CLXXV. ISOMERIZATION OF CAROTENOIDS

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(Received 8 June 1938)

It is of considerable interest to establish the limits within which the chromatographic method can be applied without danger of chemical alteration taking place in the column.

In the field of the carotenoids we were brought up against this problem [Zechmeister & Tuzson, 1938] in isolating the pigments of some fruits. The flesh of the water melon (*Cucumis citrullus*) in particular, which is rich in lycopene, sometimes gave extracts furnishing a single, very strong and homogeneous lycopene zone; in other experiments, however, carried out with the same raw material, the adsorbate showed a second layer below the lycopene ("neolycopene"), which could be washed down by means of benzene and light petroleum more easily than lycopene and differed markedly in its brownish yellow colour from the reddish lycopene zone.

A closer examination of the discrepancy between our various experiments showed that the character of the chromatogram depended on the time which elapsed between the extraction of the tissues and the beginning of the Tswett analysis. Fresh solutions furnished a single lycopene layer, older ones two coloured zones on the same part of the column; analogous results were obtained with solutions of pure crystalline lycopene. Light was thrown on these experiments by the fact that a freshly prepared solution of lycopene obviously changes its optical properties when kept. Further, this spontaneous alteration can actually be measured by means of colorimetric and spectroscopic determinations. The Tswett technique thus only furnishes a convenient demonstration of an effect already present in the solution.

The electronic configuration of the chromophore contained in the lycopene molecule becomes much less stable when the compound is dissolved. If the solution is kept at room temperature it can be easily shown that the colour intensity gradually decreases and that the extinction maxima slowly wander towards the shorter wave-lengths. Such changes must have been frequently observed by earlier workers but were probably ascribed to autoxidation. It cannot indeed be denied that simultaneous oxidative reactions do take place in the solution even under practically anaerobic conditions.

We find that autoxidation of lycopene leads partly to a colourless material and partly to coloured substances which are readily adsorbed on the highest section of the column and which cannot be reconverted into lycopene. On the other hand, neolycopene is always adsorbed below the lycopene zone and partly reverts to lycopene, if its zone is cut out and the elutriated substance is kept in a suitable solvent. The following experiments illustrate the processes indicated.

(a) A solution of 1.54 mg. lycopene (chromatographically homogeneous) in 20 ml. benzene was kept for 24 hr., diluted with light petroleum and chromatographed on $\text{Ca}(\text{OH})_2$. Two layers were obtained, containing 1.26 mg. lycopene and 0.13 mg. neolycopene. A similar experiment gave after 3 days 1.02 and 0.31 mg. respectively.

(b) 1.57 mg. pure *neolycopene* in 10 ml. benzene gave after 4 hr. 0.05 mg. lycopene and 1.49 mg. *neolycopene*; after 24 hr. 0.08 and 1.15 mg. and after 2 days 0.12 and 1.13 mg.

If the solution is heated, equilibrium between lycopene and its isomeride is reached in a much shorter time. As can be seen from Table I, 5 min. boiling under reflux is sufficient to cause noticeable effects and within 30 min. about half the lycopene undergoes rearrangement in benzene. A very similar result is obtained by heating *neolycopene* in benzene (Table III).¹

In the course of extended experiments we came to the conclusion that many former authors must have actually worked with solutions which had come to equilibrium in the course of the usual laboratory operations such as concentration, heating etc. Since *neolycopene* is transformed into ordinary lycopene by crystallization, it is understandable that its very existence has been overlooked. We may also mention that in some earlier experiments carried out by Dr Cholnoky in this laboratory, delay in the crystallization of and decrease in the colour intensity of certain lycopene solutions were observed, which could not at that time be explained.

Isomerization reactions among carotenes were first detected by Gillam & El Ridi [1935] and thoroughly described in two subsequent papers [Gillam & El Ridi, 1936; Gillam *et al.* 1937]. In the course of this extended and interesting study *pseudo- α -carotene* and *neo- α -carotene* were isolated in pure state. The formation of these new compounds was explained by the action of the adsorbent on carotenes. In such way repeated adsorptions must lead to increasing quantities of the isomeride. Without belittling the importance of the work of Gillam *et al.* we maintain that the isomerization of β -carotene is in all probability as spontaneous as that of lycopene. We claim that in no case is the phenomenon caused by the adsorption process and we are not able therefore to agree with the statement that "the analytical process itself affects the substances which it is designed only to separate".

In view of the earlier work of Gillam & El Ridi, our experiments with β -carotene were confined to the attempt to obtain sufficient information concerning the possible role of the adsorbent. As may be seen from the figures given in Table IV, the isomerization of β -carotene is directed by the same factors as the rearrangement of lycopene, though in the former case the reaction proceeds much more slowly. We find calcium hydroxide to be the most suitable adsorbent for such investigations, but since Gillam & El Ridi generally used alumina, a series of experiments was carried out with this adsorbent. Using the materials available to us we find that if a sample of β -carotene has once reached chromatographic homogeneity, little or no *pseudo- α -carotene* appears when the adsorption is subsequently repeated, provided that no considerable time elapses between each experiment and further, that all elutriations etc. are carried out not far from room temperature. Under these conditions we are unable to confirm the statement of Gillam & El Ridi that the amount of the newly formed isomeride increases with successive adsorptions.

Similar observations were made with various solutions of kryptoxanthin (Tables V-VI).

Finally we may briefly discuss the question of the natural occurrence of the new isomerides. Working under the established conditions, it was observed that rapidly prepared and chromatographed extracts (*ex* tomatoes, water melon, berries of *Tamus communis* etc.) yielded perfectly homogeneous lycopene zones.

¹ Preliminary experiments seem to show that both isomerides are much more rapidly interconverted at room temperature in the presence of a small quantity of iodine.

*neo*Lycopene was only formed if the same extracts were kept for some time. Already after 2 hr. at 20° a small amount of *neolycopene* could be demonstrated in the column. The red fruits of *Actinophleus Macarthurii* (from Java) which were conserved in ammonium sulphate for 2 years, gave only a trace of *neo*lycopene. It is obvious that in these cases lycopene occurs in the tissue under conditions which inhibit isomerization and which from this point of view may be compared with the crystalline state.

Gillam & El Ridi [1936] claim that the isomerization of β -carotene is accelerated by the presence of some impurities: "Thus the carotene fractions of butter or blood serum... are separated readily into two zones of pigments by a single adsorption... whilst with pure crystalline β -carotene it requires much longer washing or more usually elution followed by another adsorption to bring about the isomerization." We suggest that these observations could well be explained either by the original presence of *pseudo- α* -carotene in the butter and blood serum pigment or by formation of it during the lengthy course of the preparation.

EXPERIMENTAL

(Partly in collaboration with Miss I. BERGER)

Lycopene

Benzene solutions of crystalline lycopene (from various sources) were diluted with light petroleum (B.P. 60–80°), poured through a column of calcium hydroxide (25 × 5 cm.) and developed with a mixture of benzene and light petroleum 3 : 1. The main zone was cut out and eluted with benzene-alcohol. The latter was washed out and the remaining solution dried with Na₂SO₄ and evaporated rapidly *in vacuo* (bath temperature 40–45°). Under such conditions no isomerization takes place as was proved in special experiments. Working with small quantities the operation takes only 5 to 10 min.

The residue was dissolved in benzene, chloroform or acetone. If light petroleum were to be used in subsequent boiling experiments, the above evaporation could be omitted, the benzene being eliminated from the column by washing with sufficient quantities of light petroleum; the lycopene zone was then eluted with petroleum plus alcohol and the washed and dried eluate used directly for the boiling. Before proceeding to the latter an aliquot part of the solution was again chromatographed in order to check its absolute homogeneity. 20 ml. of each solution were boiled under reflux in an all-glass apparatus and the decrease of the colour intensity established by comparing directly the heated and the original solutions in a Leitz micro-colorimeter. The absorption maxima were measured with a Zeiss spectroscope.

Subsequently the cooled solutions were diluted with light petroleum and introduced into a calcium hydroxide column. (If acetone had been used, it was first eliminated by washing the solution.) In each case the chromatogram was developed with benzene and light petroleum and washed with a liberal quantity of the latter. A very good separation into two main zones was obtained, these being accompanied by much smaller layers on the upper part of the adsorbent.

The lycopene and *neolycopene* zones obtained after 30 min. boiling were eluted with light petroleum-alcohol and, after elimination of the alcohol, compared with the Kuhn & Brockmann [1932] standard in the micro-colorimeter. As the tints of the two isomerides are different (lycopene reddish, *neolycopene* brownish) it is advisable to use the solution of 145 mg. azobenzene in 100 ml. 96% alcohol for lycopene and a 10-fold dilution thereof for *neolycopene*. The results are summarized in Table I.

Table I. *Partial conversion of lycopene into neolycopene*

Solvent	mg. lycopene in 10 ml. of the original solution	Time of boiling min.	Spectrum m μ	Colorimetric value in % of the original	Amount of neolycopene formed in % of the total pigment in the column
Benzene	0.68	0	522, 487	100	
"	0.68	5	519, 484	87	
"	0.68	15	518, 485	87	
"	0.68	30	519, 485	82	55
"	0.69	0	522, 487	100	
"	0.69	5	519, 485	91	
"	0.69	15	519, 484	84	
"	0.69	30	519, 484	81	50
"	1.25*	0	521, 485	100	
"	1.25	30	519.5, 484	89	22
Chloroform	0.72	0	518, 484	100	
"	0.72	5	517, 483	89.5	
"	0.72	15	517, 482	87	
"	0.72	30	516.5, 483.5	76	39
"	0.82	0	518, 484	100	
"	0.82	5	517.5, 484	86	
"	0.82	15	517.5, 483.5	78.5	
"	0.82	30	517.5, 482.5	73	36
Acetone	0.40	0	505.5, 475	100	
"	0.40	5	505, 474	95.5	
"	0.40	15	504.5, 473	90.5	
"	0.40	30	504.5, 473	89.5	18
"	0.54	0	505.5, 475	100	
"	0.54	5	505, 474	96.5	
"	0.54	15	504.5, 473	90	
"	0.54	30	504, 473	83.5	20
Light petroleum	0.40	0	504.5, 472.5	100	
"	0.40	30	502.5, 470	86.5	44
"	0.28	0	504, 472	100	
"	0.28	30	502, 470.5	85.5	42

* This solution was not boiled but kept at 56°.

neoLycopene

This compound is much more easily soluble than lycopene itself and could not be obtained in crystalline form. Attempts to crystallize it from carbon disulphide-alcohol or benzene-methanol always led to samples which showed no perceptible difference from ordinary lycopene. The absorption bands of *neo*-lycopene show a displacement towards shorter wave-lengths (Table II).

Table II. *Absorption maxima of lycopene and neolycopene (m μ)*

Solvent	Lycopene	neoLycopene
Carbon disulphide	547, 505, 475	536, 498, 466
Benzene	522, 486, 445	512, 479, 450
Chloroform	516, 479.5, 434.5	512, 478, 447.5
Acetone	506, 475, 446	499.5, 468, 439
Light petroleum	506, 474, 445	499.5, 468, 439
Alcohol	504.5, 472.5, 445.5	500, 469, 439
Ether	503.5, 471.5, 443.5	496, 466, 438
Methanol	500, 470, 441	497, 467, 436.5

On heating *neolycopene* solutions, the colorimetric value of the liquid increases with considerable velocity (Table III) and the polyene which at first shows no evidence of heterogeneity separates into two well-defined zones on

Table III. *Partial conversion of neolycopene into lycopene*

Solvent	mg. neolycopene in 10 ml. of the original solution	Time of boiling min.	Spectrum m μ	Colorimetric value in % of the original	Amount of lycopene formed in % of the total pigment in the column
Benzene	~ 1	0	513.5, 580	100	
"	~ 1	15	518.5, 487	135.5	
"	~ 1	30	519.5, 484.5	153	~ 42

calcium hydroxide. The chromatogram is typical for the equilibrium mixture which may be obtained by starting from ordinary lycopene under the same conditions; the lycopene zone yielded a sample which gave no depression of M.P. when mixed with pure lycopene (*ex* tomatoes).

For the purposes of this work it was necessary to have a rough estimate of the colorimetric value of neolycopene, i.e. the amount had to be established which, when present in 1 ml. light petroleum, shows the same intensity as 14.5 mg. azobenzene in 100 ml. alcohol. Since neolycopene has not hitherto been obtained crystalline, the chromatographically homogeneous and colorimetrically matched solution was evaporated to dryness. The neolycopene content was about 0.003 mg. 1 ml., showing that the compound is much less coloured than ordinary lycopene, for which the corresponding figure is 0.002 mg./ml.

β -Carotene

The isomerization of this polyene takes place more slowly than that of lycopene. A solution of 50 mg. pure substance in 200 ml. light petroleum was kept for 24 hr. at room temperature and showed, on a calcium hydroxide or alumina column which was washed with the above solvent, only a slight zone of *pseudo- α -carotene*, the amount of which was about 1-2 % of the total carotene. The boiling experiments which are summarized in Table IV were carried out as

Table IV. *Partial conversion of β -carotene into pseudo- α -carotene*

Solvent	mg. β -carotene in 10 ml. of the original solution	Time of boiling min.	Spectrum m μ	Colorimetric value in % of the original	Amount of <i>pseudo-α-</i> carotene formed in % of the total pigment in the column
Benzene	1.04	0	496, 464	100	
"	1.04	5	496, 464	100	
"	1.04	15	494.5, 462.5	90	
"	1.04	30	495, 463	88	18
"	1.43	0	496.5, 465	100	
"	1.43	5	496, 464	97	
"	1.43	15	495, 463	92	
"	1.43	30	495.5, 462.5	92	21
Chloroform	0.86	0	495, 462	100	
"	0.86	5	495, 462	98	
"	0.86	30	494.5, 461.5	95.5	
"	0.86	60	494, 461.5	93	11
"	0.90	0	495, 462	100	
"	0.90	5	494.5, 461.5	98	
"	0.90	30	494.5, 462	97	
"	0.90	60	494.5, 461.5	97	12
Acetone	1.33	0	486, 455	100	
"	1.33	30	485.5, 453	~ 100	~ 0

described above, with the sole difference that the chromatogram was developed by means of pure light petroleum.

In a fairly long series of experiments we tried to establish whether the quantity of the isomeride increases or not from one adsorption to the next; the following data are typical.

(a) A solution of 100 mg. pure β -carotene in 150 ml. light petroleum (B.P. 60–80°) was chromatographed on alumina (mixture of 3 parts of Al_2O_3 Merck and 1 part standardized alumina, 25×5.7 cm.) and the column washed first with 1 l. light petroleum and then with 4 l. light petroleum plus 5% benzene. Traces of by-products fixed on the upper part of the column, as also a very small amount of *pseudo*- α -carotene, were neglected. We eluted the β -carotene zone with light petroleum-alcohol. Then the liquid was washed, dried and poured through a new column. The *pseudo*- α -carotene layer of this second chromatogram was much smaller than before, and it disappeared totally when a third chromatographic analysis was rapidly carried out.

(b) An analogous experiment with calcium hydroxide gave us a small *pseudo*- α -carotene zone. In this case, however, even the second chromatogram was composed exclusively of a single carotene layer and in spite of abundant washing no isomeride appeared.

Kryptoxanthin

As may be seen from Table V, this polyene-alcohol exhibits a tendency to spontaneous isomerization which, under similar conditions, is greater than that of β -carotene. Solutions of *neokryptoxanthin* may easily be converted into the

Table V. *Partial conversion of kryptoxanthin into neokryptoxanthin*

Solvent	mg. kryptoxanthin in 10 ml. of the original solution	Time of boiling min.	Spectrum $m\mu$	Colorimetric value in % of the original	Amount of <i>neokryptoxanthin</i> formed in % of the total pigment in the column
Benzene	0.86	0	496, 463	100	
"	0.86	5	495, 462	96.5	
"	0.86	15	494.5, 461	92	
"	0.86	30	493.5, 461	90	28
"	2.04	0	496, 463	100	
"	2.04	5	495, 462.5	97	
"	2.04	15	494, 462	91	
"	2.04	30	493.5, 461.5	88.5	30
Chloroform	0.97	0	494, 462	100	
"	0.97	5	493.5, 461	95.5	
"	0.97	15	493, 461	95.5	
"	0.97	30	493, 461	94	21.5
Acetone	1.14	0	487, 455	100	
"	1.14	30	485, 454	92	15

Table VI. *Partial conversion of neokryptoxanthin into kryptoxanthin*

Solvent	mg. <i>neokryptoxanthin</i> in 10 ml. of the original solution	Time of boiling min.	Spectrum $m\mu$	Colorimetric value in % of the original	Amount of <i>kryptoxanthin</i> formed in % of the total pigment in the column
Benzene	~ 1	0	491, 460	100	
"	~ 1	5	492, 461	103	
"	~ 1	15	493, 461	106.5	
"	~ 1	30	493, 461	110	46.5

equilibrium mixture with kryptoxanthin (Table VI). The absorption maxima of pure *neokryptoxanthin* solutions are given in Table VII.

Table VII. *Absorption maxima of kryptoxanthin and neokryptoxanthin ($m\mu$)*

Solvent	Kryptoxanthin	neoKryptoxanthin
Carbon disulphide	518, 482	510, 478
Benzene	497, 464	490.5, 459
Chloroform	494, 462.5	490.5, 458
Acetone	486, 454	480.5, 449
Light petroleum	484.5, 451.5	478, 447
Alcohol	484, 452	479, 448

SUMMARY

1. Solutions of chromatographically pure lycopene $C_{40}H_{56}$, β -carotene $C_{40}H_{56}$ or kryptoxanthin $C_{40}H_{56}O$ undergo, when kept at room temperature, a partial isomerization which manifests itself in the decrease of the colorimetric value and in the displacement of the absorption maxima towards shorter wavelengths.

2. The speed of this spontaneous isomerization, which tends towards, an equilibrium, increases on heating.

3. The interconversion is reversible.

4. Partly isomerized solutions always give two distinct layers in the Tswett column; the phenomenon is not caused by the adsorption experiment itself, but is already present in the solution. Experiments now in progress might show how far *cis*trans changes are responsible for it [cf. Gillam *et al.* 1937].

Finally we wish to thank Dr A. E. Gillam for having kindly checked some of our experiments with lycopene. This work was aided by grants from the Duke of Eszterházy and from the Széchenyi Society.

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