CLXI. THE ISOMERIZATION OF CAROTENES

III. RECONSIDERATION OF THE CHANGE

β -CAROTENE TO $\psi\alpha$ -CAROTENE

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(Received 1 July 1939)

EARLIER experiments on the chromatographic adsorption of β -carotene on Tswett columns of alumina or calcium hydroxide have shown that this pigment readily undergoes change into another carotenoid having a distinctly different absorption spectrum [Gillam & El Ridi, 1935]. Later work resulted in the isolation of a new crystalline carotenoid exhibiting an absorption spectrum different from that of β -carotene but identical with that of α -carotene. Further investigation and analysis showed this to be a carotene isomeride differing from α -carotene in certain properties and it was therefore named *pseudo- α -carotene* ($\psi\alpha$ -carotene) [Gillam & El Ridi, 1936]. A similar study of the adsorption of α -carotene indicated that this pigment could also be changed into yet another new entity (differentiated by means of its absorption spectrum) which was provisionally called neocarotene. On crystallization it was found that the absorption spectrum had reverted to that of the parent α -carotene. The properties of the crystals were however quite different from those of either α -carotene or $\psi\alpha$ -carotene, being in fact those of another new carotenoid which has been named neo- α -carotene, although the absorption spectra of all three are identical [Gillam *et al.* 1937]. At the time that these experiments were carried out it was concluded that the adsorption process itself was responsible for the isomerization of both the α and the β -carotenes, although Zechmeister & Chlcnoky [1937], Willstaedt & With [1938] and Euler *et al.* [1938] have been unable to observe isomerization of β -carotene by repeated adsorption alone.

Zechmeister & Tuzson [1938, 1, 2] have recently reported that, like the α - and β -carotenes, lycopene and kryptoxanthin readily isomerize into new carotenoids which they have named neolycopene and neokryptoxanthin respectively. At the same time these workers have also confirmed the isomerization of β -carotene into $\psi\alpha$ -carotene. Thus isomerization is definitely known to occur in at least four members of the carotenoid group, whilst, in addition zeaxanthin undergoes a change that may yet prove to be analogous [Strain, 1938]. It is therefore probable that there exists a complete series of these *isocarotenoid* pigments.

Zechmeister & Tuzson [1938, 1, 2], in studying the isomerization of lycopene, have made the important new observation that the phenomenon is not brought about by the adsorption process but is actually spontaneous and that the only action of the adsorbent is to separate the isomerides already formed. Thus they have shown that lycopene in solution slowly changes into an equilibrium mixture of lycopene and neolycopene (detected by change in absorption spectrum or by separation on adsorption), but that the change can be brought about much more quickly by heating in benzene for 30 min. These investigators have made an examination of the β -carotene- $\psi\alpha$ -carotene change sufficient to show that, like lycopene, β -carotene isomerizes spontaneously, although more slowly than does lycopene.

In view of the new approach to the problem provided by the work of Zechmeister & Tuzson and the biological importance of β -carotene as a vitamin A precursor as well as its use as an international standard for the measurement of vitamin A activity by biological methods, it seemed of importance to re-examine the isomerization of this substance into $\psi\alpha$ -carotene, particularly since the change appears to be a spontaneous one that might alter the nature of carotene preparations.

In our previous experiments [Gillam & El Ridi, 1936] we prepared $\psi\alpha$ -carotene from β -carotene by adsorption of petrol-benzene solutions of the pigment on alumina. If no separation into two coloured zones occurred the adsorbed pigment was eluted with alcohol-light petroleum, the alcohol removed and the solution *concentrated by evaporation*, then re-adsorbed on alumina. The lower zone contained $\psi\alpha$ -carotene and the upper, β -carotene, which was eluted separately, *concentrated* and re-adsorbed, when good separation into two zones was again obtained.

In planning a test experiment it seemed probable from the work of Zechmeister & Tuzson that the cause of the observed isomerization in our earlier experiments was due to the heating of the solutions during concentration between one adsorption and the next rather than to the adsorption process itself. Accordingly, it was planned to take a definite weight of carotene in light petroleum, pass it through a column of alumina, elute with benzene, concentrate *in vacuo* at not more than 25°, re-adsorb and elute as before. In order to reduce the effect of the time factor in the isomerization it was also planned to adsorb the carotene as many times as possible in one day. The experiment was finally carried out as follows.

2.3 mg. of β -carotene were dissolved in 2.5 ml. of benzene¹ and 2.5 ml. of light petroleum added. The solution was run on to a column of alumina (2.5 × 28 cm.; one part of active alumina—Merck's "Aluminium-oxyd standardisiert zur chromatographischen Adsorptionsanalyse nach Brockmann"—to two parts of inactive alumina as diluent).

The adsorbed pigment was washed down the column with light petroleum, no separation into two zones being observed. 5 ml. of benzene were then added, followed by light petroleum until the carotene was completely eluted. The solution was concentrated at 25° *in vacuo* and again adsorbed. A very faint separate zone was observed below that of the β -carotene but the percentage of $\psi\alpha$ -carotene was not determinable. The β -carotene fraction was eluted, concentrated and adsorbed a further four times, only slight separation occurring in each case. The subsequent adsorptions gave values for the percentage of $\psi\alpha$ -carotene in the total elutable pigments of 1.5, 1.47, 1.35 and 1.5 respectively (determined spectrophotometrically).

The absorption spectra of the pigments in the upper and lower zones were typical of those previously reported for β -carotene and $\psi\alpha$ -carotene respectively, i.e.

	Absorption maxima (light petroleum B.P. 70–80°)
Upper zone pigment	483 and 452 m μ
Lower zone pigment	477 and 447.5 m μ

(Data obtained on a Hilger-Nutting prism spectrophotometer.)

¹ The benzene serves the double purpose of dissolving the carotene in a smaller volume of solvent as well as of reducing the strength of adsorption of the pigment and so giving greater opportunity of separation of the two components on the column.

It is important to note that when using absorption spectra data to differentiate between carotenoids so similar as the β - and α -carotenes, the locations of the absorption maxima are very sensitive to change of solvent. Thus, if two solutions of β -carotene be made up in light petroleum, B.P. 60–80° and B.P. 40–60°, respectively, the absorption spectra of the two solutions will compare very closely with those of β -carotene and α -carotene (or $\psi\alpha$ -carotene), respectively. This is an obvious source of confusion in work on adsorption of carotenes from light petroleum solution.

The foregoing experiments make it quite clear that repeated adsorption alone is not the cause of the isomerization of β -carotene, a finding that confirms the conclusions of Zechmeister & Tuzson.

In view of the tendency of β -carotene to isomerize spontaneously into $\psi\alpha$ -carotene it is obviously important in any experimental work involving this carotenoid to have precise information regarding the stability of the pigment and the conditions affecting the change into $\psi\alpha$ -carotene. We have accordingly studied the effect of temperature on the isomerization which, from both our own and Zechmeister's observations, is obviously an equilibrium.

Effect of temperature on the change β -carotene to $\psi\alpha$ -carotene

For these experiments recrystallized β -carotene was used (M.P. 181°); intensity of light absorption at 452 m μ in light petroleum (B.P. 70–80°). $E_{1\text{ cm.}}^{1\%} = 2450$, hence order of purity = 96–98% [cf. Gillam, 1935]. The information most needed was the percentage of $\psi\alpha$ -carotene formed when β -carotene solutions are stored for varying periods of time in the refrigerator, at room temperature, and at the boiling point of the usual solvents (alcohol, light petroleum and benzene). The temperatures finally chosen for study were –2°, 20°, 40°, 60° and 80°.

–2°. Freshly adsorbed β -carotene (i.e. free from $\psi\alpha$ -carotene) was eluted with light petroleum-benzene and immediately cooled to about 0°. The solution was diluted to about 0.05% in equal volumes of light petroleum and benzene and introduced into five brown glass ampoules filled up to the neck and sealed. These were placed in the refrigerator at –2° and one ampoule opened and examined at the end of one, two, four and twelve weeks respectively. After opening each ampoule the solution was allowed to percolate through a column of alumina (active : inactive :: 1 : 1;¹ column 30 × 3 cm.) and washed down with a light petroleum-benzene mixture. The yellow-buff lower zone of $\psi\alpha$ -carotene washed out first and was collected separately from the browner β -carotene zone which followed. The amount of pigment in each of the separate solutions was determined by means of a visual spectrophotometer. A high order of analytical accuracy is not claimed, nor is it to be expected, in these evaluations owing to the easy oxidizability of the pigments, the minute amount of material in use, the method of evaluation and the sometimes slightly imperfect chromatographic separations.

The percentages of $\psi\alpha$ -carotene (on total eluted pigment) were found to be as follows:

1 week	< 1.9 %	4 weeks	< 3.0 %
2 weeks	1.7 %	12 weeks	2.5 %

The differences are not very significant but it is clear that at –2° the isomerization is very slow and that one can store β -carotene in solution for as long as

¹ Unless otherwise indicated the alumina used in all these experiments has been a mixture of one part of active plus two parts of inactive material, this composition having been found by experiment to give the best separations.

three months and be certain that less than 3–4 % of the pigment will be converted into the isomeric form.

Room temperature (ca. 20°). Pure β -carotene crystals were dissolved in light petroleum containing 30 % of benzene and adsorbed immediately. The β -carotene layer was eluted separately and the solution sealed up in brown glass ampoules filled up to the neck and kept in the dark at about 20°. After standing for varying lengths of time the percentage of $\psi\alpha$ -carotene was determined as before, the values obtained being as follows:

1 day	< 1 %	35 days	10.0 %
7 days	5.5 %	49 days	11.1 %
18 days	6.2 %		

Thus carotene solutions kept at room temperature definitely isomerize slowly if only to a few per cent. Even after standing for 7 weeks some 90 % of the pigment is, however, unchanged β -carotene (in the absence of oxygen).

40°. Solutions of β -carotene kept at this temperature gave the following values for $\psi\alpha$ -carotene content: 1 hr. 4 %; 3 hr. 5.4 %; 24 hr. 11.2 %.

60°. In 1 hr. at this temperature 7–8 % of the carotene was isomerized whilst after 3 hr. duplicate experiments showed the presence of 9.1 and 10.3 % respectively of the $\psi\alpha$ -form.

80°. At the boiling-point of light petroleum containing about 50 % of benzene (ca. 77–80°), the amount of $\psi\alpha$ -carotene formed in 1 hr. was found to be 8.0 and 9.0 % respectively, in duplicate experiments. In 3 hr. the values obtained in different experiments were 31.9, 34.1 and 27.0 % respectively, showing that at this temperature the change is relatively rapid. After concentrating solutions of β -carotene for various lengths of time at 77° values of 28.1, 32.0, 29.3 and 33.0 % were obtained in four different cases. These results give a mean value of 30.8 % and as longer boiling produces no higher values this must represent the equilibrium percentage of $\psi\alpha$ -carotene in the reversible isomerization (for these particular conditions).

As calcium hydroxide is at least as effective as alumina for the separation of the two isomerides [cf. Zechmeister & Tuzson, 1938, 2] β -carotene solutions isomerized at 80° were also adsorbed on this adsorbent. Typical values obtained were 28.5 and 29.6 % of $\psi\alpha$ -carotene, i.e. similar to those obtained with alumina.

The reverse change, $\psi\alpha$ -carotene to β -carotene

On the assumption that the spontaneous isomerization of β -carotene into $\psi\alpha$ -carotene is reversible and that the ultimate result achieved is an equilibrium between the two opposing reactions it was decided to check some of the previous values for the percentage of $\psi\alpha$ -carotene produced under given conditions of time and temperature by starting from $\psi\alpha$ -carotene instead of β -carotene.

The solution of pigment was prepared by isomerization of β -carotene by boiling the petrol-benzene solution for some 30 min. and then separating the $\psi\alpha$ -carotene by adsorption as previously described [Gillam & El Ridi, 1936] immediately before the following experiments.

One sample was stored at -2° for two months (under nitrogen) and was then adsorbed on alumina (active : inactive :: 1 : 2). Two duplicate experiments gave 95.0 and 94.0 for the percentages of $\psi\alpha$ -carotene in the mixture. The order of change (5.5 %) is thus comparable with the percentage of β -carotene isomerized

under the same conditions, i.e. 2% (*vide supra*) and the difference in the figures is consistent with the expectation that the change $\psi\alpha$ -carotene to β -carotene would be 2–3 times as fast as the reverse reaction.

After standing for 24 hr. at room temperature (*ca.* 20°) the $\psi\alpha$ -carotene solution was still found to contain 95% of this pigment, but after 2 weeks the value dropped to approximately 80%. After 2 months the $\psi\alpha$ -carotene content was found to be close to 53% thus showing that equilibrium had not yet been reached. (The ultimate value should be 30–31% of the $\psi\alpha$ -form.)

When heated in light petroleum-benzene mixture at the boiling point (*ca.* 80°) the equilibrium state was reached much more quickly from this side of the reaction than from the β -carotene side. Thus after half an hour's heating values of 30.0 and 32.5%, respectively, were obtained for the content of $\psi\alpha$ -carotene and further heating did not alter these values significantly. The mean value, 31.3%, compares reasonably well with the percentage of 30.8 found for the equilibrium value when starting from the β -carotene side of the reaction.

Separation of β -carotene and $\psi\alpha$ -carotene

Since it has been pointed out by Zechmeister & Tuzson that the conversion of β -carotene into $\psi\alpha$ -carotene is spontaneous and accelerated by heat, it follows that the best method of preparation of the latter pigment is to heat β -carotene in light petroleum or benzene for about an hour at the boiling point when the equilibrium mixture containing some 30% of the $\psi\alpha$ -form is obtained. Since it is also clear that adsorption alone does not bring about the change simple chromatography can be used to separate the two forms [cf. Gillam & El Ridi, 1936]. On the other hand crystallization of the equilibrium mixture from light petroleum yields solid β -carotene leaving the mother liquor temporarily richer in $\psi\alpha$ -carotene. On standing, or on heating, the $\psi\alpha$ -carotene percentage drops to that of the equilibrium state and is ready to deposit more β -carotene. The following experimental data illustrate these points.

(a) 30 mg. β -carotene in 75 ml. light petroleum were heated at 70–80° for 2 hr. On evaporation to crystallization-point 5 mg. β -carotene (containing <1% $\psi\alpha$ -carotene) separated. The mother-liquor contained 40% of $\psi\alpha$ -carotene but on heating at 80° for 1 hr. this value dropped to 30.2%.

(b) 27 mg. β -carotene (determined spectrophotometrically and free from $\psi\alpha$ -carotene) were partially isomerized by heating in light petroleum. ($\psi\alpha$ -carotene determined by adsorption = 26.0%.) The solution on crystallization yielded 5.5 mg. of β -carotene. The mother liquor contained 21.5 mg. of "carotene" of which 33.8% was found to be present as the $\psi\alpha$ -isomeride.

Separation and differentiation of α -carotene and $\psi\alpha$ -carotene

In the crystalline condition these two pigments are distinguishable by differences in melting point and rotation [cf. Gillam & El Ridi, 1936; Gillam *et al.* 1937]. In solution they are more difficult to differentiate, particularly as they exhibit absorption maxima at the same wave-lengths. On adsorption on alumina they separate fairly easily, the α -carotene being less strongly adsorbed [cf. Karrer *et al.* 1937]. In mixtures of β -, α - and $\psi\alpha$ -carotenes the $\psi\alpha$ -carotene is adsorbed, between the β - and α -isomerides. The two α -forms can be differentiated in solution by the fact that, on heating, α -carotene gives a mixture of α - and neo-carotenes whilst $\psi\alpha$ -carotene gives a mixture of $\psi\alpha$ - and β -carotenes, the two pairs being easily differentiated after adsorption by the location of the absorption maxima

of the eluted pigments. The following experimental data are typical and provide the support for these statements.

(Absorption maxima in light petroleum B.P. 70–80°)

It is of interest to enquire whether the isomerization of β -carotene into $\psi\alpha$ -carotene occurs in the solid state or only in solution. All the evidence goes to show that the change occurs only in solution; thus, samples of solid β -carotene kept in a refrigerator for periods of from 3 months to 2 years have yielded on solution less than 1% of $\psi\alpha$ -carotene when the solutions were examined immediately after preparation. The spontaneous change must therefore only commence immediately after the crystals are dissolved.

Further investigations are proceeding on the isomerization of α -carotene into neocarotene and neo- α -carotene.

SUMMARY

Earlier experiments on the effect of chromatographic adsorption on the α - and β -carotenes showed that these pigments readily undergo isomerization into new carotenoids. Thus, β -carotene gives $\psi\alpha$ -carotene, whilst α -carotene yields neocarotene and neo- α -carotene. It was originally thought that the actual adsorption process brought about the isomerization, but recent experiments by Zechmeister & Tuzson have shown that lycopene and kryptoxanthin can also change into new pigments and, moreover, that the change is spontaneous and not brought about by adsorption.

In the present investigation the isomerization of β -carotene has been re-examined in the light of these new observations. The suggestion of Zechmeister & Tuzson that the isomerization of β -carotene is also spontaneous and not due to adsorption, has been confirmed as also has the fact that the change is an equilibrium between two opposing reactions. The effect of temperature on the forward reaction (β -carotene to $\psi\alpha$ -carotene) has been studied and it has been found that whereas at -2° some 2.5% of the carotene is isomerized in 12 weeks, at room temperature some 11% is changed in only 7 weeks. At 80° the isomerization reaches equilibrium in less than 3 hr. when some 31% of $\psi\alpha$ -carotene is present.

The reverse change of $\psi\alpha$ -carotene to β -carotene has been found to proceed more rapidly. Even so, only 5–6% of this pigment, in solution, is changed in 2 months at -2° . At 80° the equilibrium is reached in about $\frac{1}{2}$ hr.

In order to reduce the tendency of carotene solutions to isomerize spontaneously they should therefore be stored as near to 0° as possible and concentration of the solutions should only be carried out at low temperature. When partial isomerization has occurred one adsorption on alumina or calcium hydroxide immediately before use gives β -carotene in pure form, whilst crystallization of the equilibrium mixture yields practically pure β -carotene in each crop of crystals, provided that the mother liquor is brought to equilibrium by raising to the boiling point between crystallizations.

α -Carotene and $\psi\alpha$ -carotene can be separated and differentiated by chromatographic methods with absorption spectra control.

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