UTILIZATION OF B CAROTENE IN THE PRESENCE OF LUTEIN AND LYCOPENE

Awdeh

THE CONVERSION OF β CAROTENE TO VITAMIN A IN THE PRESENCE OF LUTEIN AND LYCOPENE IN THE SMALL INTESTINE OF THE RAT.

By

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A C K N O W L E D G E M E N T

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CHAPTER I

INTRODUCTION

1. General

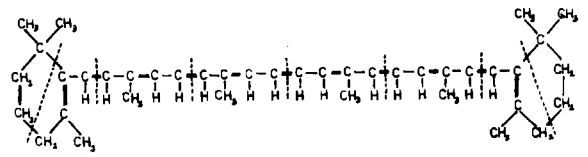
The carotenoids are a class of unsaturated fat-soluble compounds widely distributed in nature. They occur in both animal and plant species. They are yellow, orange or red pigments with a highly branched carbon skeleton composed of isoprene units (usually eight). The colour of these pigments is due to the series of conjugated double bonds present in the molecule (see fig. 1A).

Wachenroder[1] isolated carotene from carrots in 1826 and the carotenoids, of autumn leaves were extracted by Berzelius[2] in 1837. Similar pigments were shown later[3,4] to be widely distributed in the green parts of plants.

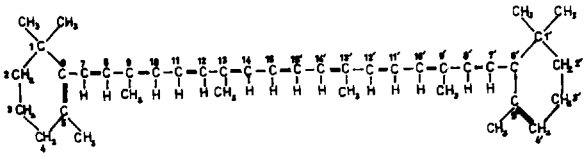
2. Nomenclature and Numbering

Carotenoids are divided into two main groups: (1) Carotenes which are hydrocarbons, and are soluble in nonpolar solvents, such as petrol and (2) xanthophylls which contain oxygen and are very soluble in alcohols.

The most common method for designating the carbon atoms of the carotenoid molecule was proposed by Karrer[5] and recommended by the "Union International de Chimie" in 1948. In this system the carotenoid molecule is divided into two halves, one half of the molecule being designated by plain numbers, the other half by similar numbers with a superscript. If the molecule is asymmetrical e.g. α carotene, the half containing the β ionone residue is designated by plain numbers (see fig. 1B).



A



B

FIGURE 1. A. STRUCTURAL FORMULA OF β CAROTENE SHOWING EIGHT ISOPRENE UNITS

FIGURE 1. B. NUMBERING SYSTEM OF THE CARBON ATOMS IN A TYPICAL CAROTENOID (α CAROTENE)

3. Plant Carotenoids

a. Occurrence

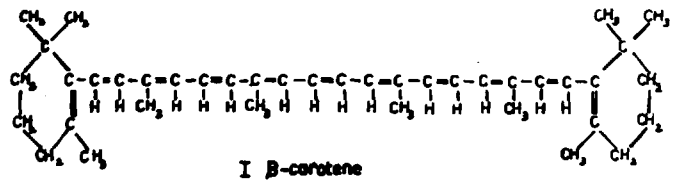
A very wide variety of carotenoids are present in plants,[6] most being derivatives of either β carotene (I), α carotene (II), γ carotene (III), lycopene (IV), or lutein (V), (see fig.). The concentration of individual carotenoids varies in different parts of the plant.

In the photosynthetic green tissue of all plants so far examined[7] β carotene and lutein (3,3 dehydroxy α carotene) predominate. Hydroxy β carotenes, epoxy β carotenes and α carotene are present in smaller amounts. Lycopene and its derivatives are completely absent. There is little variation in the carotenoid composition of green leaves among different plants.

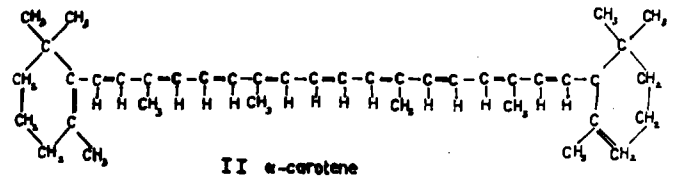
In fruits there are large variations in both the composition and nature of the carotenoids present. Some fruits contain only a trace of carotenoids e.g. apples, others such as red palm have a large quantity of β carotene and its derivatives. Tomatoes and some other fruits contain large quantities of lycopene. Light, temperature, and the nature of the soil have been shown to affect the quantity of carotenoid present.[8] Roots rarely contain carotenoids, the exceptions, the most important being carrots, contain mainly β carotene. The carotenoids present in algae and bacteria vary widely. Generally α and β carotene and their derivatives predominate in algae, while derivatives of lycopene predominate in bacteria.[10]

b. Biosynthesis

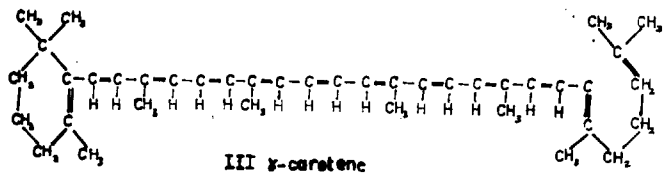
In plants, carotenoid synthesis takes place throughout the whole active life of the plant and is at its greatest at the period of



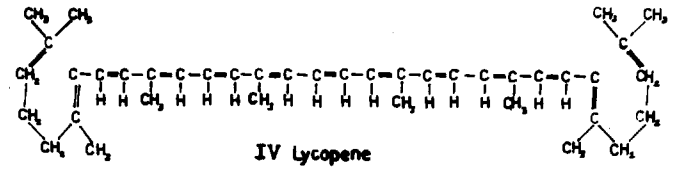
I β-carotene



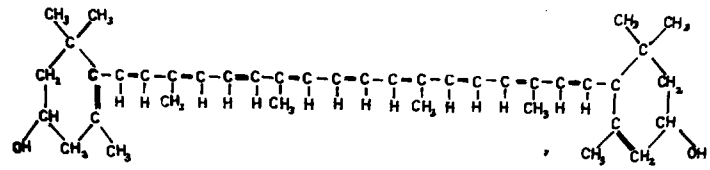
II α-carotene



III γ-carotene



IV Lycopene



V Lutein

FIGURE 2. STRUCTURAL FORMULAE OF COMMONLY OCCURRING CAROTENOIDS.

maximum growth. The nature of the carotenoids synthesized does not however change appreciably throughout the whole period of the plant's life.

By using C14- labelled acetate, Grob and Butler[11] demonstrated its incorporation into β carotene. Later the incorporation of radioactive mevalonic acid into the carotenoids of tomatoes[12] and carrot root preparations[13] was demonstrated. In cell-free extracts of tomatoes and carrots the incorporation of labelled acetate and mevalonic acid into carotenoids was achieved. ATP, CoA, Mn^{++} and DPNH were required when acetate was the substrate but not when mevalonic acid was employed. Although the exact mechanism of carotenoid synthesis is not known, it appears to be similar to that of steroids. A complete scheme for the carotenoid synthesis starting with 2 carbon units has been postulated,[14] and although many of the predicted intermediates have been isolated from carotenoid-producing tissue, many parts of the suggested scheme remain to be verified.

c. Function

The association of chlorophyll and the carotenoids in all green tissues suggests that the carotenoids play a role in photosynthesis. As early as 1880[15] work was carried out which indicated that this was so. A major function of carotenoids in photosynthesis was demonstrated by Blinks et al[16] who showed that light of wave lengths most strongly absorbed by the carotenoids were the most effective in promoting photosynthesis in the brown alga, (see fig. 3).

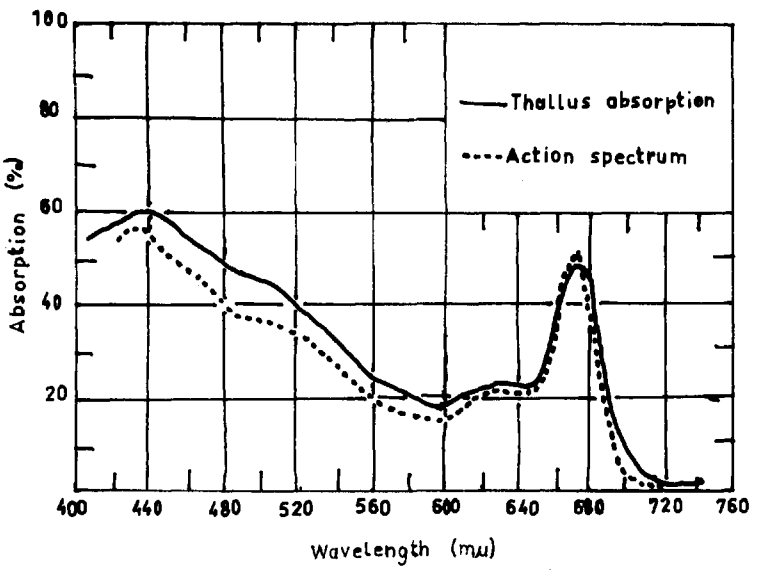


FIGURE 3. RELATIONSHIP BETWEEN THE ACTION SPECTRUM FOR PHOTOSYNTHESIS WITH THE ABSORPTION SPECTRUM IN THE BROWN ALGA[16]

It was at first thought that the function of carotenoids in photosynthesis was based on their ability to bind oxygen.

$$\text{Carotene} + O_2 \rightleftharpoons \text{Xanthophyll}$$

This was shown to be unlikely by Willstater[17] who demonstrated that the ratio of xanthophyll to carotene did not change under differeht conditions of oxygenation and illumination. Further evidence against this early hypothesis is that, although the main carotene in green leaves is β carotene its corresponding xanthophyll has only been found in small amounts. Similarly, the reduced form of lutein, ∝ carotene is not a major component of such tissue although lutein is the major xanthophyll present.

A more likely function of carotenoids in photosynthesis is their ability to absorb light energy in the blue region of the visible spectrum and transfer this energy to chlorophyll. It has been demonstrated[18] that the light absorbed by the carotenoids excites chlorophyll fluorescence.

4. Animal Carotenoids

a. Occurrence

All animal carotenoids must be of dietary origin, since it has never been shown that animals are capable of synthesizing carotenoids from smaller units.[19] Many animals can however modify ingested carotenoids. Some animals e.g. the rat, do not accumulate carotenoids as such in their tissues. Other animals e.g. cattle, accumulate only carotenes. Others e.g. man, absorb unselectively both xanthophylls and carotenes. Mammalian carotenoids are found mainly in the blood, liver, adipose

tissue, and milk. Although the body is capable of converting several of the carotenoids to vitamin A, carotenoids per se have never been shown to have any physiological function.

Birds differ from mammals in their ability to store xanthophylls preferentially. The main deposits of these pigments are in skin, body fat and feathers. Considerable amounts are found in eggs, lutein being the main pigment in egg yolk.[20]

b. Fate of carotenoids in the animal body

It was not until 1930 that the relationship between carotenoids and vitamin A was demonstrated. Moore[21] showed that carotene was metabolized in the animal body to form vitamin A which was stored in the liver. It was soon realized that not all carotenoids possess vitamin A activity. β carotene was found to be twice as active as α carotene[22] for this purpose. The inactivity of lycopene and lutein indicated that the presence of an unsubstituted β ionone residue was essential for vitamin A activity.* (see Table 1.)

Table 1.

Some Naturally Occuring Carotenoids and their Vitamin A Activity

Name		Biological Potency
β carotene	=	100%
α carotene		53%
γ carotene		43%
Lutein		0
Lycopene		0

*Some epoxy derivatives of β carotene were reported to have slight Vitamin A activity (29).

There is great variation in the manner in which different animals deal with dietary carotenoids. Man absorbs and accumulates all carotenoids unselectively[23] while goats and sheep do not accumulate any of the carotenoids.[24] Other animal fall in between these two groups. All animals, except for the carnivores[25], share the common ability of converting provitamin A carotenoids to vitamin A.

Although a great deal of work is done on the fate of the provitamin A carotenoids, the metabolism of non provitamin A carotenoids in the animal appears not to have been studied.

1. Mechanism of conversion of β carotene into vitamin A

It is now well established that the major site for the conversion of provitamin A carotenoids to vitamin A is the small intestine[26,27] although other tissues are capable of this conversion.[28] The structural requirement for the formation of vitamin A from a carotenoid is the presence in the molecule of an β ionone ring and a vitamin A side chain. The importance of stereo configuration of the carotenoid molecule in the potential provitamin activity is shown in Table 2.

Table 2

Vitamin A activity of β carotene isomers [29]

Structure	% Biological activity
all-trans	100
15 - cis	30 - 50
9 - cis	38
9, 13 - dicis	53
11, 11´-dicis	30

The resemblance of the structure of vitamin A to β carotene and the fact that β carotene was twice as active as α carotene[30] as a vitamin A precursor suggested that the molecule of B carotene was split at the central double bond yielding two molecules of vitamin A. (i.e. central fission).

Later when vitamin A was obtained in pure cyrstalline form[31] and its biological activity compared with that of β carotene, it was found to be twice as active weight for weight.[32] This suggested that one molecule of vitamin A was formed from one molecule of B carotene.

This result could not be explained in terms of central fission since one molecule of β carotene should produce two molecules of vitamin A. An alternative scheme of conversion was proposed,[33] in which the fission of the carotene molecule occurred asymmetrically by a mechanism of terminal oxidation. In support of the terminal oxidation hypothesis it was pointed out[34] that the central double bond of a conjugated system is more stable than the terminal one due to resonance. Many inorganic oxidants attack the terminal double bond of β carotene yielding long chain aldehydes.[35]

However, no intermediate compounds between B carotene and vitamin A have been detected in experimental animals given large doses of B carotene.[36] Moreover some experiments have shown[37,38,39] that the efficiency of conversion of B carotene to vitamin A could exceed 50% thus favouring the central fission hypothesis.

Many experiments carried out to settle this controversy were inconclusive mainly because the conversion reaction is very slow.

For many years attempts to obtain an *in vitro* system that will convert β carotene into vitamin A were unsuccessful.[40] Recently a limited success was achieved with intestinal loops which could convert only 2.5% of the β carotene into vitamin A.[41,42] This conversion required oxygen and an agent to form an emulsion between B carotene and the buffer.

Attempts to prepare the active enzyme "carotenase" that will carry out this conversion have not yet succeeded.[43,44]

The hypothesis of terminal oxidation requires that the terminal double bond should be attacked. This would be followed by a step-wise degradation leading to vitamin A. (see Fig. 4). Many of the required hypothetical intermediates have been prepared synthetically and their potency measured by the amount of vitamin A stored in liver.[36] The low yield, (less than 4%), of vitamin A obtained from these compounds did not provide the expected support for the terminal oxidation hypothesis. Finally, the metabolism of uniformly labelled B carotene has been studied in the rat.[45,46] About 3% of the label appeared in respiratory CO_2 within the first day after dosing. Sterols and fatty acids were found to be labelled as well as vitamin A indicating that some degradation of B carotene takes place during conversion.

ii. Conversion of β carotene to vitamin A in the presence of lutein and lycopene.

The effect of non-provitamin A carotenoids on the efficiency of conversion of β carotene to vitamin A is another subject of controversy.

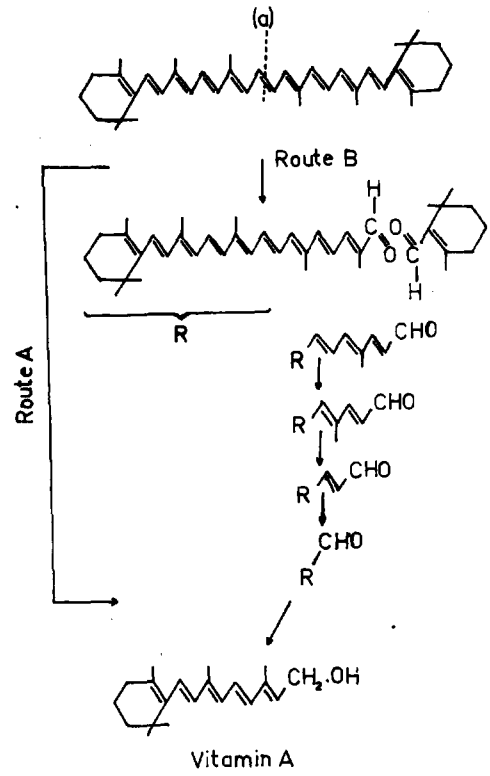


FIGURE 4. THE TWO POSSIBLE MECHANISMS FOR THE CONVERSION OF β CAROTENE TO VITAMIN A.

Sherman[47] compared the mortality rate of two groups of vitamin A depleted rats in which the first group were given 2 ug of β carotene daily and the second group the same dose of β carotene together with 5 ug of lutein. His conclusion was that the addition of lutein improved the utilization of β carotene in the rat by acting as an antioxidant.

Kemmerer et al[48] approached this problem by feeding larger quantities of B carotene and lutein to rats for 14 days. The efficiency of conversion was estimated by measuring vitamin A in the livers of these animals at the end of the period. This work showed that the group of rats receiving β carotene alone stored 20% more vitamin A than the other group.

Using the weight gain of rats as an index of vitamin A status, Callison et al[49] reported that lutein up to a level of 250 ug per day had no effect on B carotene utilization.

Other workers[50] reported that, in rats fed β carotene, small amounts of lutein increased liver storage of vitamin A while large amounts caused a decrease.

In the chick Vavich et al[51] found no effect when using 65 ug/day of β carotene but at a level of 130 ug/day liver stores of vitamin A were reduced in the presence of lutein.

Lycopene was found[52] to increase rat liver stores of vitamin A when given in small amounts with β carotene, but larger doses had no effect. The results of these experiments are summarized in Table 3.

Table 3

Comparison of the effect of lutein and lycopene on the utilization of β carotene reported by previous workers

Carotenoids tested	Methods of Bioassay	Animal used	Number of animals in each group	Number of groups	Results	Reference
2 ugβ carotene 5 ug lutein/day	Mortality rate	rat	4,16	2	Lutein improve β carotene utilization	47
60 ug β carotene 60 ug lutein	Liver storage	rat	6	2	Lutein decreased utilization of β carotene by 20%	48
6.3 - 12.6 ug β carotene 63 - 252 ug lutein	Growth rate	rat	6 - 10	18	Lutein had no effect in any combination tested	49
15 - 60 ug β carotene 5 - 500 lutein	liver storage	rat	3 - 14	18	Small amounts of lutein increased storage but large amounts decreased storage of vitamin A	50
65 - 130 ug β carotene 100 - 600 ug lutein	Liver storage	chick	10 - 15	10	Lutein had no effect when β carotene was 65 ug/day and reduced storage when β carotene was 130 ug/day.	51
30 ug β carotene 25 - 300 ug lycopene	Liver storage	rat	4 - 9	6	Small amounts of lycopene increased utilization of β carotene. Large amounts had no effect	52

It is clear from the table that the results of experiments to investigate the effect of lutein and lycopene on the utilization of B carotene in the animal body are contradictory. Since these investigations were carried out, study of the methods of bioassay of vitamin A shows clearly that under the experimental conditions described by these workers the results are unreliable.

Owing to the wide individual variations between rats, results based on mortality rate have no significance unless large numbers of animals are used. The numbers of rats used in the experiment of Sherman[47] were very small.

The growth rate method for the estimation of vitamin A potency depends on the increase in weight of depleted rats while receiving daily doses of the material to be tested. This method is reliable only when the dosage is close to the daily requirement of the animal. In the rat the reliable range is between an equivalent of 0.2 ug to 1.2 ug of vitamin A per day.[53] In the experiment reported by Callison et al[49] the amounts of β carotene used (6.3 - 12.6 ug/day) were greatly in excess of this range. Under these conditions it is unlikely that any effect of lutein would be detectable.

Measurement of liver stores, now generally accepted as the most reliable method for vitamin A bioassay, is known to be unsatisfactory in the rat if the material assayed has a potency of less than 500 I.U. of vitamin A.[54] The quantity required for accurate bioassay in the chick is even greater than that required for the rat. Below these

levels the liver does not store vitamin A linearly. In the four experiments[48,50,51,52] which used vitamin A liver stores to measure the effect of lutein on the conversion of β carotene, the amount of β carotene fed was lower than the required minimum.

Thus in the light of the recent critical examination of vitamin A bioassay methods it is not surprising that the results of previous workers were inconclusive.

In the study of human vitamin A deficiency carried out in the Nutrition Research Laboratory it was frequently found that serum from children suffering from vitamin A deficiency contained high levels of non provitamin A carotenoids, mainly lutein, with almost no β carotene, and vitamin A levels close to zero. Since β carotene is always associated with lutein in green leaves, it was difficult to explain the absence of both β carotene and vitamin A from the serum of these children. It seemed possible that the high levels of lutein from dietary origin may have had some inhibitory effect upon the absorption of β carotene.

It was therefore decided to carry out a preliminary study on the effect of lutein and lycopene, the two major non provitamin A carotenoids occurring in food, on the absorption and conversion of β carotene to vitamin A in the animal body. It seemed likely that this investigation would also give some information on the fate of non provitamin A carotenoids in the animal body.

The rat was chosen as an experimental animal because vitamin A studies on this animal are well documented. Since the presence of high levels of carotenoids have been shown by the author[55] to affect the estimation of vitamin A in biological materials, the rat, not absorbing any of the ingested carotenoids as such, is an ideal animal for such a study.

CHAPTER II

MATERIALS AND METHODS

1. Animals

In all experiments described, rats of the Wistar strain bred at the American University of Beirut were used.

2. Diets

Vitamin A-free diet for the rats was prepared from the following:

18%	Egg albumin
15%	Brewers yeast
53%	Wheat starch
10%	Olive oil (carotene free)
4%	Salt mixture (USP XIV)
1.5mg%	Vitamin E
3ug%	Vitamin D

3. Preparation of lycopene

Lycopene was prepared by the procedure of Sandoval et al.[56] 3 Kgs of canned tomato paste were dehydrated with 4 liters of methanol, filtered and the solid pressed dry. The filter cake was extracted twice with 4 liters of methanol: carbon tetrachloride 1:1. The carbon tetrachloride layer was washed several times with water to remove traces of methanol, dried over sodium sulfate and evaporated to dryness *in vacuo*.

The residue was dissolved in 75 ml of hot benzene, 45 ml of hot methanol were added dropwise, the solution was cooled and

the red crystals were collected and recrystallized from benzene : methanol. Lycopene was further purified by chromatography on a calcium hydroxide column.

In petroleum ether the absorption spectrum of the material prepared was identical with that described for lycopene.

4. Preparation of lutein

Lutein was isolated from green leaves by a modification of the method described by Karrer.[57] About 10 kgs of fresh lettuce leaves were air dried at room temperature for two days. The semi-dry leaves were further dehydrated by homogenizing them in a Waring blender with 5 liters of methanol. The remaining solid was treated overnight with 5% methanolic KOH and extracted with ethyl ether. The ether layer was separated and washed several times with water until neutral. It was then evaporated to dryness in vacuo. The residue was dissolved in 2 liters of petroleum ether-methanol 1:1, and after adding 100 ml of water to the mixture, the methanol water layer contained most of the lutein. The methanol water extract was shaken with petroleum ether to remove traces of carotenes and dried in vacuo. The residue was redissolved in ethyl ether and applied to a deactivated Aluminum oxide column (containing 5% water) and eluted with 5% methanol in ethyl ether. The material prepared by this method showed absorption maxima identical with those described for lutein.

5. Estimation of carotene and vitamin A in serum[58]

To 0.1 ml of serum is added 0.1 ml of alcohol and 0.15 ml of petroleum ether (40 - 60°C). Thorough mixing is obtained by holding the tube against a rapidly rotating drill. The tubes are centrifuged for 5 minutes at 3000 rpm. The carotene concentration is determined by measuring the optical density of the petroleum layer at 450 mu.

0.1 ml of the petroleum layer is evaporated under a stream of nitrogen at room temperature. The residue is dissolved in 0.01 ml of chloroform and treated with 0.1 ml of chloroform: Trifluoroacetic acid 2:1. The optical density of the resulting colour is measured at 620 mu exactly 30 seconds after the addition of the chromogen. Calculations are based on standard curves for pure B carotene and vitamin A.

6. Estimation of B carotene and vitamin A in liver.

The method used to estimate carotene and vitamin A in the liver was a modification of the method described for serum. About 1 gm of liver was cut into small pieces and accurately weighed. To this was added 5 mls of 5% aqueous KOH and a few crystals of pyrogallol as an antioxidant. The mixture was heated in a boiling water bath until the tissue was completely digested (approximately 30 minutes). 5 ml of ethanol were added and the material extracted with 10 ml. of petroleum ether (40-60°C). The carotene content was estimated by measuring the optical density of the petroleum layer at 450 mu. 2 ml of the petroleum layer were evaporated under a stream of nitrogen at room temperature. The residue was

dissolved in 0.2 ml of chloroform and treated with 2 ml of chloroform:trifluoroacetic acid 2:1. The optical density of the resulting colour was measured at 620 mu exactly 30 seconds after the addition of the chromogen. Calculations were based on standard curves for pure β carotene and pure vitamin A.

7. Preparation of oily solutions containing (a) β carotene (b) β carotene and lutein, (c) β carotene and lycopene.

a) 20.75 mgs of pure β carotene were dissolved in 25 ml of hot benzene, 5 ml of cotton seed oil were added and thoroughly mixed. The benzene was completely evaporated on a water bath under reduced pressure. The remaining oily solution contained 2500 ug of β carotene per 0.6 ml.

b) 20.75 mgs of β carotene and 333 mgs of lutein were dissolved in 100 ml of hot benzene, 4.6 ml of cotton seed oil were added and thoroughly mixed. The benzene was completely evaporated under reduced pressure, and the remaining oily solution contained 2500 ug of β carotene and 40 mg of lutein per 0.6 ml.

c) This was prepared similarly to (b) except that lutein was replaced by an equal quantity of lycopene.

8. Preparation of emulsions of β carotene, lutein and lycopene

10 mg of β carotene and 1 ml of cotton seed oil were dissolved in 10 ml of benzene. The benzene was evaporated and the remaining oil mixed with 1 ml of Tween 80. 8 ml of water were added and the mixture vigorously shaken. Emulsions containing lutein and lycopene were similarly prepared.

Dilutions of these emulsions with either normal saline or Krebs-Ringer were prepared and the final concentration of the carotenoid measured just before use. The emulsions so formed were

stable for at least three hours at room temperature.

9. Preparation of Krebs-Ringer solution[59]

This solution was prepared from the following:

NaCl	0.9%	100 parts
KCl	1.15%	4 parts
$MgSO_4$	3.82%	1 part
Phosphate buffer pH	7.4	
0.1M		20 parts

10. Preparation of thin layer chromatography plates[60]

A uniform film 0.25 mm thick of aluminum oxide* mixed with water 1:2, was spread on glass plates (20 x 20 cms) by the use of a special apparatus.* 20 minutes after the film had hardened the plates were dried for 30 minutes at 110°C in an oven and immediately put into a dessicating cabinet over $CaCl_2$.

11. Detection of vitamin A by the use of thin layer chromatography

This technique was employed to detect traces of vitamin A in petroleum extracts of intestinal homogenates before and after incubation with B carotene. 25 ul of the extract was applied on a thin layer plate, prepared as previously described. On the same plate a similar spot containing 1 ug of vitamin A was applied. The plate was developed by 5% acetone in petroleum ether (40 - 60°C)

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*Supplied by Shandon Scientific Company Ltd. London.

and dried at room temperature. When viewed under the U.V. lamp the vitamin A spot exhibited a greenish fluorescence at Rf = 0.3.
As a further test the plate was sprayed with 10% $SbCl_3$ in chloroform. Vitamin A gave a blue spot which faded after about a minute.

CHAPTER III

RESULTS

1. Provitamin A Activity of β-Carotene in the Presence of Lutein and Lycopene.

The proportion of orally fed vitamin A that is deposited in liver of rats is constant over a range of 500 - 10,000 I.U. A bioassay for vitamin A and provitamin A compounds based on the liver storage of rats was described by Ames et al.[54]. It is the most accurate method for bioassay of vitamin A yet described. In our work this method was used to study the effect of lutein and lycopene on the utilization of β carotene.

The rats used for our feeding experiments were fed by mothers receiving a vitamin A and carotene-free diet during the nursing period and weaned onto this diet 21 days after birth. 6 sets each composed of 3 male litter mates were used. From each set one rat was taken at random to form group A., B and C. Twenty days after weaning the weight of the animals was stationary and they were presumed to be completely depleted of vitamin A. At this stage no eye changes had occurred.

All the animals were given orally, over a period of 3 days, 2500 ug of β carotene dissolved in cotton seed oil To the carotene solution fed to each rat of group B was added 40 mg of lutein, and to that of group C, 40 mg of lycopene.

On the fourth day the animals were sacrificed after blood had been taken from the heart, and the livers were immediately removed and weighed. The sera and livers were then stored at - 10°C until analysed. This was never longer than a week.

The results of the analysis of liver and serum are listed in table 4. It is clear from the results that there is no significant difference between vitamin A liver stores of groups A, B or C. These results also indicate that only traces of the carotenoids are absorbed as such.

2. The measurement of rate of disappearance of B carotene, lutein and lycopene from intestinal loops of anaesthetized rats.

This classical in vivo method which has been used by many workers to measure the rate of disappearance of test substances from the intestine[61] was used in our work to compare the behaviour of lutein and lycopene with that of B carotene in the small intestine under similar conditions.

Male rats weighing 200 - 300 gms, raised on a stock diet, were fasted for 24 hours. They were then anaesthetized by intraperitoneal injection of Nembutal (4.5 mg/100 gm body weight). A further injection of 1 mg/100 gm Nembutal was given 45 minutes later. The abdomen was opened and a glass cannula, connected to a burette, was introduced into the lumen of the small intestine about 10 cms below the stomach. A second cannula was similarly introduced 15 cms below the first (see fig. 5). The upper cannula

Table 4

Comparison of vitamin A content and Carotenoid Content of the Livers and Sera of rats fed B carotene with ar without Lutein and Lycopene.

Litter number	GROUP A B carotene only				GROUP B B carotene and lutein				GROUP C B carotene and lycopene			
	Vitamin A ug/liver	Carotenoids ug/liver	Serum vitamin A ug%	Serum carote-noids ug%	Vitamin A ug/liver	Carote-noids ug/liver	Serum Vitamin A ug%	Serum carote-noids ug%	Vitamin A ug/liver	Carote-noids ug/liver	Serum Vitamin A ug%	Serum carote-noids ug%
I	359	44	27	7	475	48	21	9	388	31	33	20
II	362	17	18	13	536	31	21	11	577	55	27	2
III	327	22	11	27	333	20	23	6	306	13	29	3
IV	410	9	9	13	350	15	12	17	490	40	25	10
V	735	24	26	5	642	40	19	8	530	56	12	11
VI	565	34	26	5	484	34	28	12	555	44	24	11
Mean	459	25	20	12	470	31	21	11	474	40	25	9
Standard deviation	± 159	±12	±8	±8	±116	±12	±5	±4	±106	±16	±7	±7

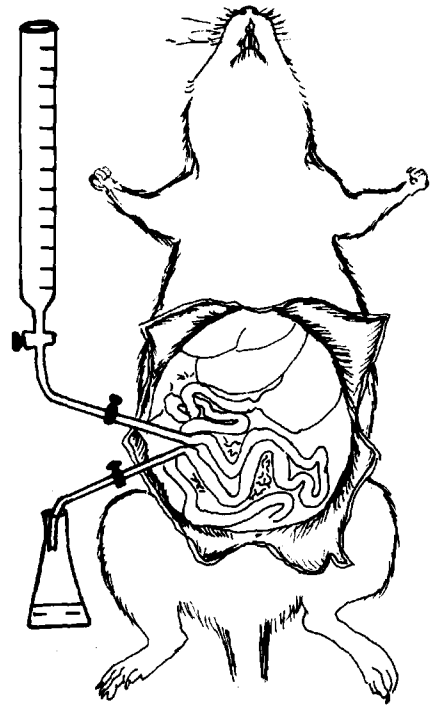

FIGURE 5. Isolated loop in intact rat.

was connected by a short length of rubber tubing to a burette containing normal saline at 37°C. The flow from the burette was controlled by a screw clip. The flow from the bottom cannula was similarly controlled. The section between the two cannulae was first washed out with 100 ml of normal saline and then a known quantity of B carotene emulsified in 1 ml of saline containing 1% glucose was injected into the upper cannula through the rubber tubing and washed into the intestine with 2 ml of saline from the burette. 15 minutes later, the contents of this section of the intestine were emptied into a flask through the bottom cannula by flushing with 50 ml of saline. The contents of the flask were extracted with petroleum ether which was made up to a known volume and the carotenoid content measured spectrophotometrically. 5 minutes after the removal of B carotene the procedure was repeated using lutein and then lycopene.

This experiment was carried out on five rats, the order of introduction and the quantity of the carotenoids being changed. At the end of each experiment t he intestinal loop was removed from the animal, homogenized and extracted with petroleum ether and the carotenoid content was estimated as described previously. Before each animal was killed, a sample of blood was drawn from the heart for serum carotenoids measurement. The results of this experiment are presented in Table 5.

Table 5

Disappearance of three Carotenoids after 15 minutes in intestinal loops of rats

Rat Number*		Carotenoid introduced into the lumen	Carotenoid recovered from the lumen after 15 minutes	Difference between second and first column	Serum carotenoid at the end of each experiment	Carotenoid recovered from intestinal tissue
		ug	ug	ug	ug	ug
I	B carotene	6	0	6	2	0
	Lycopene	4	0	4		
	Lutein	4	0	4		
II	Lutein	33	1	32	7	9
	Lycopene	42	0	42		
	B carotene	38	20	18		
III	B carotene	85	47	38	7	12
	Lutein	105	14	91		
	Lycopene	-	-	-		
IV	Lycopene	160	7	153	10	27
	B carotene	123	74	49		
	Lutein	136	19	117		
V	Lutein	363	19	344	9	48
	Lycopene	260	27	233		
	B carotene	184	134	50		

*The carotenoids are listed in the order in which they were introduced in the intestine of each animal.

3. Disappearance of carotenoids from media incubated with intestinal segments.

The method used has been previously described by Shamma's et al.[62] Normal male rats weighing 200 - 300 gm each, were fasted for 24 hours and anaesthetized with Nembutal (5 mg /100 gm). The middle section of the small intestine was removed from the animal, washed with chilled Krebs - Ringer, and slit along its whole length. The intestine was then put on a paraffin plate bathed in Krebs-Ringer and cut into 6 mm segments with a special cutter.[62] 10 randomly chosen segments were placed into each of three 50 ml flasks. To the first flask 10 mls of Krebs-Ringer solution containing a known amount of β carotene emulsified with Tween 80 was added. To the second a similar solution containing lutein instead of β carotene was added. To the third lycopene was added.

An identical set of 3 flasks was prepared, each flask containing 10 boiled segments of intestine to act as blanks. All the flasks were placed in a shaker at a temperature of 37°C and gently agitated at a rate of 30 strokes per minute. 0.1 ml of each of the solutions was withdrawn at 10 minute intervals over a period of one hour and the carotenoid content of the solution measured as previously described. At the end of the experiment the intestinal segments were thoroughly washed and only traces of carotenoids could be detected. The results of this experiment are presented in Table 6 and Fig. 6.

Table 6

Rate of disappearance of 3 carotenoids incubated with rat intestinal segments

Time in minutes	Lutein				Lycopene				β Carotene			
	Concentration with live tissue ug%	Concentration with boiled tissue ug%	Difference ug%	% uptake	Concentration with live tissue ug%	Concentration with boiled tissue ug%	Difference ug%	% uptake	Concentration with live tissue ug%	Concentration with boiled tissue ug%	Difference ug%	% uptake
0	330	330	0	0	246	246	0	0	250	250	0	0
10	198	308	110	33	159	240	81	33	220	250	30	12
20	110	312	202	61	-	238	-	-	190	240	50	32
30	82	300	218	66	90	230	140	57	170	243	73	29
40	68	290	222	67	69	235	166	67	170	228	58	23
50	65	285	220	67	60	221	161	65	170	226	56	22
60	50	290	240	73	60	226	166	67	161	224	63	25

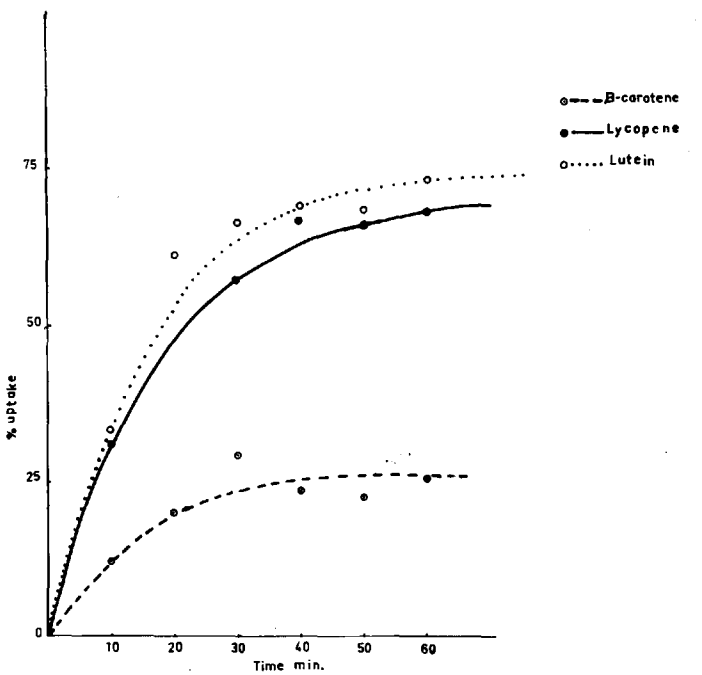

FIGURE 6. Uptake of lutein, lycopene and B carotene by intestinal segments of the rat.

4. Intestinal Homogenates

The whole small intestine, weighing 8 gm, of a vitamin A depleted rat was removed under anaesthesia, washed with chilled 0.1 M phosphate buffer pH 7.4 and homogenized in a Waring blender with 100 ml of the same buffer. The homogenate was centrifuged for 30 minutes at 5000 rpm and 0°C. 5 ml portions of the supernatant liquid were incubated at 37°C with β carotene, lycopene and lutein. 0.1 ml samples were withdrawn from each of the three flasks at 5 minutes intervals and pipetted into 0.1 ml of ethanol to stop the reaction. The carotenoids in each of these samples were estimated. The results of this experiment are shown in Table 7 and Fig.7.

After 30 minutes of incubation the reaction was stopped in the remaining homogenates by the addition of 4 ml of ethanol. Each mixture was extracted with petroleum ether and a sample of each petroleum extract was applied on thin layer plates and developed as previously described. There were no differences between the patterns obtained from the homogenates incubated with lutein, lycopene and β carotene after developing with sulfuric acid. Vitamin A was not detected in any of the extracts.

Table 7

Rates of Disappearance of three carotenoids incubated with extract of rat intestine.

Time in minutes	β carotene present in the medium	Lutein present in the medium	Lycopene present in the medium
	ug	ug	ug
0	56	51	58
5	49	39	48
10	46	31	36
15	43	23	30
20	41	25	29
25	41	23	20
30	38	21	20

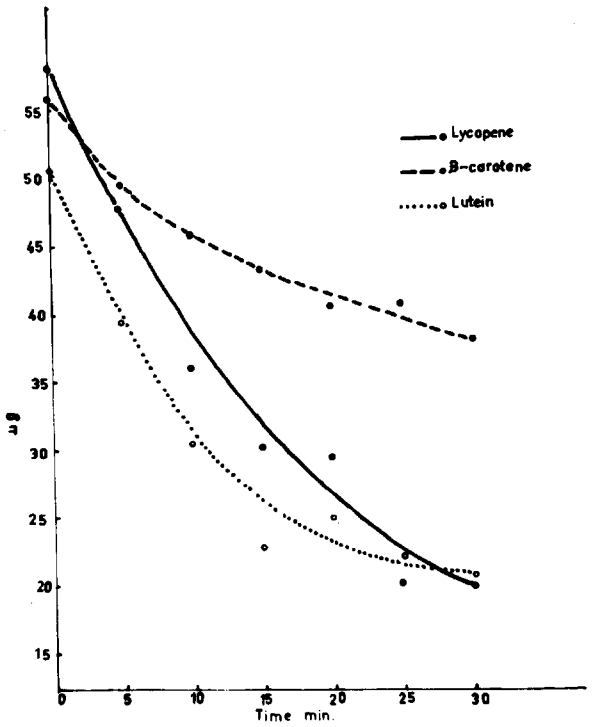

FIGURE 7. Rate of disappearance of lutein, lycopene and β carotene incubated with cell-free extracts of the small intestine of the rat.

CHAPTER IV

DISCUSSION

The feeding experiment was planned to investigate the effect of lutein and lycopene on the conversion of β carotene to vitamin A. In this experiment (Table 4) no significant effect of either lutein (t = 0.22) or lycopene (t = 0.26) on the utilisation of β carotene at the levels used was found, indicating that the enzyme system involved in the conversion is either specific for β carotene or that the levels of lutein and lycopene used were not sufficient to saturate the system.

The fact that only traces of carotenoids were found in the liver, serum and adipose tissue, the sites most likely to accumulate such compounds, indicates that they were either not absorbed at all or were completely metabolised during their passage through the gut. β carotene is transformed in the wall of the small intestine into vitamin A. The fate of the other carotenoids is not known although it seems unlikely that their metabolism is similar to that of β carotene, since Ames et al[63] could not detect α"vitamin A" after feeding carotene. Yet they showed that α"vitamin A" is readily stored in the animal body.

The second experiment (using intestinal loops) was designed to determine whether lutein and lycopene were absorbed in the small intestine of the rat, and to compare their rate of uptake, if any, with that of β carotene. The results (Table 5)

show clearly that lutein and lycopene are absorbed through the gut wall and that their rate of absorption is faster than that of β carotene. The disappearance of these carotenoids from the lumen of the small intestine and their absence from the blood indicates that they were actively metabolized by the animal.

The above experiment did not definitely prove that lutein and lycopene are metabolized in the small intestine. It is possible that they are removed by the blood stream to other organs where they could be actively metabolized. Incubation of these carotenoids with intestinal segments was used to settle this point. It also made possible the measurement of their rate of disappearance. The results of this experiment (table 6) clearly demonstrate that the intestinal segments were actively converting the carotenoids to colourless products. The rates of conversion of lutein and lycopene were found to be similar and about 3 times that of β carotene (Fig. 6). This difference is difficult to explain on the basis of chemical structure since lycopene and β carotene are both carotenes while lutein is a xanthophyll. This suggests that there may be more than one mechanism involved in the metabolism of the carotenoids.

An attempt was made to detect the metabolites resulting from the three carotenoids under study by incubating each carotenoid with a cell-free extract of the small intestine. This was not possible with the previous experiment since the use of whole tissue would have made the detection of such material difficult.

The time study indicated that the cell-free extract was behaving towards the carotenoids in a manner similar to that found in the intestinal segments experiment, (Table 7). However, at the end of this experiment, thin layer chromatography did not show any difference between the three incubation mixtures, except for the residual carotenoids. Moreover, it appeared that there was no difference between these preparations and a similar one without any carotenoid. Thin layer chromatography is capable of separating vitamin A and similar compounds which would be expected to result from lutein and lycopene if they were broken into fragments similar to vitamin A. As such compounds were not detected one may conclude that these carotenoids were not converted to such compounds under the condition of the experiment.

The complete absence of vitamin A from the preparation which contained β carotene can only be explained by assuming that the system responsible for the conversion of β carotene to vitamin A was absent from the preparation. Therefore the disappearance of β carotene from our cell-free system cannot be explained by its conversion to vitamin A. It seems possible that under our experimental conditions β carotene was metabolized in a fashion similar to that of lutein and lycopene, the fate of which is unknown.

From these experiments it is evident that in the rat lutein and lycopene as well as β carotene are absorbed in the small intestine and do not appear in the animal body. Moreover lutein and lycopene are metabolized in the small intestine at a faster rate than β carotene. The final product is unknown but appears to be unrelated to vitamin A. β carotene is shown to be capable of undergoing similar reactions to yield products other than vitamin A.

It seems reasonable to belive that carotenoids are metabolized by at least two different systems:

1. Provitamin A carotenoids ——→ Vitamin A
2. Provitamin and nonprovitamin A carotenoids ——→ unknown products.

From our work with intestinal homogenates it seems that β carotene could be acted upon by the second system as well as by the first. Lutein and lycopene are not acted upon by the first system because even at high concentrations they did not inhibit the conversion of β carotene to vitamin A. Furthermore the products which would be expected to result from lutein and lycopene if they were acted upon by the first system have never been detected in animal tissue.

The main objection to the central fission hypothesis for the conversion of β carotene to vitamin A i.e. the fact one molecule of β carotene does not produce two molecules of vitamin A in the

animal body can now be explained by assuming the presence of another enzyme system capable of degrading β carotene to products other than vitamin A. This assumption also explains the wide range of yields of vitamin A from β carotene reported by previous workers,[32,37,38,39] because the relative enzyme activity could vary in different animals.

Since the enzyme responsible for the conversion of β carotene to vitamin A appears to be very specific for β carotene, and possibly other provitamin A carotenoids, there is little possibility that the presence of other carotenoids will have any inhibitory effect on the utilization of β carotene.

The results of this work are very preliminary leaving many obvious questions to be answered: such as the fate of non-provitamin A carotenoids and of the part of β carotene that is not converted to Vitamin A. Considerable information could obviously be obtained by the use of C - 14 labelled carotenoids. Unfortunately no labelled carotenoid is available commercially and even unlabelled material of this nature is very limited. So far no one has synthesized a labelled carotenoid. The few workers who used labelled β carotene prepared their material biosynthetically. It was beyond the capabilities of our laboratory to prepare material in such a fashion since owing to the very low content of carotenoids in plant material, the expense involved is prohibitive.

Although the results of this work, based on the rat, cannot be directly applied to man, it is likely that the enzyme system responsible for conversion of β carotene to vitamin A is similar in both species. Our experiments indicate that this system does not act on nonprovitamin carotenoids, and any undesireable effect of these on the utilisation of β carotene seems unlikely.

SUMMARY

In the work presented here, the utilization of β carotene by the rat was studied in the presence of two nonprovitamin A carotenoids, lutein and lycopene. The vitamin A content of the rat liver was used as a measure of the efficiency of conversion of β carotene to vitamin A. The results indicated that nonprovitamin A carotenoids had no effect on the utilization of β carotene.

The behaviour of lutein and lycopene in intestinal loops of anaesthetized rats, intestinal segments and intestinal homogenates was studied and compared with that of β carotene. It was shown that in the intestine, lutein and lycopene are metabolized at a higher rate than β carotene.

This work suggests that in the rat intestine there are at least two systems for dealing with the carotenoids: (1) A specific system which acts only on provitamin A carotenoids to produce vitamin A, and (2) another system which acts on carotenoids in general yielding unknown products.

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