CCXLIII. THE ISOMERIZATION OF CAROTENES BY CHROMATOGRAPHIC ADSORPTION.

I. *PSEUDO- α -CAROTENE.*

BY ALBERT EDWARD GILLAM AND MOHAMMED SHAFIK EL RIDI.

From the Chemistry Department, Victoria University, Manchester.

(Received 21 July 1936.)

IN an earlier examination of the carotene of butter by the chromatographic adsorption method [Gillam & Heilbron, 1935] it was found that the pigment separated readily into two clearly-defined coloured zones when adsorbed on alumina from light petroleum solution. The colour, location and general appearance of the zones together with the absorption spectra of the separately eluted pigments indicated that, in addition to β -carotene, appreciable amounts of the α -isomeride were also present. α -Carotene has since been independently detected in butter, using magnesium oxide as adsorbent instead of alumina [Strain, 1935]. A comparison of the adsorption of butter carotene on each of these two adsorbents has since shown that chromatographic analysis on alumina makes the α -carotene content of butter appear much greater than when magnesium oxide is used.

In order to explain these anomalous results we have carried out adsorption experiments on pure β -carotene using, primarily, aluminium oxide as adsorbent. The results show that the process of adsorption itself separates β -carotene into two zones of pigment having properties exactly comparable with those obtained when gross butter carotene is used. Subsequent experiments have shown that the lower zone contains a new carotenoid pigment having absorption maxima (in the visible) different from those of β -carotene but identical with those of α -carotene. It is thus clear that when the carotene fraction of butter is adsorbed on alumina the second pigment is not wholly α -carotene, as was at first thought, but contains a considerable amount of a new compound.

These phenomena have already been reported briefly [Gillam & El Ridi, 1935, 2], but since then, starting with pure β -carotene, we have prepared the new pigment, which we propose to call *pseudo- α -carotene*. The present paper describes its preparation and properties.

Table I summarizes these properties in comparison with those of α - and β -carotene examined under the same conditions [cf. also, Kuhn & Brockmann, 1931; Kuhn & Lederer, 1931, 1, 2; Karrer & Walker, 1933; Strain, 1934, 2].

The probable structure of pseudo- α -carotene.

The method of preparation of *pseudo- α -carotene* from β -carotene and the analytical data indicate that the substance is a hydrocarbon carotenoid, of formula $C_{40}H_{56}$, produced by isomerization of β -carotene.

Preliminary feeding tests on rats, for which we are indebted to Mr A. L. Bacharach of Glaxo Laboratories, Ltd., show clearly that the substance possesses strong vitamin A activity when fed at the rate of 2γ per rat per day. From this it can be inferred that the compound contains at least one structural unit made up of a β -ionone ring and four ethylene linkages conjugated with it, this unit being—as far as our knowledge goes—specifically associated with vitamin A

Table I. *Properties of pseudo- α -carotene.*Compared with those of the α - and β -carotenes.

	<i>pseudo-α-Carotene</i>	β -Carotene	α -Carotene
Formula	$C_{40}H_{56}$	$C_{40}H_{56}$	$C_{40}H_{58}$
Melting-point	166° (uncorr.)	182° (uncorr.)	183.5° (uncorr.)
Rotation	Nil	Nil	+377° (benzene)
Absorption maxima ($m\mu$):			
Carbon disulphide	507, 477	514, 484	507, 476
Chloroform	486, 456	491, 463	487, 456
Petroleum (70–80° B.P.)	477, 446	484, 452	477, 447
Pyridine	493, 461	—	—
Ethyl alcohol	478, 447	—	—
Antimony trichloride:			
Colour	Blue	Blue	Blue
Absorption maxima ($m\mu$)	591 only	591, 541	591, 541, 492
Analyses. Found:*			
Carbon %	89.3	89.5	89.0
Hydrogen %	10.0	10.1	10.6
Partition, 90% methyl alcohol-petrol	Epiphasic	Epiphasic	Epiphasic
Hydrogenation (double bonds)	10.9	10.9	11.0
Adsorption on alumina	Slowly produces another zone above the main <i>pseudo-α-carotene</i> zone (β -carotene?)	Slowly forms a lower zone of <i>pseudo-α-carotene</i>	Slowly produces a lower zone below the main zone, different from <i>pseudo-α-carotene</i>
Adsorption affinity	Less than that of β -carotene	Greater than either α - or <i>pseudo-α-carotene</i>	Less than β - and similar to that of <i>pseudo-α-carotene</i>

* $C_{40}H_{56}$ requires 89.55, 10.45.

activity [cf. Kuhn & Brockmann, 1935]. Quantitative hydrogenation of the pigment shows that it contains a total of eleven ethylene linkages but absorption spectra data indicate that only ten of these are conjugated. The absorption maxima in the visible are identical with those of α -carotene, lutein and β -oxy-carotene, and the chromophore responsible for the absorption should therefore be the same in each case, i.e. one double bond in a β -ionone ring and nine others in a straight chain conjugated with it [Karrer, Morf & Walker, 1933; Kuhn & Brockmann, 1932, 1; Karrer, Zubrys & Morf, 1933; Kuhn & Lederer, 1931, 1]. The eleventh double bond must be isolated from the conjugated system (cf. α -carotene Formula I).

Owing to the similarity of the compound to α -carotene the question arises whether, in fact, it is identical or not. The evidence is as follows:

(a) *pseudo- α -Carotene* melts at 166° (uncorr.) $\pm 1^\circ$ only in four different crystalline preparations. Contrast α -carotene, m.p. 187° (corr.) [Karrer & Walker, 1933], and our own specimen (*vide supra*), m.p. 183.5° (uncorr.).

(b) It has no detectable rotation; contrast α -carotene $[\alpha]_{\text{D}} = +380^\circ$. This absence of optical activity, however, might be due to racemization which is to be expected from the manner of its preparation from inactive β -carotene.

(c) By adsorption on alumina *pseudo- α -carotene* is partially re-converted into a substance identical with, or closely similar to, β -carotene.

Genuine α -carotene, on the other hand, behaves quite differently on adsorption, for whereas *pseudo- α -carotene* is converted into a pigment having absorp-

tion maxima at longer wave-lengths, α -carotene is converted into another pigment with absorption maxima at shorter wave-lengths.

Pigment	Adsorption on alumina	Absorption maxima ($m\mu$) (petroleum B.P. 70–80°)		Pigment present
		484	452	
<i>pseudo</i> - α -Carotene	Upper zone	484	452	β -Carotene
	Lower zone	477	446	<i>pseudo</i> - α -Carotene
α -Carotene	Upper zone	477	447	α -Carotene
	Lower zone	470	441	"Neocarotene" (<i>vide infra</i>)

This property clearly differentiates *pseudo*- α -carotene from α -carotene, hence we conclude the former to be a new pigment and not the racemic form of α -carotene.

By analogy with the known structural formula of α -carotene [Karrer, Morf & Walker, 1933] (I)

I.

it is probable that *pseudo*- α -carotene has a similar structure. From its method of preparation and properties it is to be inferred that the mechanism of formation is a displacement of one terminal double bond out of the conjugated system of β -carotene (11F). As this is more likely to be displaced to the 3' 4' position than to the 4' 5' or 5' 6' positions the resulting formula is probably the same as that of α -carotene (cf. Formula I). The closely similar properties (without actual identity) of *pseudo*- α -carotene and α -carotene make it probable that the difference is due to a rearrangement of the double bonds (leaving a group of ten conjugated) or to geometrical isomerism. The final decision on this point must await further work.

The effect of other adsorbents on β -carotene.

Having found that adsorption on alumina results in the isomerization of β -carotene, other adsorbents commonly used for the separation of carotenoids were tested for this property. Thus, calcium hydroxide, which has been used successfully for the separation of α -carotene from β -carotene [Karrer, Walker, *et al.* 1933] and the purification of the former [Karrer & Walker, 1933], has been found to convert β -carotene into *pseudo*- α -carotene exactly as does alumina, but it gives a smaller yield each time.

Magnesium oxide which has also been used [Strain, 1934, 1, 2] for the separation of α - and β -carotenes was similarly tested by repeatedly adsorbing β -carotene, in light petroleum solution, upon it. The β -carotene showed no evidence of separation into two zones even after four adsorptions. Furthermore, if a mixture of β -carotene and *pseudo*- α -carotene prepared by repeated adsorption of β -carotene on either alumina or calcium hydroxide is adsorbed on magnesium oxide the chromatogram shows no evidence of heterogeneity. There is reason to believe, however, that even if it does not produce any separable *pseudo*- α -carotene from β -carotene as do the other two adsorbents, after repeated adsorption on magnesium oxide, β -carotene begins to show displacement of its absorption bands

towards shorter wave-lengths. Conversely, adsorption of *pseudo- α -carotene* displaces the maxima back again to wave-lengths not quite as long as those of β -carotene. The following data are typical.

Adsorption of pigments on magnesium oxide.

	Absorption maxima in petroleum (B.P. 70–80°) ($m\mu$)	
Pure β -carotene	483	452
Pure β -carotene, after 1 adsorption	480	450
Pure β -carotene, after 2 adsorptions	479	449
<i>pseudo-α-Carotene</i>	476	446
<i>pseudo-α-Carotene</i> , after adsorption	479	448
"Neocarotene" (by adsorption on alumina)	470	441
"Neocarotene" (by adsorption on MgO)	472	443

We believe these differences in the positions of the absorption maxima to be significant but they are approaching the limits of detection by the method of photographic or visual absorption spectrophotometry unless high dispersion spectrographs are used.

Adsorption experiments with α -carotene.

We have carried out a few preliminary experiments on the effect of chromatographic adsorption on α -carotene (pure crystalline material *ex* palm oil; analytical data in Table I). Like β -carotene this material also suffers change on adsorption on alumina or calcium hydroxide and separates into two zones of pigment which, after elution, exhibit absorption maxima as follows:

	Carbon disulphide $m\mu$		Petroleum (B.P. 70–80°) $m\mu$	
Upper zone	507	477	477	477.5
Lower zone	501	470	470	441

The lower zone clearly contains a pigment distinctly different from either β - or α -carotene and which we propose to call *neocarotene*. The absorption maxima characteristic of this pigment are closely comparable with those reported for a yellow substance, occurring in small quantity at the bottom of the column, when α - and β -carotene mixtures are adsorbed on calcium hydroxide [Karrer & Walker, 1933]. It is now clear that this material must have been formed from α -carotene during the actual adsorption. α -Carotene is readily changed into neocarotene by adsorption on magnesium oxide (contrast β -carotene on this adsorbent).

Pending the results of a fuller examination of the properties of neocarotene which we are now conducting discussion of its probable structure is necessarily only conjecture, but the absorption spectrum, identical with that of taraxanthin [Kuhn & Lederer, 1931, 1], violaxanthin [Kuhn & Winterstein, 1931; Karrer *et al.* 1933], and α -oxycarotene [Karrer *et al.* 1934], suggests a straight chain of nine conjugated double bonds as the chromophoric grouping. From its method of preparation from α -carotene—strictly analogous with the preparation of *pseudo- α -carotene* from β -carotene—and from the light absorption data it is probable that it has the structure of α -carotene (Formula I) but with the β -ionone double bond displaced, probably into the 3, 4 position, thus leaving an α -ionone

ring at each end and nine double bonds in conjugation. If this is correct neither half of the molecule will contain the half β -carotene structure essential for growth-promoting (vitamin A) activity, and the substance should therefore be biologically inactive, a forecast which we shall test when the material is available.

In considering the possible causes of the observed fact that when apparently pure β -carotene is repeatedly adsorbed on alumina it separates into two zones of pigment having different absorption spectra there appeared to be two possible explanations:

(a) that the original β -carotene contained *pseudo*- α -carotene as an impurity or,

(b) that the *pseudo*- α -carotene was formed from β -carotene.

The impurity hypothesis is definitely precluded by the fact that the carotene fractions remaining after one or more yields of *pseudo*- α -carotene had been removed were identical with each other, and with the original β -carotene. The following experimental results illustrate the point.

Fraction	M.P. (° C.)	Absorption bands petroleum (B.P. 70–80°) ($m\mu$)		C (%)	H (%)
		484	452		
Original β -carotene	182	484	452	89.5	10.1
After one adsorption (no separation)	—	484	452	—	—
After two adsorptions:					
Upper zone	183	484	452	88.8	11.0
Lower zone	166	477	446	89.3	10.0
Readsorption of upper pigment only:					
Upper zone	—	483	452	—	—
Lower zone	—	476	447	—	—

There is thus no doubt that *pseudo*- α -carotene is not present as an impurity but is produced directly from β -carotene.

A further interesting point about this isomerization of both α - and β -carotenes is that it appears to be reversible, for whereas by separating the pigment formed by adsorption and readsorbing the unchanged pigment several times it can be wholly converted into the corresponding isomeride, if either of the new pigments is readsorbed on alumina or calcium hydroxide it is reconverted largely into what is apparently its parent substance. Thus we have converted β -carotene into *pseudo*- α -carotene and then back again. The properties of the resulting pigment are very similar to those of β -carotene.

Reversal of isomerization by adsorption.

Sample	Source	Adsorption on	M.P. ° C.	Absorption bands petroleum (B.P. 70–80°) $m\mu$	
				484	452
β -Carotene	Grass	—	182	484	452
<i>pseudo</i> - α -Carotene	β -Carotene	Alumina	166	477	446
β -carotene?	<i>pseudo</i> - α -Carotene	Alumina	175	480	452

Despite the fact that the process β -carotene $\rightleftharpoons$ *pseudo*- α -carotene will proceed in either direction on adsorption although not to completion, *pseudo*- α -carotene once formed does not apparently revert spontaneously to β -carotene even when kept for several months in light petroleum solution (in the absence of oxygen).

The basic or amphoteric nature of the adsorbents so far examined is probably a significant factor in producing these changes which are almost certainly due to displacements of one double bond out of the respective conjugated systems. It is clear however that the ease of this displacement is affected by the presence of impurities. Thus the carotene fractions of butter or blood serum, even after removal of sterols by freezing, are separated readily into two zones of pigment by a single adsorption on alumina [Gillam & El Ridi, 1935, 1], whilst with pure crystalline β -carotene it requires much longer washing, or more usually elution followed by another adsorption, to bring about the isomerization.

The possibility of these phenomena being due to oxidation is precluded by the following considerations.

(a) β -Carotene can be converted into *pseudo- α* -carotene by adsorption even when oxygen is rigorously excluded and when the adsorbent has been previously ignited in a stream of nitrogen to remove some or all of the adsorbed oxygen.

(b) The analytical data show that *pseudo- α* -carotene contains no oxygen (or only a trace due to autoxidation).

(c) If *pseudo- α* -carotene were an oxidized carotene derivative it would be adsorbed above β -carotene in the adsorption columns and not below it.

In view of the observation that certain adsorbents can isomerize α - and β -carotenes during the actual process of chromatographic analysis the technique as a whole must be more critically regarded. Originally due to Tswett [1906] this method of separation of pigmented substances by adsorption on suitable powders has been applied most successfully to the study of carotenoid pigments, notably by Kuhn and his collaborators [Kuhn & Brockmann, 1932, 2; Winterstein, 1933; Zechmeister, 1934]. Whilst there can be no doubt that in the vast majority of cases of the application of the method to carotenoid problems the separations obtained are genuine, substances undergoing adsorption are sometimes changed in the process, e.g., the formation of a red material by adsorption of vitamin A concentrates on alumina or calcium hydroxide [Castle *et al.* 1933].

The isomerization of α - and β -carotenes by adsorption on alumina, described above, is thus another exceptional example where the analytical process itself affects the substances which it is designed only to separate. In view of these cases it is important to realize that, following the usual procedure of chromatographic analysis, spurious separations can occur even if only rarely.

EXPERIMENTAL.

Preparation of pseudo- α -carotene. 200 mg. of pure β -carotene (*ex* cocksfoot grass; M.P. 180–182°; rotation, nil [Pollard, 1936]), made available through the kindness of Prof. A. C. Chibnall and Dr A. Pollard, were dissolved in light petroleum (B.P. 70–80°) and poured through a 50 × 6 cm. column of alumina (Brockmann's standardized *ex* Merck, diluted 1:3 with inactive alumina) and washed down with light petroleum (with or without the addition of benzene). The first adsorption, as a rule, did not show appreciable separation, but after elution with light petroleum-alcohol, followed by water washing, drying and another adsorption, a clear separation into two zones was usually obtained. By continued washing the lower zone was washed out of the column and collected separately. The unchanged β -carotene was eluted, recovered and reabsorbed as before (4–5 times) until changed completely into the *pseudo- α* -form. In this way 200 mg. of β -carotene produces about 80–100 mg. of *pseudo- α* -carotene in some 4 litres of solvent, the remainder of the β -carotene being lost by oxidation, incomplete elution etc. Concentration of the solution by evaporation under reduced pres-

sure in nitrogen with subsequent filtration, to remove alumina, and cooling, gave a crop of crystals which was further purified by recrystallization from light petroleum. Adsorption methods could not be used on account of the reversion to β -carotene.

ANALYSES. Micro-analyses (Pregl's method) gave the following results:

pseudo- α -Carotene. (1) Found: C, 89.3; H, 10.0%; $C_{40}H_{56}$ requires C, 89.5; H, 10.45%. (2) Found: C, 89.2; H, 9.9%.

β -Carotene. Found: C, 89.48; H, 10.1%.

α -Carotene. Found: C, 89.0; H, 10.6%.

ROTATIONS. Determinations carried out in red light (Pointolite lamp and Wratten 29 red filter giving only rays near the cadmium line at 6438 A.U.).

α -Carotene. 10 cm. 0.105% in benzene + 0.396°; $[\alpha]_{ca} = +377^\circ$.

pseudo- α -Carotene. 10 cm. 0.15% in benzene + 0.002° $[\alpha]_{ca} = +1.3^\circ \pm 5^\circ$.

HYDROGENATION. Microhydrogenations, for which we are indebted to Mr H. Jackson, gave the following results: Solvent, decalin-acetic acid mixture; Catalyst-PtO₂ (for details of method cf. Jackson & Jones [1936]).

pseudo- α -Carotene. 2.522 mg. absorb 1.215 ml. of H₂ at 766.5 mm. and 21.2°; double bonds, 10.9.

The pseudo- α -carotene for hydrogenation was specially prepared using light petroleum and benzene free from thiophen. When less pure solvents were used the pigment contained sufficient sulphur to poison the catalyst and stop the hydrogenation either wholly or in part.

β -Carotene ex *cocksfoot grass*. 1.582 mg. absorb 0.780 ml. of H₂ at 754.5 mm. and 17.5°; double bonds, 11.0.

α -Carotene ex *Palm oil*. 1.470 mg. absorb 0.725 ml. of H₂ at 15.6° and 755 mm.; double bonds, 11.0.

ABSORPTION SPECTRA of the pigments were determined on a Hilger-Nutting visual Spectrophotometer, whilst objective confirmation was obtained photographically on a Hilger E₃ quartz spectrograph fitted with a Spekker photometer.

SUMMARY.

Following up previous work on the carotene of butter where it was found that adsorption on alumina or calcium hydroxide separates the pigment into two parts which simulate α - and β -carotene, respectively, it has now been found that what was apparently α -carotene is largely made up of a new pigment, spectroscopically identical with it.

The new pigment—pseudo- α -carotene—has been prepared by repeated chromatographic adsorption of β -carotene on alumina. It has m.p. 166°, is isomeric with β -carotene, and on readsorption is reconverted partially into this compound. Although spectroscopically identical with α -carotene it differs in having no rotation. It further differs in adsorption properties and hence is probably not a racemic form of α -carotene. It is suggested that the mechanism of formation is a displacement of a terminal double bond out of the conjugated system by the adsorption process.

Preliminary experiments with α -carotene indicate that this also can be changed by adsorption into another carotenoid ("neocarotene") the absorption maxima of which are displaced even further towards the violet from those of α -carotene. Its properties are being further examined.

It is pointed out that in using the chromatographic method for the separation of carotenoids care must be taken to differentiate between genuine separations and transformations brought about by the process itself.

We wish to acknowledge our indebtedness to Prof. I. M. Heilbron for his interest and advice, to Messrs Lever Bros., Ltd. (Dept. CTD/F) for a generous supply of palm oil unsaponifiable matter, and to Mr B. Lythgoe for a sample of α -carotene. Our thanks are also due to Messrs Imperial Chemical Industries for a grant, and to the University of Cairo for facilities enabling one of us (M. S. El R.) to participate in the work.

REFERENCES.

- Castle, Gillam, Heilbron & Thompson (1933). *Biochem. J.* **28**, 1702.
Gillam & El Ridi (1935, 1). *Biochem. J.* **29**, 2465.
—— (1935, 2). *Nature, Lond.*, **136**, 914.
—— & Heilbron (1935). *Biochem. J.* **29**, 834.
Jackson & Jones (1936). *J. Chem. Soc.* (in Press).
Karrer, Morf & Walker (1933). *Helv. chim. Acta*, **16**, 975.
—— Solmssen & Walker (1934). *Helv. chim. Acta*, **17**, 417.
—— & Walker (1933). *Helv. chim. Acta*, **16**, 641.
—— Schöpp & Morf (1933). *Nature, Lond.*, **132**, 26.
—— Zubrys & Morf (1933). *Helv. chim. Acta*, **16**, 977.
Kuhn & Brockman (1931). *Hoppe-Seyl Z.* **200**, 255.
—— (1932, 1). *Ber. dtsh. chem. Ges.* **65**, 894.
—— (1932, 2). *Hoppe-Seyl. Z.* **206**, 41.
—— (1935). *Liebigs Ann.* **516**, 95.
—— & Lederer (1931, 1). *Hoppe-Seyl. Z.* **200**, 246.
—— (1931, 2). *Hoppe-Seyl. Z.* **200**, 108.
—— & Winterstein (1931). *Ber. dtsh. chem. Ges.* **64**, 326.
Pollard (1936). *Biochem. J.* **30**, 382.
Strain (1934, 1). *Science*, **79**, 325.
—— (1934, 2). *J. biol. Chem.* **105**, 523.
—— (1935). *J. biol. Chem.* **111**, 85.
Tswett (1906). *Ber. dtsh. bot. Ges.* **24**, 316.
Winterstein (1933). *Klein's Handbuch der Pflanzen-Analyse*, **4**, 1403.
Zechmeister (1934). *Carotinoide.* (Julius Springer, Berlin.)