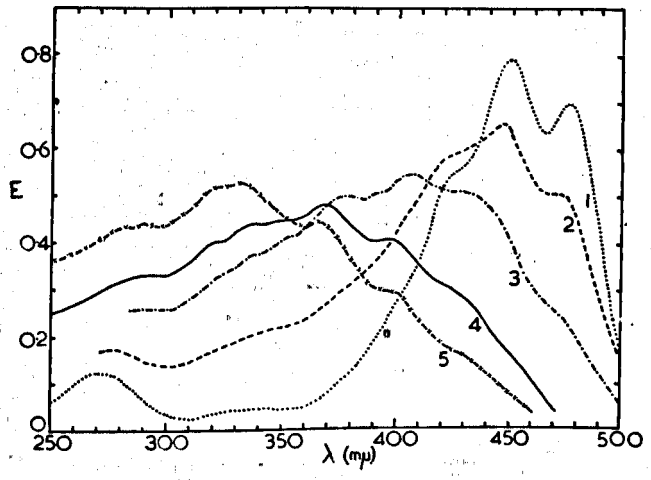

Fig. 1.

Spectrum 1 : β-carotene,
Spectra 2 - 5 : gross oxidation products of reactions in which the reagent concentrations and period of the reactions were varied.

It will be seen (Fig. 1.) that the initial stages of the reaction are characterized spectroscopically by an increase in extinction in the 420 mμ. region and a decrease in the 475 mμ. region (Curve 2). In a later stage (curve 3) the main absorption occurs in the 400 - 440 mμ region due to the β-apo-carotenals and at the same time there is an increased absorption in the 300 - 370 mμ region, indicating the formation of vitamin A aldehyde. The next spectrum (curve 4) shows an increased formation of vitamin A aldehyde (λ max. in 370 mμ region), while the absorption due to the β-apo-carotenals is now decreasing but that due to smaller fragments is increasing (300 - 340 mμ region). The subsequent stage represented in curve 5 shows that the maximum absorption is now due to the small molecular weight fragments absorbing in the 280 - 350 mμ region, while the concentration of vitamin A aldehyde has fallen and the β-apo-carotenals are now only apparent as shoulders at 400 and 430 mμ.

Thus it appears from these results that the oxidation is initiated by an attack on the terminal bonds of the β-carotene molecule with the formation of the long chain β-apo-carotenals. These then appear to be progressively oxidized further from the open chain end with the production of smaller and smaller fragments. The fact that long chain dialdehydes are also produced indicates

that terminal oxidation of β-carotene may occur simultaneously at both ends of the molecule and that oxidation of the β-apo-carotenals may sometimes occur at terminal bonds near the β-ionone end of the chain. The course of the H_2O_2/OsO_4 oxidation of β-carotene can, therefore, be represented as follows:

β-carotene

↓ slow

β-apo-8'-carotenal

↓ v. fast.

β-apo-10'-carotenal

↓ fast

β-apo-12'-carotenal

↓ slow

vitamin A aldehyde

↓ slow

β-ionylidene acetaldehyde

↓

further degradation products.

The relative rates of the different reactions are based on the yields of the β-apo-carotenals and vitamin A aldehyde obtained. It is, however, very difficult to make an accurate assessment of the dynamics of the process because of the complicated nature of

the reaction mixture and the lack of knowledge of the relative stabilities of the various products towards the attacking reagents.

An alternative oxidation mechanism which might be considered is that the reagents attack double bonds at random positions all along the chain. This type of mechanism would result in the production of a number of short chain fragments immediately upon the onset of the oxidation. However, the spectroscopic evidence favours the progressive oxidation process so this type of mechanism must be regarded as being unlikely.

The oxidation mechanism which has been put forward has an important bearing on the question of the biological conversion of β-carotene into vitamin A which will be discussed fully in the next section.

PART III.

THE CONVERSION OF β-CAROTENE INTO VITAMIN A *IN VIVO*.

CHAPTER VIII.

INTRODUCTION

INTRODUCTION.

Probably the most important role which the carotenoid pigments play in the metabolism of the animal organism is the capacity of certain of them to be converted into vitamin A. In fact, all animals are dependent ultimately for their source of vitamin A on the ability of plants to synthesize these carotenoid precursors which are then converted into the physiologically active vitamin in the body. There is, as yet, no clear evidence that animals can synthesize vitamin A de novo, although it is difficult to account for the high concentrations of the vitamin stored by certain fish on the basis that it ultimately had a purely vegetable origin.

Naturally, much attention has been paid to the chemical relationships between the carotenoid pro-vitamins A and vitamin A and to the biochemical problems of trying to establish the site and the mechanism of the conversion process. The purely chemical problems have now been fully worked out and in recent years, it has been clearly shown that the intestinal wall is

the most important site at which the transformation occurs. In comparison, however, comparatively little is known concerning the enzyme systems involved or the mechanism of the process. Indeed, the present state of knowledge of this problem is confused and controversial. For this reason, the present investigation on the mechanism of the conversion in vivo of provitamins A into vitamin A, was undertaken. The carotenoid which has been selected for this study is β-carotene, since it is the most important provitamin A on account of its widespread distribution in the plant kingdom and because of the fact that β-carotene possesses the greatest biological potency of all the vitamin A-active carotenoids.

The Relation between the Carotenoids and Vitamin A.

The association of vitamin A activity with the green parts of plants was demonstrated early in the present century by a number of workers, but at first, the activity could not be related to any particular plant pigment. In 1919, however, Steenbock showed that the vitamin A activity was confined to the carotenoid pigments, the main activity being in the carotene fraction, but none in the xanthophyll fraction. Later, Steenbock et al. (1921) claimed that carotene, after repeated crystallizations,

exhibited considerable vitamin A activity. This result was subsequently confirmed by von Euler et al. (1928) and by Moore (1929). Moore (1930) also showed that after administering carotene to a vitamin A-depleted rat, vitamin A appeared in the liver. Thus it appeared that the vitamin A activity of carotene was due to its conversion into vitamin A in the body.

The implication of this important observation was that there must be a structural relationship between carotene and vitamin A. That this was indeed true was proved by the extensive researches of Kuhn and Karrer and their co-workers (see Karrer and Jucker, 1950) into the constitution of the carotenoids and vitamin A.

From this work the conversion of β-carotene into vitamin A could be visualized simply as a hydrolytic fission of the central double bond (Karrer et al., 1931) to produce two molecules of vitamin A. In a comparison of the biological potencies of the various carotene isomers it was found that α- and γ-carotenes are only about half as active as β-carotene (Kuhn et al., 1933) and lycopene is completely inactive (Karrer and Jucker, 1950). Thus it appears that the first essential for vitamin A activity is that the carotenoid must possess at least one β-ionone ring. Furthermore, the β-ionone ring must be unsubstituted, as shown by the complete inactivity of zeaxanthin and lutein and the

relative activity of cryptoxanthin, which is about half that of β-carotene (see Goodwin, 1951a). This criterion has to be modified somewhat in the case of the β-carotene epoxides since β-carotene 5, 6; 5', 6' diepoxide possesses vitamin A activity (Karrer *et al.*, 1945). Whether the epoxide is active *per se* or is reduced to β-carotene *in vivo* remains to be proved.

To summarize; for a carotenoid to exhibit vitamin A activity it must contain an unsubstituted β-ionone ring together with an intact polyene chain so that its conversion *in vivo* will give rise to a complete molecule of vitamin A.

It remains to be seen how far this definition will have to be modified in the light of future research. Already it has been shown that the β-ionone ring can undergo modification without destroying activity, as in the epoxides, but even more strikingly in the case of vitamin A_2. This possesses a dehydro-β-ionone ring (Farrar *et al.*, 1952) and is active *per se* (Shantz *et al.*, 1946), although the activity is only 40% of that of vitamin A_1. Also, Heilbron and co-workers (1949) have synthesized vitamin A acid analogues containing demethylated β-ionone rings. These were found to exhibit growth-promoting properties, but only 0.1% of that of vitamin A. In conclusion, mention must be made of the unidentified factor in lard which can elicit a biological

response similar to that of vitamin A (Lowe and Morton, 1953). This factor is probably quite distinct from vitamin A so that its mode of action cannot be explained in terms of a general structural similarity between the two compounds as in the previous cases.

It appears then that caution must be exercised in attempting to define vitamin A activity. It may well be, as Heilbron et al. (1949) pointed out, that "the failure of a carotenoid to promote growth may be attributable to its inability to undergo fission in the required manner rather than to the inherent inactivity of its breakdown products."

The Site of Conversion of Carotene into Vitamin A.

The work of Moore (1930, 1931) demonstrated unequivocally that carotene is converted into vitamin A in the animal body and that the vitamin A formed is stored in the liver, while smaller amounts are found in the lungs, kidneys and intestines. This, and the fact that unchanged carotene was found to be present in the intestine, many days after the cessation of its administration, led Moore (loc. cit.) to conclude that the conversion occurred in the liver and not during the digestive process.

However, attempts by subsequent workers to prove that the liver was the site of the conversion, produced conflicting results. Olcott and McCann (1931) incubated liver tissue from vitamin A-deficient rats with carotene and from spectroscopic evidence claimed that vitamin A had been formed. They suggested that an enzyme "carotenase" was present in the liver, which could effect the transformation. Similar experiments by other workers confirmed this result but others (e.g., Rea and Drummond, 1932) were unable to demonstrate the conversion. The claims of a positive result were criticized by Woolf and Moore (1932) who pointed out the uncertainty in detecting the small amounts of vitamin A which were claimed to have been formed.

Equally conflicting were results obtained using intact animals. Many workers claimed that carotene administered parenterally could give rise to the formation of vitamin A, while others concluded that carotene administered by this route is completely lacking in vitamin A activity (see Bieri and Pollard, 1953a). The evidence, however, was weighted in favour of the observations that the intestinal absorption produced a more favourable biological response than did the introduction of carotene into the circulation.

It was not until 1946, when Sexton et al. compared the

biological activity of carotene when given orally and injected, that it was suggested that the site of conversion was probably the intestine and not the liver. This was substantiated by the fact that despite the considerable accumulation of carotene in the liver of rats which had received injections of carotene, the symptoms of vitamin A deficiency still persisted.

Final confirmation that the conversion does in fact occur mainly in the intestinal wall came almost simultaneously from three groups of workers (Mattson, Mehl and Deuel, 1947; Thompson, Ganguly and Kon, 1947; Glover, Goodwin and Morton, 1947, 1948a). These findings have since been substantiated by other workers, and besides the rat and the goat, the intestinal conversion has been shown to occur in chicks, sheep and cattle. It is now apparent that the failure of the earlier workers to demonstrate the presence of vitamin A in the intestine is due to the relatively slow rate of the conversion process, coupled with the rapid removal of the vitamin A from the intestine as soon as it is formed.

Thus it has been clearly established that the intestinal wall is the main site of the conversion of β-carotene and other provitamins A into vitamin A. Up till now, however, all attempts to demonstrate the conversion under <u>in vitro</u> conditions have

resulted in failure (Bieri and Pollard, 1953a).

That the intestine is not the only site of the conversion is suggested by the recent work of Bieri and Pollard (1953b, 1953c) who found that formation of vitamin A can occur when an aqueous dispersion of carotene is injected. Furthermore, the conversion still occurs when either the small intestine or more than half the liver of a rat is removed. These observations have been confirmed recently by Kon and his associates (see Goodwin, 1954). Thus it appears that the enzyme system capable of breaking down the carotene molecule is more widespread than at first thought.

The Absorption and Metabolism of β-carotene.

In any discussion relating to the biological potency of β-carotene or other provitamins A, it is necessary to take into account the numerous factors which affect the absorption of the pigments and the efficiency of the process of conversion into vitamin A. These factors include mainly, the degree of stability of the carotenoids towards oxidation in their passage through the gastro-intestinal tract, the physical state in which they are present in the intestine, and conditions which influence the efficiency of the conversion system.

The carotenoids are readily susceptible to oxidation because of the presence of the conjugated double bond system. For this reason much attention has been paid to the possibility of an oxidative destruction of the carotenoids taking place in the alimentary tract prior to absorption and of the means of preventing it. In spite of this work, however, comparatively little is known of these processes apart from a few empirical observations.

It has been known for some time that vitamin E, presumably behaving as an antioxidant, has a beneficial effect on the utilization of vitamin A (Hickman, 1943). It was found also that the tocopherols had a marked effect on the vitamin A activity of carotene as measured by the growth response of rats (Harris et al., 1944). These workers found that 1.2 μg. β-carotene daily was able to support life but when 0.5 mg. tocopherol daily was given in addition, 23 g. more growth was produced after 36 days. The synergistic effect appeared to be greater on β-carotene than on vitamin A and it was concluded that the sparing action of the tocopherols is due to a "repression of oxidation in or near the gastro-intestinal tract" (Hickman et al., 1944). Although it is probably undoubtedly true that the tocopherols and other antioxidants such as laurylhydroquinone, do have an

appreciable effect on the stabilization of carotene in the lumen of the intestine, it is likely that they also have an effect on the further metabolism of carotene after absorption. That the latter may be true is implied by the work of Johnson and Baumann (1948a) who found that large amounts of tocopherol fed with carotene to rats reduced the amount of vitamin A in the liver. Similar results were obtained by High et al. (1954) who suggested that large amounts of antioxidants may in addition to preventing oxidative destruction, may also inhibit the oxidative process which is probably involved in the conversion of β-carotene to vitamin A. It can be seen, therefore, that the picture presented by Hickman and co-workers (loc. cit.) is by no means clear cut since antioxidants can still exert an influence on β-carotene after it has been absorbed. The significance of this fact will be dealt with more fully when the mechanism of the transformation of β-carotene into vitamin A is discussed.

With regard to the agents responsible for the gastro-intestinal oxidation of carotenoids, little is known. Hove (1943) reported the presence of an oxidase in rat stomach which could oxidize carotene in the presence of methyl-linoleate, which suggests that the enzyme behaves in similar way to soya bean lipoxidase. This enzyme, however, was not found in the intestine.

It is not clear, therefore, whether the oxidative process involved is caused by an enzyme or is a straightforward chemical oxidation or both. There have been no reports of the existence of any products of the oxidation. In fact there has been no clear proof that a large scale oxidative destruction of carotenoids in the gastro-intestinal tract does occur at all! Most of the experimental evidence for the occurrence of oxidative destruction has been based on results obtained by feeding antioxidants and as already mentioned, these may have a protective action on β-carotene and vitamin A after absorption. Experiments in which the faeces have been examined after feeding carotenoids have shown that considerable amounts still persist unchanged. In fact, Johnson and Baumann (1948a) could not find any change in the amount of β-carotene excreted after the administration of tocopherol. The importance of the gastro-intestinal destruction of the carotenoids may, therefore, have been over-emphasized. Whilst it is not denied that a small amount of destruction does occur it is probably of no great significance except at very low levels of intake of β-carotene.

β-carotene is not very well absorbed by mammals and large amounts may be excreted in the faeces. Vitamin A, however, is very readily absorbed. The chief factors which

affect the absorption of β-carotene are, the amount and form in which it is ingested, the nature of the other food constituents, and the dietary history of the animal.

It is fairly well established that the presence of fat in the diet aids the absorption of carotene, presumably because it can dissolve in the fatty globules which enter the mucosal cells of the intestine. Bile salts and other substances which have a detergent action, also facilitate absorption. There are a number of reports that aqueous dispersions of carotene stabilized with surface-active agents such as "Tween" are more efficiently absorbed than solutions in oil (Hebert and Morgan, 1953). In general, it may be said that the factors involved in the absorption of fats are also essential for the absorption of carotene. The presence of mineral oils, however, tends to reduce the amount of carotene absorbed (Burns et al., 1951a), presumably because the carotene is easily soluble in the oils which are not absorbed.

The amount of carotenoids fed determines to a large extent the amount which will be absorbed but as the dose level rises, the amount absorbed probably tends towards a limit such that further increases in the dose will not cause an appreciable increase in absorption.

The fact that some animals appear to accumulate large amounts of carotenoids in their tissues, while others do not, would appear to indicate that the ability of absorption varies in different animals. On the other hand, since all mammals have a general capacity for converting certain carotenoids into vitamin A, the accumulation of carotenoids in the body may be related to the degree of efficiency of the conversion process. Mammals can be differentiated into three groups according to the way they treat absorbed carotenoids: (a) those which accumulate indescriminately in their tissues, carotenes and xanthophylls, (e.g., man); (b) those which accumulate mainly carotenes (e.g., cattle, horses); and (c) those which accumulate neither (e.g., sheep, goats, rats), (Goodwin, 1954). Animals of group (c), which is the largest group, may be said to possess the most efficient system for the conversion of carotenoids into vitamin A, and also an efficient "xanthophyll oxidase" system for the conversion of xanthophylls into colourless products (the latter have not yet been detected). In animals of the other groups, these systems are either both deficient, or the efficiency of one with respect to the other may be reduced (Goodwin, loc. cit.)

The rat, belonging to group (c) has an extremely efficient system for the conversion of carotenoid precursors into vitamin A

and is, therefore, eminently suitable as an experimental animal for studying the mechanism of this process.

The results obtained will, in general, be applicable to other mammals. In future, therefore, any discussion will refer to results obtained using rats, unless otherwise stated.

After the absorption of the carotene, the greater part of it is transformed into vitamin A in the gut wall, where it is then esterified. The vitamin A ester is then carried mainly via the lymphatic system into the blood, and thence to the liver where it is stored. In a vitamin A-deficient animal the vitamin A ester is hydrolyzed to the free physiologically active vitamin, which is probably rendered water-soluble by combining with protein. After the immediate physiological needs of the animal have been met and the blood level of vitamin A rises to a certain value (40 - 50 i.μ./100 ml. plasma) the excess vitamin A is stored in the liver where it is re-esterified. Any residual vitamin A ester which may be dissolved in neutral fat is probably taken up mainly by the Kupffer cells of the liver. Unchanged carotene which enters the circulation is probably also taken up by the Kupffer cells (Glover, 1947).

It has been postulated by Glover (1947) that only the vitamin A stored in the hepatic cells can be made available for

the physiological requirements of the animal, whereas the vitamin A ester held by the Kupffer cells cannot be utilized and is probably destroyed. The relative concentrations of the vitamin in the two types of cell appears to depend upon the rate at which vitamin A enters the body; at low levels of intake the greater is the amount stored in the true storage cells, whilst at high levels, deposition in the Kupffer cells will increase. This is illustrated well in a comparison of the relative depositions of vitamin A after administering β-carotene and preformed vitamin A (MacQueen, 1949). In the case of the animals fed β-carotene the rate of intake of vitamin A is controlled by the rate of the conversion process so that a greater proportion of the vitamin A is stored in the true storage cells than when the free vitamin is fed.

THE CONVERSION OF PROVITAMINS A INTO VITAMIN A.

Soon after the elucidation of the chemical structures of β-carotene and vitamin A, interest was focussed on the mechanism of the conversion of β-carotene into the vitamin in the animal body. The conclusion drawn by Karrer and his associates in 1931 that the β-carotene molecule simply adds on water to form two molecules of vitamin A, has persisted unchallenged for many years and it still appears in the text-books as the probable mechanism of the *in vivo* conversion process. At best, Karrer's visualization of the mechanism can only be regarded as a much simplified general overall result of the process. More recent observations have suggested that the steps leading to the formation of vitamin A probably involve an oxidation-reduction type of reaction, an idea which is more in keeping with the established pattern of biochemical degradations and syntheses.

(i) Evidence obtained by comparing the biological potencies of β-carotene and vitamin A.

Apart from any considerations regarding the actual mechanism, which will be discussed later in greater detail, Karrer's scheme for the conversion also postulates that it is the central double bond of the β-carotene molecule which

undergoes fission, thereby forming two molecules of vitamin A. Implicit in this view, is the fact that , weight for weight, β-carotene should have the same biological potency as vitamin A. This, however, was found not to be the case. Most of the workers in this field have reported results which indicate that the biological activity of β-carotene, as measured by the growth response test, is only half that of an equal amount of vitamin A (Hume, 1951).

Pure crystalline vitamin A was first isolated by Holmes and Corbet in 1937. Its biological activity was measured against the International Standard preparation of β-carotene and it was found to have an activity corresponding to 3×10^6 i.u./g. Now the β-carotene contains by definition 1.667×10^6 i.u./g. so it appeared that vitamin A was approximately twice as active in promoting the growth of vitamin A-depleted rats. This result was confirmed by Mead, Underhill and Coward (1939). Furthermore, in a number of collaborative trials carried out in different laboratories to establish vitamin A acetate as the new International Standard, the unanimous result was that 0.6 μg. β-carotene was biologically equivalent to 0.3 μg. vitamin A (Hume, 1951). There is, therefore, a wealth of evidence to suggest that β-carotene is not quantitatively

converted into vitamin A but that the conditions of the conversion process are such that only one molecule of vitamin A can be produced from each molecule of β-carotene. Alternatively, it might be argued that only half of the β-carotene can be converted, the other 50% being lost due to non-absorption or to an oxidative destruction. This, however, is unlikely since this type of process would be extremely variable and would not account for the general result that β-carotene always has half the potency of vitamin A. It is significant that values either below or above this figure have not been reported, except in at least one case.

These results, therefore, do cast serious doubt on the central bond fission hypothesis, which implies the formation of two molecules of vitamin A from one molecule of β-carotene. If central fission does occur, then one-half of the molecule must in some way be destroyed or converted into some substance which is not vitamin A, but such a process is difficult to conceive. What is much more likely is that the initial split of the molecule occurs at one of the terminal double bonds followed by a progressive degradation of the fragment until vitamin A is formed.

This latter type of conversion mechanism has, however, not been universally accepted. Many workers are still loath to

discard the central fission hypothesis for a number of reasons. The most important of these is based on results of experiments where β-carotene was fed to vitamin A-depleted rats, together with an antioxidant such as tocopherol. The numerous observations which claimed that antioxidants have a beneficial effect upon the utilization of β-carotene (vide supra) prompted Koehn (1948) to reinvestigate the problem of the conversion by comparing the biological potencies of β-carotene and vitamin A in the presence of tocopherol. He fed β-carotene and vitamin A to vitamin A-deficient rats at dose levels of the order of 1 μg. per day; in addition, the animals received daily doses of 1 mg. of α-tocopherol. The results of several tests showed that almost identical growth responses were produced by equal weights of both substances. Koehn concluded, therefore, that in the presence of adequate amounts of tocopherol, β-carotene is quantitatively converted into vitamin A by a fission of the central double bond. The only criticism which can be levelled at this work is that the control animals were not given doses of tocopherol.

Koehn's results were confirmed by Burns, Hauge and Quackenbush (1951b). These workers also found that the amount of tocopherol fed was a critical factor in determining the efficiency of the growth response due to β-carotene. The optimum

daily dose appeared to be 1 mg. but a dose of 0.5 mg. or 2 mg. decreased the efficiency of utilization significantly. The validity of the results of these authors in purporting to confirm the work of Koehn (1948) must, however, be held in doubt since they employed a basal diet containing 5% lard, a substance which is known to have a vitamin A-replacing effect (Lowe and Morton, 1953).

An anomalous and unexplained result of the work of Burns et al. (loc. cit.) is that the rats which had received β-carotene lived longer than those which had received the same amount of vitamin A, when both groups were replaced on the vitamin A-deficient diet.

These claims that β-carotene is quantitatively converted into vitamin A by central fission must, at the moment, be accepted with some reserve. As already mentioned, the specific role of the tocopherols in the metabolism of carotene and vitamin A has not yet been clearly worked out. It is now becoming evident that the antioxidant activity of the tocopherols is not only confined to the protective effect in the gastro-intestinal tract but it may also have an inhibitory action on the oxidation process which is probably involved in the conversion of carotene into vitamin A.

In this connexion, growth promoting activity and liver storage tests yield some quite different results in the biological assay of the various carotenoids. For example, α-carotene yielded lower stores of vitamin A than its growth promoting power would lead one to expect, (Johnson and Baumann, 1947) while cryptoxanthin appeared to be twice as potent in causing storage of vitamin A as in promoting growth (Johnson and Baumann, 1948b). These results indicate that growth stimulation and liver storage may not always be parallel phenomena, so that it is not always possible to evaluate completely the conversion process for a particular carotenoid in terms of the results of such tests.

(ii) The evidence for the existence of a "carotenase" enzyme.

Soon after the carotene-vitamin A relationship was established it was postulated that the conversion was probably effected by a specific carotenoid-splitting enzyme which was termed "carotenase" (Olcott and McCann, 1931). Although there is no convincing evidence for the presence of such an enzyme the idea is still widely held, especially as it is more in conformity with the central double bond fission hypothesis. It is difficult, however, to reconcile the existence of carotenase in the light of experimental results if it is assumed that the enzyme is specific.

The work of Zechmeister and his school on the biological activities of the stereoisomeric provitamins A has shown that the all-trans carotenoids possess the greatest vitamin A activity, whereas the cis-isomers have considerably less activity (Zechmeister, 1949). It may be assumed, therefore, that when the molecule is in the cis-form it loses the rod-like nature of the all-trans molecule and becomes bent so that it does not "fit" the enzyme readily and consequently the conversion will be less efficient. On the other hand, Kemmerer and Fraps (1945) found that neo-β-carotene U (peripheral mono-cis form) was partially transformed into all-trans β-carotene in the intestine of the rat. In the opinion of Kemmerer (1952) "the reported findings of biological activity of the cis-neo-isomers of carotenes must be viewed with caution since it is possible that at least part of the biological activity of these may be attributed to in vivo transformation." This view is probably more consistent with the concept of the existence of a carotenoid-splitting enzyme, since only a molecule possessing a special stereoisomeric configuration or correct shape will fulfill the conditions of stereochemical specificity of the enzyme.

Zechmeister (loc. cit.), however, allows the hypothetical enzyme greater flexibility and assumes that the division of activity

is not between active trans-compounds and inactive cis-isomers but rather between active provitamin A molecules whose overall shape does fit into the enzyme system and inactive molecules which do not fit. However, it is difficult to see how this argument can be applied to the provitamin A activity of compounds whose molecules are both shorter and longer in length than the all-trans carotenoids. The shorter chain compounds are the β-apo-carotenals, produced by the stepwise degradation of β-carotene, and these were shown to be vitamin A-active by von Euler et al. (1938a, 1938b). More recently a longer chain homologue of β-carotene has been synthesized, known as 16, 16' homo-β-carotene ($C_{42}H_{58}$). This substance was shown to possess approximately 20% of the activity of all-trans β-carotene (Deuel et al., 1952). It is interesting to note that because of the introduction of two extra carbon atoms, 16, 16' homo-β-carotene does not possess a centrally placed double bond and also that both the all-trans and the di-cis compounds have equivalent biological potencies. In order to account for these results the specificity of carotenase must be redefined. Since the length of the provitamin A molecule does not appear to be critical, all that can be said of the specificity of the enzyme is that it requires the substrate to possess at least one β-ionone ring, together with an intact side chain, with the

ring acting as the anchoring group at a point on the enzyme surface.

The evidence for the existence of a specific provitamin A-splitting enzyme is therefore extremely slender. Up till now the enzymic conversion has not been conclusively demonstrated under in vitro conditions (Bieri and Pollard, 1953a). This may be because the enzyme is very sensitive to non-physiological conditions or that the necessary co-factors are not present in sufficient concentrations.

The possibility that the conversion process is a non-specific oxidation of vitamin A precursors, rather than a specific enzymic reaction, has not been suggested by many workers. This type of mechanism has, in fact, more points in its favour than the carotenase hypothesis and will be discussed in detail later.

(iii) Factors which influence the efficiency of the conversion process.

In general, all mammals have the ability to convert carotenoid precursors into vitamin A but the efficiency of the process varies considerably according to the species. It is not clear why these species differences should exist but they are probably dependent to some extent upon the variation in the capacity of the absorption of carotenoids in different animals. At the moment the factors which control the conversion process in any one particular

species are of more immediate interest because of the possible light they may throw on the mechanism of the conversion.

The influence of the tocopherols and other antioxidants on the conversion reaction have already been discussed.

The question whether there is a central hormonal mechanism controlling the rate of the conversion has not yet been settled. There have been suggestions (e.g., Glover et al., 1948) that the vitamin A concentration in the blood may control the secretion of a hormone which is responsible for the activation of the conversion mechanism. In this connexion, much attention has been paid to the influence of the thyroid hormone on carotene metabolism. This work has been reviewed by Goodwin (1952). Many workers have pointed out that thyroidectomy produces symptoms of vitamin A deficiency and similar results were obtained by dosing animals with anti-thyroid substances such as thiouracil. It was generally concluded that the thyroid hormone exerted a direct influence on the enzyme system involved in the conversion reaction. The problem was reinvestigated by Cama and Goodwin (see Goodwin, 1952) who came to the conclusion that the thyroid hormone probably does not directly control the conversion reaction but that it affects primarily the extent of the absorption of carotene from the intestine. This action is not specific for carotene since the

thyroid probably also controls general absorption in accordance with the metabolic requirements of the animal.

The effect of insulin has also been studied. Bauereisen (1939) claimed that insulin stimulated the conversion of β-carotene into vitamin A. This has been confirmed by Sobel et al. (1953) who found that alloxan diabetic rats stored only one-quarter as much vitamin A after a fixed dose of carotene as did non-diabetic controls, the conclusion being that there is an impairment of the conversion system in alloxan diabetes. These authors did not discuss the possibility that carotene absorption may have been affected although they stated that diabetes did not affect the absorption of preformed vitamin A.

With regard to the effect of other hormones, there is a report that cortisone in large doses appears to inhibit the conversion process (Clark and Colburn, 1953).

Apart from isolated reports of the effect of certain pathological conditions, such as diseases of the liver (see Rosenberg, 1942) which seem to diminish the rate of the conversion process, there does not seem to be any single factor which has a specific action on the system.

(iv) The mechanism of the conversion reaction.

As previously stated, the hypothesis put forward by Karrer et al. (1931) that the mechanism of the conversion of β-carotene and other provitamin A carotenoids into vitamin A involves simply a hydrolytic fission of the central double bond must now be viewed with doubt in view of the fact that this type of mechanism is not consistent with the present state of knowledge concerning similar biochemical reactions. Degradations and syntheses in biological systems usually proceed by oxidation-reduction type mechanisms (e.g., the metabolism of fatty acids) while reactions involving a hydrolytic fission of double bonds are unknown. It is hardly likely that the carotenoids are unique in this respect so it is probable that the conversion mechanism is also an oxidation-reduction type of reaction. The possibility that this type of process is involved was put forward by Hunter (1946). He suggested that the first stage of the conversion was probably an oxidative attack on the central double bond to give vitamin A aldehyde or a product which is subsequently converted into this. Vitamin A alcohol is then presumed to be formed in a later stage by a reduction of the vitamin A aldehyde so formed. This idea received support from the experimental work of Glover et al. (1948b) who found that vitamin A aldehyde is immediately reduced to vitamin A

alcohol on entering the gut wall. This proved that there is an efficient enzyme system present in the intestinal wall which can effect the second stage of the reaction process. The same authors (1948a) also put forward a reaction sequence taking into account the dynamics of the mechanism as follows: (a) the oxidation of β-carotene to vitamin A aldehyde; (b) the reduction of the aldehyde to the alcohol; (c) the absorption and esterification of vitamin A alcohol by its removal from the intestine into the blood stream.

It was found that processes (b) and (c) are extremely rapid. The amount of vitamin A found in the intestinal wall during the conversion of β-carotene is, however, very small, so it appears that reaction (a), the breakdown of β-carotene to the aldehyde, is probably a slow reaction compared with (b) and (c). Thus the rate of the transformation of β-carotene into vitamin A, therefore, is probably controlled by the initial oxidative step or steps leading to the formation of vitamin A aldehyde. This is in agreement with the observations of other workers (see Glover et al., 1948a) who found that the rate of deposition of vitamin A in the liver is greater when preformed vitamin A is fed than when β-carotene is administered. Up till now, however, the initial oxidative stages of the process have not been demonstrated so this

type of mechanism cannot yet be accepted unequivocally. The difficulty in establishing the oxidation-reduction reaction lies mainly in the fact that the vitamin A aldehyde formed in the oxidation stage probably has only a transient existence due to the rapidity with which the reduction to vitamin A alcohol occurs. Nevertheless, in spite of the lack of equivocal proof at the moment, this type of oxidation-reduction mechanism is a much more likely process than the simple hydrolytic mechanism and it will be accepted as a working hypothesis.

The question which remains to be considered is the initial point of oxidative attack on the carotenoid molecule. In the work which has just been discussed it was assumed that it is the central double bond which is split. It will be seen, however, that an initial asymmetric oxidative fission of the molecule would be more in accordance with the established facts and with physico-chemical theory.

The results obtained from a study of the chemical oxidation of β-carotene have clearly shown (vide supra) that the initial point of attack of the reagent is at a double bond at one end or near one end of the conjugated chain, giving rise to long chain aldehydes, the β-apo-carotenals. Further oxidation to vitamin A aldehyde then appears to proceed by a progressive oxidation of the

terminal double bonds of these aldehydes from the open chain end. Thus it seems that the central double bond of the β-carotene molecule is not the most vulnerable position for oxidative attack in spite of its appearing to possess sterically the least hindered disposition. As already shown, this apparent anomaly is resolved when the stability of the β-carotene molecule is considered from physico-chemical grounds. It can be shown that in a compound possessing a linear conjugated chain that, because of resonance, each double bond loses some of its double-bonded character to the neighbouring single bonds (Zechmeister et al., 1943). This effect increases towards the centre of the system so that the central double bond will possess the least double-bonded character and, therefore, the maximum stability.

It is not unreasonable to suggest that if an oxidation mechanism for the in vivo conversion of β-carotene into vitamin A is accepted, then this process may follow a similar pathway to the chemical oxidation of β-carotene, viz., that the oxidation is initiated at a terminal double bond of the conjugated chain, followed by a progressive oxidation leading to the formation of vitamin A aldehyde, which is then reduced to vitamin A by the dehydrogenase enzyme system.

CHAPTER IX.

THE METABOLISM OF THE β-APO-CAROTENALS.

THE METABOLISM OF THE β-APO-CAROTENALS.

A. THE CONVERSION OF THE β-APO-CAROTENALS INTO VITAMIN A IN VIVO.

The hypothesis which has been put forward is that the in vivo transformation of β-carotene into vitamin A proceeds by a progressive degradation of the molecule from one end of the conjugated chain. In order to test this hypothesis experimentally, the first point to establish was whether substances intermediate between β-carotene and vitamin A are converted into vitamin A. The β-apo-carotenals were chosen for this purpose since they are most likely to be possible intermediates, or closely related to intermediates in an "end-on" type of oxidation mechanism.

It was shown by von Euler et al. (1938a) that the β-apo-carotenals are vitamin A active as measured by the growth response induced in vitamin A-depleted rats; β-apo-8'-carotenal and β-apo-8'-carotenal oxime are active in daily doses of 5 μg.,

whereas for β-apo-12'-carotenal, 20 μg. daily doses are required to produce the same biological response. In another series of experiments, von Euler et al. (1938b) extracted the livers of rats which had received daily doses of 50 μg. β-apo-8'-carotenal. The liver lipid showed an inflexion between 320 and 330 mμ and this, together with the blue colour formed with $SbCl_3$, was taken as proof that vitamin A had been formed. There was no evidence for the formation of any other transformation products of the carotenal. These workers, therefore, did not show conclusively that vitamin A had been formed since the product was not isolated or characterized spectroscopically and the absorption maximum of the blue colour was not measured. Hence it was considered that these findings should be confirmed and extended.

In the present work, the β-apo-carotenals were fed to vitamin A-depleted rats and the growth response compared with rats fed equivalent doses of β-carotene. At the end of the experiment the livers were removed and the lipids examined spectroscopically.

Experimental.

Preparation of β-apo-carotenals and β-carotene.

The β-apo-carotenals were prepared by the H_2O_2/OSO_4 oxidation of β-carotene (vide supra). These were purified by

repeated chromatography.

The β-carotene was obtained by chromatographic purification of the commercial product (vide supra).

Appropriate quantities of these substances were then dissolved in a suitable volume of arachis oil such that one drop, measured by a special pipette, contained the required dose. The solutions were stored in the dark at 0°.

Animals.

For each set of experiments groups of male hooded rats from the same litter were selected. These were usually weaned directly on to the vitamin A-defidient diet (Table 1.) After about 5 - 6 weeks, growth had ceased and the animals were then ready for the experiment. During the experiment the animals were dosed daily and their weights were taken at suitable intervals. At the end of the experiment the rats were anaesthetized and killed; the small intestines, livers and kidneys were then removed for analysis.

Table 1.

Composition of Vitamin A-deficient Diet.

Ingredient.	Percentage by weight.
Starch	65
Casein (ethanol extracted)	18
Marmite	8
Arachis oil	5
Salt mixture*	4

*Prepared according to method of Osborne and Mendel (1913).

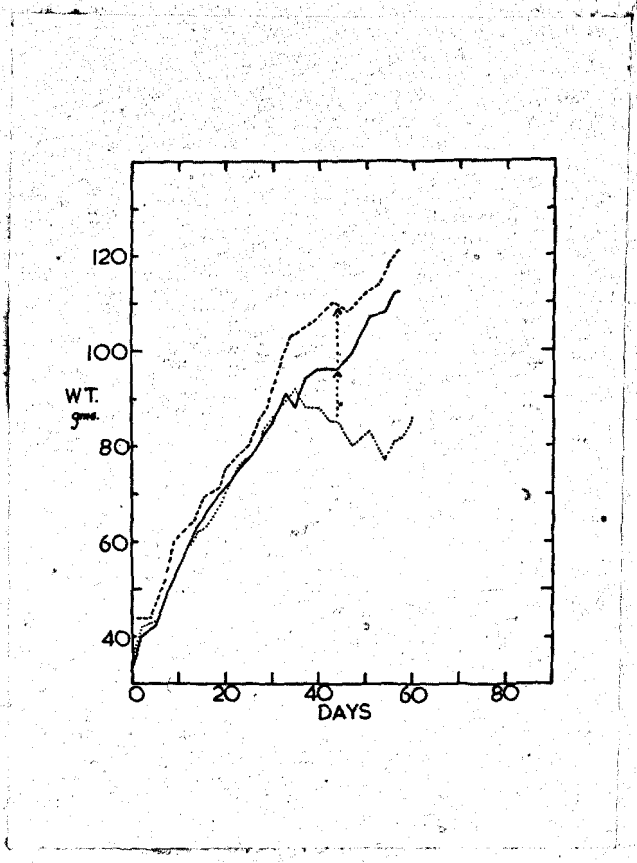

Fig. 1.

Growth response curves of rats given daily doses of:

(i) 43 μg. β-apo-8'-carotenal ——

(ii) 43 μg. β-carotene ' - - - - -

(iii) no dietary supplements (controls) .....

Preparation of tissue extracts and analytical methods.

These have been described fully in Chapter I.

In two separate experiments the biological activities and conversion into vitamin A of β-apo-12'-carotenal and β-apo-8'-carotenal were investigated.

(i) Conversion of β-apo-8'-carotenal into vitamin A.

Six male rats from the same litter were used. After 44 days on the vitamin A-deficient diet growth had ceased (weight 90 - 110 g.). Two rats were given daily doses of 43 μg. β-apo-8'-carotenal and another two, daily doses of 43 μg. β-carotene. The remaining two rats were used as controls and received only the basal diet (10 g. daily). The growth response over a period of 11 days was measured and a typical result is shown in Fig. 1. It will be seen that an almost identical growth response was produced by both substances.

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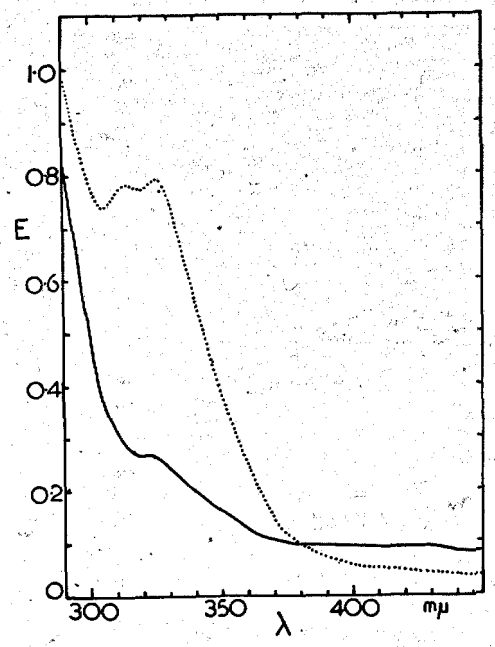


Fig. 2.

Spectra of liver lipids (in cyclohexane) of rats fed equivalent daily doses of:-

(i) β-apo-8'-carotenal ——

(ii) β-carotene .....

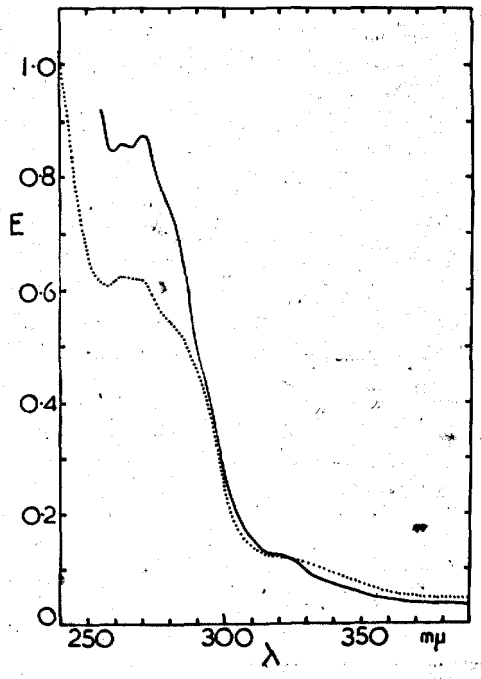
Fig. 3.

Control rats.

(i) Intestinal lipid ——

(ii) Liver lipid .....

(in cyclohexane)



The spectra of the liver lipids (in cyclohexane) of the β-apo-8'-carotenal and β-carotene fed animals are shown in Fig. 2. For comparison Fig. 3. shows the spectrum of the liver lipid of the control animals.

It can be seen that both the liver lipids of the dosed animals show a peak in the region 323 - 325 mμ, but the lipid of the β-carotene fed animal shows in addition a peak at 314 mμ.

The results of the analyses of the lipids are given in Table 2.

Table 2.

Vitamin A stored in the livers of groups of rats which had received daily doses of 43 μg. β-apo-8'-carotenal and β-carotene, respectively.

Substance fed.	Total wt. of livers (g.)	Total vitamin A (μg.)		Vitamin A. μg./rat.	Vitamin A % stored of total substance fed.
		U.V.	$SbCl_3$.		
Controls.	6.5	0	0	0	
β-apo-8'-carotenal.	9.3	27	17	13.5; 8.5	1.7 - 2.8
β-carotene.	8.8	186	145	101; 72	15 - 20

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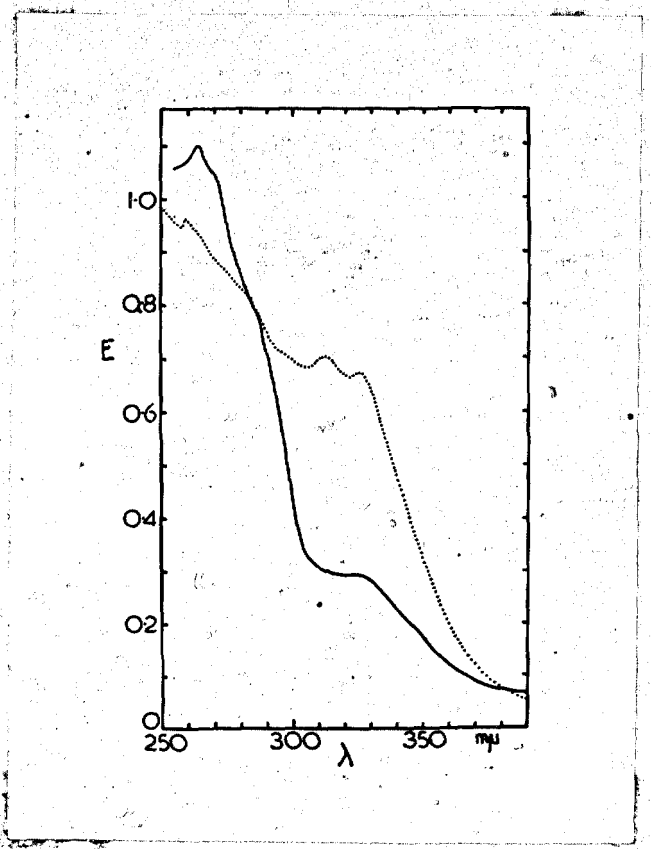

Fig. 4.

Spectra of liver lipids (in cyclohexane) of rats fed equivalent daily doses of:-

(i) β-apo-12'-carotenal ——

(ii) β-carotene .....

(ii) Conversion of β-apo-12'-carotenal into vitamin A.

The 12 male rats used in this experiment had been weaned on to a vitamin A-low diet (includes light white casein containing traces of vitamin A and β-carotene; see Lowe and Morton, 1953). After five months on this diet they were placed on the vitamin-free diet (12 g. daily) until constant weight (170 - 200 g.) was attained. Daily doses of 100 μg. β-apo-12'-carotenal and β-carotene were then given to two groups each consisting of three rats. The remaining six rats were used as controls. After 9 days the animals were killed and the tissues analyzed.

It was found that in both groups of rats receiving the supplements there were no significant increases in weight over the period of the experiment.

The spectra of the liver lipids (in cyclohexane) are shown in Fig. 4. and the results of the analysis in Table 3.

Table 3.

Vitamin A stored in the livers of group of rats which had received 9 daily doses of 100 µg. β-apo-12'-carotenal and β-carotene, respectively.

Substance fed	Total wt. of liver (g.)	Total vitamin A (µg.) U.V.	Total vitamin A (µg.) $SbCl_3$	Vitamin A. µg. per rat.	Vitamin A. % stored of total substance fed.
Control.	-	0	0	0	-
β-apo-12'-carotenal.	16.0	96	78	32; 26	3 - 3.5
β-carotene*	12.3	310	239	155; 119	13 - 17

*One rat from this group died before the end of the experiment.

The remaining portions of the liver lipids were saponified and the unsaponifiable extracts chromatographed on 8 g. alumina (3 parts activated, 1 part deactivated). The spectroscopic properties of the fractions obtained are described in Table 4.

Table 4.

Chromatography on alumina (25% deactivated) of (i) liver unsap. of β-carotene fed animals, and (ii) liver unsap. of β-apo-12'-carotenal fed animals.

Zone No.	Eluant. (1% ether in light petroleum)	Absorption maxima (mμ.) (in light petroleum) (i)	(ii)
1.	0 - 2	258	Infl. at 260, 275, 290.
2.	4	-	-
3.	6	Infl. at 250, 275, 285.	Infl. at 250, 275, 285.
4.	8	-	Infl. at 275, 285.
5.	10 - 12	Infl. at 330.	Infl. at 285.
6.	14 - 16.	310, 325.	-
7.	20 - 28	310, 324.	Infl. at 285, 325.
8.	50 - 100	-	Infl. at 325.

In both these experiments the intestinal lipid of all groups of animals (including the controls) exhibited a slight maximum at 406 mμ. (in cyclohexane). In addition, the intestinal

lipid of one of the control groups showed a maximum at 492 mμ. These substances have not been identified.

Discussion.

The presence of vitamin A in the livers of rats which had received daily doses of β-apo-8'-carotenal and β-apo-12'-carotenal was proved by inspection of the spectrum of the liver lipids and by the fact that λ max. of the $SbCl_3$ colour test occurred at 620 mμ. Also, a definite growth response was produced by β-apo-8'-carotenal and the increase in weight was equivalent to that produced by the same amount of β-carotene. In the case of the other experiment no significant increases in weight occurred both in the group of rats which received β-apo-12'-carotenal and that which received β-carotene. This may be attributed to the fact that the animals used had been fed the basal diet ad libitum for a long period prior to the experiment. Thus the 12 g. daily ration on which they were placed may have been insufficient for their requirements.

Although it has been shown that the β-apo-carotenals are converted into vitamin A in the rat, the results obtained indicate that the efficiency of the conversion process, compared

with that for β-carotene, is relatively low. The amount of vitamin A stored in liver based on the total amount of β-apo-carotenal fed is only about 3%, whereas the comparable figure for β-carotene is 17 - 20%. Thus the efficiency of the conversion of the β-apo-carotenals into vitamin A is only 20 - 30% of that for β-carotene. It should be noted, however, that the figures obtained for the animals fed β-carotene are higher than those found by other workers in this laboratory (Glover, 1947). The reason for this wide difference in the storage levels of vitamin A may be explained as follows. As will be shown later, the first stage in the metabolism of the β-apo-carotenals is a reduction to the alcohols. When these are present in relatively large amounts, some may be transported to the liver before the conversion into vitamin A can be completed. In the liver, the β-apo-carotenols may be stored preferentially in the Kupffer cells where they are probably oxidatively destroyed (MacQueen, 1949). The vitamin A derived from β-carotene, however, is probably released slowly and thus will be stored mainly in the true storage cells.

Another significant difference between the two sets of results is that the spectrum of the liver lipid obtained from

the animals fed β-carotene shows a maximum at 314 mμ. (see Figs. 1 and 3.).

It was found that by chromatographing the liver unsaponifiable material, the vitamin A fractions contained the same substance absorbing at 310 mμ. (see Table 4). The nature of the substance is not known but it is very likely that it is some degradation product of β-carotene since it occurs neither in the livers of the control animals nor in those of the animals fed β-apo-carotenals.

B. INVESTIGATION OF THE INTERMEDIATE STAGES IN THE CONVERSION OF β-APO-CAROTENALS INTO VITAMIN A.

Although it was established in the previous experiments that the β-apo-carotenals are converted into vitamin A in vivo no intermediate products in the process of the conversion could be detected. Thus this evidence alone is not unequivocal proof that the normal conversion of β-carotene proceeds by a step-wise degradation of the molecule from one end, although it could be used as an argument against the existence of a specific carotene-splitting enzyme, for the reasons already stated. The next stage of the investigation was directed, therefore, towards an attempt to try to isolate and identify the intermediate compounds in the process of the transformation of the β-apo-carotenals into vitamin A. This problem was tackled by feeding to rats, large doses (0.5 to 3 mg.) of the β-apo-carotenals, and after 5 - 6 hours, when the concentration of metabolites in the small intestine should be optimal, removing the intestines and livers for analysis.

Experimental.

The β-apo-carotenals were administered to the rats as aqueous dispersions stabilized with Tween 80. The dispersions were prepared as follows.

The β-apo-carotenal was dissolved in 1 ml. ether and 1 ml. of an ethereal solution of Tween 80 (5 drops Tween 80 in 20 ml. ether) added. 2 ml. Water was then added and the mixture shaken. The ether was evaporated *in vacuo* with continuous shaking. The resulting aqueous dispersion was stable over a long period.

The rats which had previously been maintained on a vitamin A-low diet, were fasted for 12 hours prior to dosing. The dose was then administered by mixing the dispersion with about 2 g. of the diet which was quickly consumed by the animal. Five to six hours later the animals were sacrificed and the intestines, intestinal contents and livers extracted and analyzed as previously described. The intestinal and liver lipids were then chromatographed and the fractions obtained examined spectroscopically.

Results.

(i) The metabolism of β-apo-8'-carotenal.

0.45 mg. β-apo-8'-carotenal (λ max. 452 mμ.) was administered

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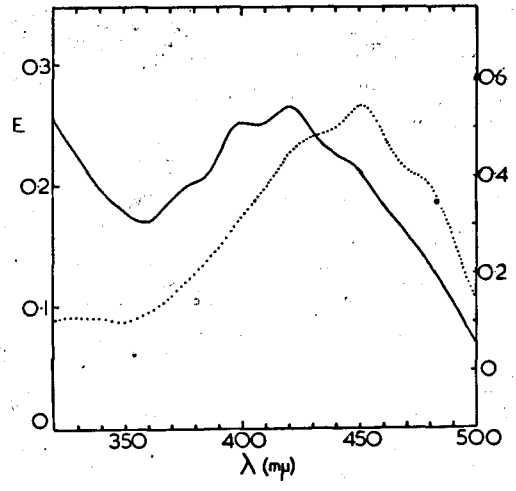

Fig. 5.

Metabolism of β-apo-8'-carotenal

(i) Intestinal lipid ———

(ii) β-apo-8'-carotenal fed .....

(in light petroleum)

to one rat. The animal was killed six hours later.

The spectroscopic properties of the various tissue extracts were as follows:-

Intestinal lipid. λλ max. 401, 421 mμ.; inflexions 375, 448 mμ. Fig. 5. shows the spectrum of the intestinal lipid together with that of the β-apo-8'-carotenal fed (λ max. 451, shoulders at 430 and 475 mμ.).

Liver lipid. λλ max. 421, 443 mμ.; broad shoulder at 321 mμ. The spectrum is shown in Fig. 6.

Intestinal contents. λλ max. 395, 421 mμ.; shoulder at 444 mμ.

The liver unsap. and the intestinal lipid were each chromatographed on 6 g. deactivated alumina. The chromatograms and spectroscopic properties of the fractions are described in Tables 5 and 6.

Table 5.

Chromatography on deactivated alumina of the intestinal lipid of a rat fed 0.45 mg. β-apo-8'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)
1.	colourless	100% light petroleum.	Steep end absorption.
2.	"	2 - 4% ether.	" " "
3.	red band.	6 - 10% ether.	424, 446; shoulders 400, 475.
4.	continuation of 3.	12 - 14% ether.	452, shoulder 475.
5.	colourless.	16% ether.	-
6.	yellow band.	18 - 24% ether.	-
7.	colourless.	50 - 100% ether.	-
8.	yellow band.	ethanol and acetic acid (equal vols.)	410, 425, 440; shoulder 475.

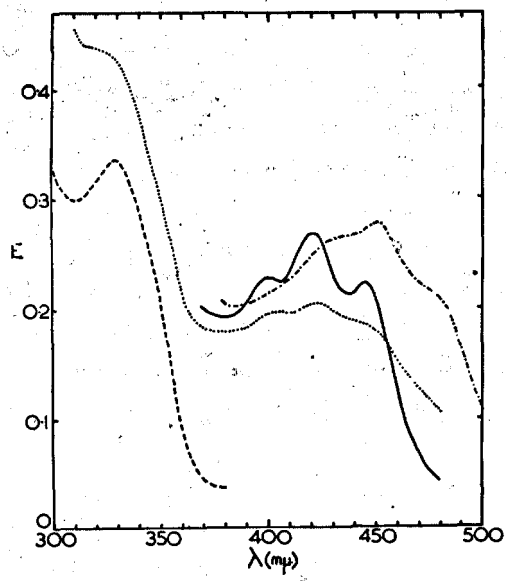

Fig. 6.

Metabolism of β-apo-8'-carotenal:-

(i) Gross liver lipid .....

(ii) Fraction 2. - · - · -

(iii) Fraction 3. -------

(iv) Fraction 4. ———

Table 6.

Chromatography on deactivated alumina of the liver unsap. of a rat fed 0.45 mg. β-apo-8'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)	
1.	colourless.	4% ether.	-	
2.	pale yellow.	4% "	450.	See Fig. 6.
3.	colourless.	8% "	330.	See Fig. 6.
4.	pale yellow.	8% "	399, 421, 444	See Fig. 6.
5.	colourless.	12 - 20% ether.	-	
6.	colourless.	50 - 100% "	-	
7.	colourless.	100% ethanol.	-	

The main intermediate products of the metabolism of β-apo-8'-carotenal as indicated by chromatography of the liver unsap. (Table 5. and Fig. 5) appear to be: (i) fraction 2, which is probably unchanged β-apo-8'-carotenal; (ii) fraction 3, which is probably vitamin A although its λ max. (330 mμ.) seems rather high; and (iii) fraction 4, which is undoubtedly β-apo-carotenol by analogy with the other carotenols and theoretical considerations (vide supra).

Fraction 3 of the intestine also appears to contain β-apo-8'-carotenol, probably as the ester, along with the carotenal.

(ii) The Metabolism of β-apo-12'-carotenal.

Two rats were each dosed with 1.5 mg. β-apo-12'-carotenal (λ max. 408 mμ.) Six hours later the animals were killed and the tissues removed for analysis. The spectroscopic properties of the tissue extracts were as follows:-

Intestinal lipid. λ max. 405 mμ.; broad inflexion 360 - 380 mμ. and small inflexions at 325 and 345 mμ. (Fig. 7.)

Liver lipid. λλ max. 325, 393 mμ.; inflexion at 375 mμ.

Intestinal contents. λ max. 407 mμ.; (spectrum characteristic of β-apo-12'-carotenal.)

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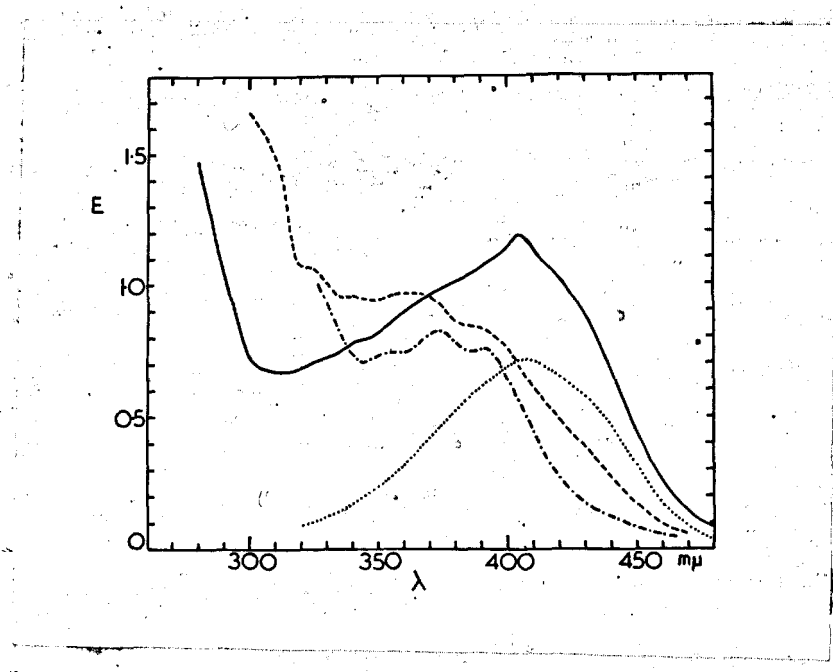

Fig. 7.

Metabolism of β-apo-12'-carotenal.

(i) β-apo-12'-carotenal fed .....

(ii) Intestinal lipid, ——————

(iii) Intestine Fraction 6. - - - - -

(iv) Liver Fraction 5. -·-·-

(in light petroleum)

The intestinal and liver lipids were each chromatographed on 10 g. deactivated alumina. The chromatograms and the spectroscopic properties of the fractions are described in Tables 7 and 8.

Table 7.

Chromatography on deactivated alumina of the intestinal lipid of two rats fed a total of 3 mg. β-apo-12'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)
1.	pale yellow.	100% light petroleum.	Steep end absorption; no maxima.
2.	colourless.	2 - 12% ether.	
3.	pale yellow.	12% ether.	
4.	orange band.	16 - 20% ether.	408.
5.	continuation of 4.	20% ether.	408.
6.	yellow band.	28% "	340, 365; shoulders 325, 390. (see Fig.7.)
7.	yellow band.	100% ethanol.	-
8.	yellow band.	equal vols. ethanol/acetic acid.	405; inflexions 345, 360, 380, 430.

Table 8.

Chromatography on deactivated alumina of the liver lipid of two rats fed a total of 3 mg. β-apo-12'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)
1.	pale yellow.	100% light petroleum.	Steep end absorption; no maxima.
2.	colourless.	2 - 10% ether.	
3.	colourless.	16 - 18% "	
4.	orange band.	20% ether.	405.
5.	yellow band.	32% "	374, 392; shoulder 355. (see Fig. 7.)
6.	yellow band.	100% "	323; shoulders 370, 390.
7.	yellow band.	ethanol/acetic acid.	shoulder 400; inflexion 420.

The main intermediate product of the metabolism of β-apo-12'-carotenal appears to be β-apo-12'-carotenol. This appears in the liver fraction 5. (see Fig. 7) with λλ max. 374 and 392 mμ. (spectrum of pure substance has λλ max. 372, 391 mμ; vide supra). Also, correction for irrelevant absorption in intestine fraction 6 (Fig. 7) gives a curve with λλ max. 327, 372, 392 mμ, which indicates

the presence of β-apo-12'-carotenol along with vitamin A. Thus as in the case with β-apo-8'-carotenal, the major part of the absorbed β-apo-12'-carotenal is reduced to the alcohol in the intestine. Before the conversion of all the absorbed material into vitamin A can be completed, some of the carotenol and also the carotenal can pass through unchanged to the liver.

A significant feature of the chromatography of the intestinal and liver lipids in this experiment was the presence on the columns of very strongly adsorbed yellow pigments which could only be removed with ethanol and acetic acid mixtures. (A similar zone was noticed in the chromatography of the intestinal lipid in the previous experiment). The λ max. of the intestinal pigment was 405 mμ and that of the liver pigment (after arbitrarily correcting for irrelevant absorption) 403 mμ. These maxima are close to that of pure β-apo-12'-carotenal (λ max. 408 mμ) and the general shape of the curves show a similar likeness. In order to dispose immediately of any possibility of a combination of the aldehyde with Tween 80, a small portion of the dispersion was chromatographed but there was no evidence of a strongly adsorbing component. It was evident, therefore, that the substance was either a transformation product

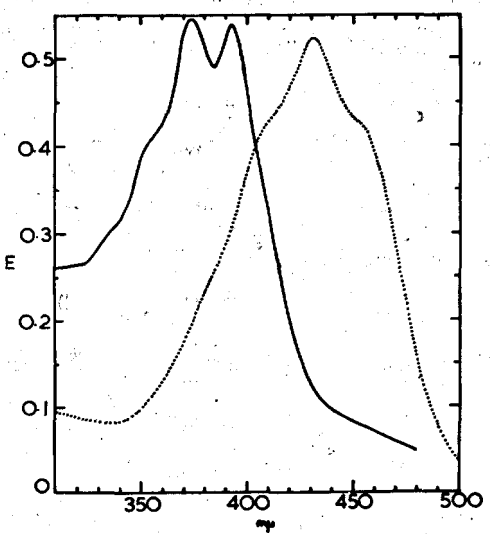

Fig. 8.

Metabolism of β-apo-10'-carotenal.

(i) β-apo-10'-carotenal fed .....

(ii) Intestinal lipid ——

(in light petroleum)

of the aldehyde (e.g., an acid) or the β-apo-12'-carotenal in combination with a substance (e.g., a phospholipid) with strong adsorbing properties. In order to investigate these possibilities, the intestinal fraction 8 was saponified. On shaking with ether it was found that all the pigment remained in the aqueous layer; the latter was then acidified with dilute acetic acid and the pigment extracted with light petroleum. The λ max. was measured and found to be 402.5 mμ. The substance was then dissolved in ethanol and methylamine solution (4 drops) added. The λ max. was then 386 mμ; on acidification (4 drops conc. HCl) the λ max. shifted to 396 mμ. Thus it appears that the substance is not β-apo-12'-carotenal since the methylamine derivative of the latter exhibits λ max. 403 mμ in alkali solution and shifts to λ max. 486 mμ on acidification (Pitt et al., 1955).

(iii) The metabolism of β-apo-10'-carotenal.

1.45 mg. β-apo-10'-carotenal (λ max. 432 mμ) was fed to one rat. Five and a half hours later the animal was killed. The spectroscopic properties of the tissue extracts were as follows:

Intestinal lipid. λλ max. 373, 393 mμ.; inflexion at 355 mμ. (Fig. 8.)

Liver lipid. shoulders at 328 and 400 mμ.

Intestinal contents. shoulders at 365 and 390 mμ.

Stomach contents. λ max. 431 mμ; shoulders at 405 and 450 mμ.

The intestinal and liver lipids were each chromatographed on 6 g. deactivated alumina. The chromatograms and spectroscopic properties of the fractions are described in Tables 9 and 10.

Table 9.

Chromatography on deactivated alumina of the intestinal lipid of a rat fed 1.45 mg. β-apo-10'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)
1.	colourless.	100% l.petroleum.	Steep end absorption; no maxima.
2.	"	1 - 12% ether.	
3.	"	12 - 20% "	
4.	orange band.	20% ether.	355, 374, 394; inflexion 406.
5.	colourless.	100% "	325, 373, 394.
6.	yellow band.	ethanol/acetic acid.	no maxima.

Table 10.

Chromatography on deactivated alumina of the liver lipid of a rat fed 1.45 mg. β-apo-10'-carotenal.

Zone No.	Description.	Eluant.	Absorption maxima (mμ.) (in light petroleum)
1.	colourless	100% l.petroleum	steep end absorption; no maxima.
2.	colourless.	1 -12% ether.	
3.	yellow band	12 - 20% "	inflexion at 325.
4.	colourless	20% "	-
5.	colourless	100% "	sharp peak at 330; shoulder at 400.
6.	yellow band	ethanol/acetic acid.	-

The main intermediate product of the metabolism of β-apo-10'-carotenal is β-apo-12'-carotenol. This is indicated by the spectrum of the intestinal lipid (Fig. 8) and by the spectrum of fraction 4 of the intestinal lipid (Table 9). The spectrum of the latter also shows a slight inflexion at 406 mμ, which is probably due to the presence of a smaller amount (approximately one-third of total) of β-apo-10'-carotenol. This is also confirmed by the fact that the ratio of the peaks E.374/E.394 is smaller than that for pure β-apo-12'-carotenol. There was no evidence for the presence of any residual

β-apo-10'-carotenal, either in the intestinal or liver lipids which means that all the β-apo-10'-carotenal absorbed had been efficiently transformed into β-apo-12'-carotenol.

The main product in the liver lipid was a substance absorbing maximally at 330 mμ, but there was also evidence of a small amount of β-apo-12'-carotenol (liver lipid and fraction 5 exhibited shoulders at 400 mμ.).

Discussion.

The results of these experiments show that the first stage in the metabolism of the β-apo-carotenals is reduction to the β-apo-carotenols in the wall of the intestine. In the case of the β-apo-8'- and 12'-carotanals, the greater part of these substances absorbed is reduced to the corresponding alcohols. The alcohols and unchanged aldehydes were also found in the liver which means that these substances can enter the circulation and be transported to the liver before the transformation into vitamin A can be completed in the intestine. The reduction of the β-apo-carotenals is probably effected by the same enzyme system (alcohol dehydrogenase) which is responsible for the reduction of vitamin A aldehyde to vitamin A alcohol (Glover, Goodwin and Morton, 1948b).

The first stage in the metabolism of β-apo-10'-carotenal is different from the other carotenals; it appears to be transformed mainly into the next lower alcohol in the series, i.e., β-apo-12'-carotenol, by the loss of two carbon atoms. The reason for this is probably due to the difference in structure. Both the β-apo-8'- and 12'-carotenals have a methyl group in the α-position with respect to the aldehyde group, whereas in β-apo-10'-carotenal the terminal methyl group is in the γ-position. Thus the terminal double bond of β-apo-10'-carotenal is free from the steric hindering effect of a methyl group and the molecule, therefore, readily undergoes β-oxidation to β-apo-12'-carotenal which is then immediately reduced to the corresponding alcohol. Once this relatively more stable substance is formed, where the methyl group in the α-position protects the terminal double bond, further degradation of the molecule is a slower process and some of the alcohol is able to pass to the liver unchanged.

This argument may also explain why, compared with β-apo-10'-carotenal, the in vivo reduction of β-apo-8'- and 12'-carotenals is less efficient resulting in the passage of the unchanged aldehydes to the liver. Thus the position of the terminal methyl-group can also influence the reaction rate of the enzymic reduction.

In all these experiments there was evidence for the formation of a shorter chain compound. This appeared in the liver as a substance with an absorption maximum at 330 mμ. This does not correspond with the λ max. 325 mμ (in light petroleum) for vitamin A, although the cis-isomer of vitamin A (neo vitamin A) shows a maximum in this region (Robeson and Baxter, 1947). It has already been shown that the β-apo-carotenals are converted into vitamin A when these substances are fed to animals over a protracted period, so it appears likely that the substance absorbing at 330 mμ is either vitamin A or a substance closely related to it (e.g., an isomer).

C. AN ATTEMPT TO ISOLATE INTERMEDIATES IN THE BREAKDOWN OF β-CAROTENE TO VITAMIN A.

One of the greatest difficulties in accepting the asymmetric fission hypothesis is the fact that, up till now, no intermediates between β-carotene and vitamin A have been detected or isolated. The reason for this, however, may be due to the dynamics of the process; i.e., the reaction rates may be such that the "build-up" in concentration of any particular intermediate is impossible. Thus, due to the low concentration and transient existence of any such intermediate, its detection by the ordinary physico-chemical methods would be difficult. The application of a radioactive tracer technique using ^{14}C-β-carotene appeared, however, to offer a greater chance of success.

It has already been shown that the β-apo-carotenals are the most likely intermediates in a terminal double bond fission conversion mechanism since (a) they are converted into vitamin A in the body, and (b) they appear to undergo degradation by a process of β-oxidation. Thus if these substances are intermediates, then by administering them to animals along with ^{14}C-β-carotene it seems possible there would be an exchange of a radioactive intermediate as a carotenal or carotenol with

the inactive preformed substance. If, therefore, the substance on re-isolation was radioactive, this would be strong evidende in favour of the β-apo-carotenals being intermediates in the process of the conversion of β-carotene into vitamin A.

Experimental and Results.

The ^{14}C-β-carotene was prepared by the detached leaf culture method. The specific activity was 0.04 μc./mg.C.; this was equivalent to a count rate of 27 c.p.m./μg. using the gas counter.

Three male rats which had been maintained on a vitamin A-free diet were used. These were dosed with a total of 0.5 mg. ^{14}C-β-carotene and 2 mg. β-apo-10'-carotenal. Six hours after dosing the animals were killed and the small intestines and livers removed for analysis.

The spectrum of the intestinal lipid indicated that approximately 200 μg. β-apo-12'-carotenol were present. The lipid was saponified and the unsap. chromatographed on 8 g. alumina (3 parts activated, 1 part deactivated). The carotenol zone was eluted with 20 - 50% ether in light petroleum; the yield of β-apo-12'-carotenol was only 18 μg. Thus a considerable destruction of the carotenol had occurred

on the column. It appeared also that the fraction was contaminated with a colourless crystalline material which was probably a sterol.

The whole fraction was combusted to CO_2 and the radioactivity determined using the gas counter by the method previously described. 8.4 mg. Carbon as CO_2 were obtained, thus confirming the presence of an impurity. The specific activity of the material was 5×10^{-4} μc./mg. C. (equivalent to a count rate of 0.46 c.p.m./μg.C.) and the total activity 4.2×10^{-3} μc. Since the total activity of all the ^{14}C-β-carotene fed was only 2×10^{-2} μc. and the specific activity, 4×10^{-2} μc./mg.C., it seems that these results are rather high and that most of the activity must be due to the colourless sterol-like component which was probably formed from the radioactive colourless impurity associated with the ^{14}C-β-carotene (vide supra). Thus it cannot be said with certainty that the β-apo-carotenol did in fact possess activity or whether the activity obtained was merely due to the presence of other contaminating substances.

The specific activity of the sterol fraction of the intestinal lipid of a rat dosed with ^{14}C-β-carotene was determined in a second experiment in which ^{14}C-β-carotene (0.5 mg.) and

β-apo-12'-carotenal (2 mg.) were administered to two rats. The lipid was saponified and the sterols precipitated as the insoluble digitonides. These were assayed using the end-window counter. The specific activity was 1.2×10^{-3} μc./mg.C. This is higher than the specific activity of the β-apo-12'-carotenol fraction assayed in the previous experiment. Thus it appears that the colourless impurity present in the ^{14}C-β-carotene is either a sterol or a substance which can act as a sterol precursor in the animal body. The presence of this substance, therefore, presents a serious difficulty in interpreting the results of this type of experiment. For this reason, attempts were made to try to separate the β-apo-12'-carotenol from sterols using other chromatographic techniques.

The chromatographic method used was that described by Ashby and Kodicek (1954). Whatman No.3. paper was prepared by soaking the paper in a 5% (v/v) solution of liquid paraffin in light petroleum and then allowing to dry thoroughly. The material to be separated was then pipetted on to a strip of this paper which was then placed in an air-tight chamber containing 95% methanol as the developing solvent. The chromatogram was allowed to develop overnight and the

paper strip was then dried and sections cut out for analysis.

In some trial experiments, mixtures of vitamin A, β-apo-12'-carotenol and 7-dehydrocholesterol were subjected to this technique. It was found, however, that the degree of resolution obtained was not very great and that a considerable destruction of the carotenol occurred. Various modifications of the technique were tried, e.g., chromatography at 0^{o} in a nitrogen atmosphere; increasing the water content of the methanol from 5% to 10%; using paper impregnated with a 10% paraffin solution, but all were without success.

Although other possible techniques of separation were not attempted, it was considered that the extreme lability of β-apo-12'-carotenol would be a serious drawback in any method. The other alternative method which would probably meet with more success would be to use pure ^{14}C-β-carotene. This would necessitate the removal of the colourless impurity from the β-carotene by chemical or physical means without affecting the latter. This problem would be facilitated if the nature of the impurity was known.

Because of these difficulties it was not possible to complete these experiments satisfactorily; the results obtained, therefore, are inconclusive.

D. POSSIBLE MECHANISM OF THE CONVERSION OF β-CAROTENE INTO VITAMIN A IN VIVO.

The two possible ways in which β-carotene may be converted into vitamin A are (a) by a central fission of the molecule, and (b) by a stepwise degradation from one end of the molecule. As already shown, the former mechanism is based largely on *ad hoc* assumptions and there is little experimental evidence in its support. The second type of mechanism, however, is more in accordance with the results of previous workers and the results of the foregoing experiments have provided further confirmation. In addition, it has been possible to extend and elaborate the hypothesis to include a possible reaction mechanism.

It has been shown that β-apo-10'-carotenal readily undergoes β-oxidation in the intestine because the terminal double bond is unrestricted. It appears, however, that once the α-methylated compound is formed further breakdown to vitamin A is a relatively slower process, due to the steric effect of the methyl group. In vitamin A, the terminal methyl group is in the β-position so that further oxidation from the open chain end of the molecule is probably entirely

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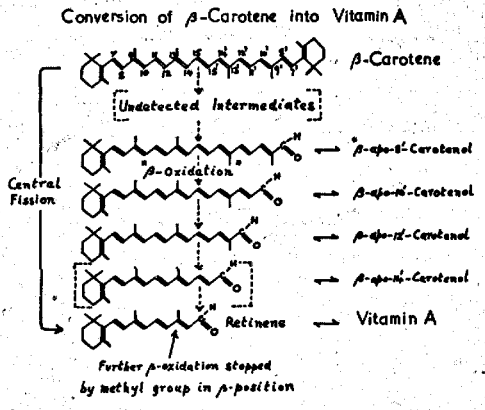

Fig. 9.

Possible mechanisms of the conversion of β-carotene into vitamin A in vivo.

eliminated. This idea of a β-oxidation mechanism which is controlled by the positions of the branched methyl groups receives support from observations on the metabolism of branched chain fatty acids. Kuhn and Livada (1933) found that α-methylated n-phenyl fatty acids undergo β-oxidation, whereas β-methylated fatty acids are oxidized with difficulty and are mostly excreted unchanged in the urine. This was confirmed by Carter et al. (1939) who reported that β-methylated fatty acids are not oxidized but are excreted in part in the form of α, β-unsaturated acids. More recently Weitzel (1951) has studied the metabolism of α-methylated fatty acids and has shown that these undergo β-oxidation.

Thus the pattern of the degradation of β-carotene is probably as follows (see Fig. 9.). After the initial attack at one end of the molecule, a two-carbon unit linked with coenzyme A (such as acetyl-CoA) is split off alternately with a three-carbon unit (such as propionyl-CoA). The relative position of the terminal methyl group will influence the rate of the reaction at each stage until vitamin A aldehyde is reached. Here, the terminal methyl group is in the β-position so that further degradation from the open chain end of the molecule will be stopped and the aldehyde will be reduced to

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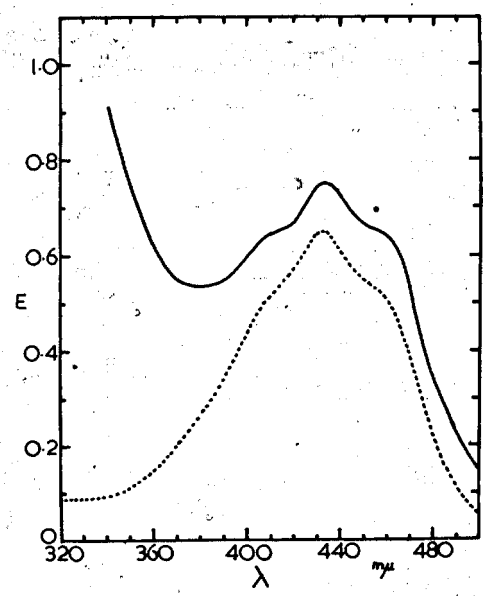

Fig. 10.

Comparison of the pigment isolated from horse intestine (Festenstein, 1951).

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with pure β-apo-10'-carotenal .....

vitamin A alcohol. Subsequent oxidation and metabolism of vitamin A will probably be then initiated at the β-ionone ring end. The latter appears to be the part least stable to chemical oxidation judging by the ease with which it forms an epoxide (see Kemmerer, 1952).

Up till now there has been no evidence for the occurrence of the various intermediates suggested in this reaction sequence. Festenstein (1951), however, has reported the presence of certain unidentified pigments in horse intestine whose spectroscopic and chromatographic properties seem to be identical with those of β-apo-10'- and 12'-carotenals. One of these pigments exhibits λ max. at 405 mμ (cf. β-apo-12'-carotenal; λ max. 408 mμ) and another has λ max. 433 mμ. with inflexions at 410 and 460 mμ (cf. β-apo-10'-carotenal, λ max. 432 mμ, shoulders at 410 and 454 mμ.). The spectrum of the latter substance is reproduced in Fig. 10. along with β-apo-10'-carotenal for comparison. These substances are not found in green plant materials and it is considered that they may be identical with these β-apo-carotenals. The reason why they are found in horse intestine and not in the rat is probably due to the fact that the former is a large organ, having many mucosal cells, so that an intermediate normally present in small quantities

will be readily detected. Thus, these findings may constitute confirmatory evidence for the conversion mechanism suggested. However, further investigations will have to be carried out in order to characterize these substances fully.

The mechanism of the conversion of β-carotene into vitamin A which has been put forward is thus based on a reasonable amount of experimental evidence. It is considered that this mechanism is in accordance with the known facts concerning the biochemical degradation of other substances (e.g., fatty acids) since it involves merely the participation of the normal β-oxidation enzyme system without the necessity of introducing special hypothetical enzymes (e.g., carotenase). In this way, the results that β-carotene can be transformed into vitamin A in tissues other than the intestine (Bieri and Pollard, 1953b) can be readily explained.

Thus the degradation of β-carotene and other provitamins A into vitamin A can be looked upon as a special case of the oxidation of long chain molecules such as fatty acids, where the structural features of the molecule (methyl branching and alicyclic end groups) impose a certain restraint on the course of the metabolism to produce the biologically active compound with the requisite structural configuration (i.e., vitamin A).

It would be interesting to see whether a similar mechanism operates in the case of other carotenoids, such as the xanthophylls and lycopene. It seems possible that in the case of the xanthophylls, the presence of -OH groups may have a profound influence on the mechanism of oxidation. This is suggested by the fact that after the administration of zeaxanthin to a rat, there is no evidence for the storage of any chromogenic material in the liver (Patel, Mehl and Deuel, 1951). On the other hand, the biological potency of cryptoxanthin compared with β-carotene has been found to be as high as 59% (Deuel et al., 1945). Thus it seems that the end of the chain containing the -OH group may be attacked preferentially. In the case of zeaxanthin, which contains an -OH group at both ends, the molecule may be readily attacked from both ends, resulting in a complete oxidation. The latter type of mechanism almost certainly occurs with lycopene, whose chain is open at both ends, which may explain why this substance is biologically inactive although one of its oxidation products (probably erroneously described as vitamin A_2 aldehyde) is reported to possess activity (Meunier, 1951).

Thus the asymmetric fission hypothesis provides a fresh approach to the general problem of the animal metabolism of

carotenoids and may in addition lead to a greater understanding of the metabolism of other methyl-branched molecules such as the sterols.

In conclusion, mention may be made of the possible nature of the enzyme system concerned in the oxidation of β-carotene. The view that has been favoured in the present investigation is that the enzyme is not a specific carotenase which effects a simple hydrolytic fission of the central double bond of the molecule but rather an enzyme or enzyme system, which catalyzes an oxidative degradation of the molecule. The question which arises is, whether the enzyme is a specific "carotene-oxidase" or a non-specific oxidase. The fact that the β-apo-carotenals and retinene are reduced by the highly unspecific enzyme, alcohol dehydrogenase, makes the concept of a non-specific oxidase not untenable. In either case, the main difficulty is to onceive how β-carotene, a lipophilic substance with no active groups, can come into combination with the enzyme. It has been shown, however, that carotene can be transported in the blood in the form of a water-soluble protein complex (Palmer and Eckles, 1914; Ganguly et al., 1952) which proves that linkage with proteins can occur. It would be interesting to see if the phospholipids are the mediators in this combination.

Once the initial oxidative fission has been effected with the formation of an aldehyde, the further enzymic stages of the oxidation are more easily envisaged since the aldehyde group is extremely reactive and condenses readily with $-NH_2$ and -SH groups on proteins (Wald, 1954).

An alternative enzymic oxidation mechanism which is worthy of consideration is the possibility that the oxidation of β-carotene is coupled with that of other substances. It has been reported that carotenoids can be co-oxidized during the oxidation of unsaturated fatty acids by soya bean lipoxidase (see Bergström and Holman, 1948). The existence of lipoxidase type enzymes in animal tissues is doubtful, but it has been shown that ordinary iron-porphyrins catalyze the oxidation of multiply unsaturated fatty acids without an enzyme system (see Breusch, 1948). Since the β-carotene conversion reaction appears to be inhibited in the presence of large amounts of tocopherol (High et al., 1954) it might be expected that this should provide a lead to the type of oxidative process involved. There is, however, little information on the mechanism of the in vivo antioxidant action vitamin E. Recently Tappel (1954) has studied the problem and concluded that tocopherol functions as an inhibitor of

unsaturated fatty acid oxidation catalyzed by haematin.

Thus it seems possible that the oxidation of β-carotene *in vivo* could be coupled with unsaturated fatty acid oxidation with an iron-porphyrin compound acting as catalyst.

CHAPTER X.

SUGGESTIONS FOR FUTURE RESEARCH.

SUGGESTIONS FOR FUTURE RESEARCH

It is hoped that the work presented in this thesis will arouse a sufficient stimulus for further investigation, not only along the same lines, but in related fields of study. In the brief summary which follows are a few suggestions of possible lines of future research, which could be taken up to develop the various aspects of the present work.

1. The Synthesis of ^{14}C-labelled β-carotene and vitamin A.

   (i) The possibility of obtaining more actively labelled β-carotene by extending the life of detached leaf cultures or by the use of algae, might be considered.

   (ii) Attempts could be made to obtain pure β-carotene by separating it from the colourless impurity. A study of the chemical nature of the impurity might suggest a suitable chemical method of separation.

(iii) Comparatively large amounts of ^{14}C-vitamin A with a moderate specific activity may be obtainable by a direct chemical synthesis.

2. The Chemical Oxidation of β-carotene.

The main outstanding problem in this study is confirmatory evidence for the chemical constitutions assigned to the oxidation products. This would necessitate a determination of various physical and chemical properties (e.g., preparation of derivatives, C and H analyses).

3. The Conversion of β-carotene into Vitamin A in vivo.

(i) A further attempt to isolate intermediates of the conversion process might be undertaken, using pure ^{14}C-β-carotene.

(ii) A study of the metabolism of β-methylated analogues of vitamin A (e.g., β-ionylidene acetaldehyde) would be interesting, since it should provide confirmatory evidence for the hypothesis connecting stability towards oxidation and the position of the terminal methyl group.

(iii) A study of the enzyme system involved in the conversion process. This would possibly include a reinvestigation of the conversion reaction under *in vitro* conditions.

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