CCIX. STUDIES ON CAROTENOIDS[1].

I. THE CAROTENOIDS OF *DIOSPYROS* FRUITS.

II. THE CAROTENOIDS OF ARBUTUS FRUITS (*ARBUTUS UNEDO*).

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(*Received May 20th, 1935.*)

I. THE CAROTENOIDS OF *DIOSPYROS* FRUITS.

The plants of *Diospyros* are cultivated in Portugal and Spain in numerous varieties, the fruits of which are very difficult to distinguish. It is desirable to investigate their carotenoid content because of their significance in nutrition.

Karrer *et al.* [1932] have isolated lycopin and zeaxanthin from the fruits of *Diospyros Kaki*.

We are indebted to Prof. Dr Carisso, director of the Botanical Garden of Coimbra for a quantity of fruits of *Diospyros costata* which were collected in an immature state from one tree in November 1934 and kept till maturity in a room at 20°.

The fruits were dried with alcohol, which extracted a small quantity of yellow water-soluble colouring matter, and the carotenoids were then totally extracted with a mixture of light petroleum and acetone. When shaken with light petroleum and alcohol (90 %) the pigment passes entirely into the upper layer, indicating the absence of free xanthophylls[2]. After saponification, however, the greater part of the pigment passes into the lower layer.

The colouring matters contained in the upper layer were adsorbed from a solution in light petroleum on to activated aluminium oxide in the apparatus recently described by Winterstein and Schön [1934]. The developed chromatogram showed four distinct zones, from which we isolated cryptoxanthin, lycopene and β-carotene in the crystalline state and determined the presence of α-carotene by spectroscopy. The cryptoxanthin, which still contained a small quantity of lycopene, was separated from the latter by a second adsorption.

In the chromatographic analysis of the xanthophyll fraction by means of calcium carbonate a broad yellow uniform zone develops, which fills the greater part of the column and consists of zeaxanthin. The highest part of the column contains a very small zone of intensive yellow colouring distinctly different from that of zeaxanthin. From this zone we have isolated a small amount of a xanthophyll which forms yellow-brown stellate groups of crystals. The spectrum, the reaction with hydrochloric acid (blue coloration with 25 % HCl) and the behaviour on adsorption, show that it consists of violaxanthin.

[1] In this paper we publish results of our studies on the Carotenoids of Portuguese plants, which were carried out under the direction of the Director of the Institute, Prof. Dr A. de Morais Sarmento.

[2] As cryptoxanthin accompanies the hydrocarbons at this stage in the upper layer, we cannot decide as to the state of this xanthophyll in the fruit.

It is noteworthy that the main part of the vitamin A activity of *Diospyros* fruits depends on the presence of cryptoxanthin, which forms 70 % of the "hydrocarbon fraction," whilst carotene is only present in small amount. An analogous case is reported by Kuhn and Grundmann [1934, 1] with regard to yellow maize.

An interesting fact in regard to the biogenesis of the carotenoids of *Diospyros* fruits is that more than 90 % of the pigment, *i.e.* zeaxanthin, cryptoxanthin and β-carotene, belongs to the same steric group [Kuhn and Grundmann, 1934, 2], whilst α-carotene, lycopene and violaxanthin—the structure of the latter being still uncertain—form only a smaller part. Zechmeister and Cholnoky [1934] made similar observations on the colouring matters of the pimento.

Experimental.

Isolation of the carotenoids of the fruits of Diospyros costata.

15 kg. of the ripe fruits were crushed by hand and placed in 5 litres of alcohol for 2 days. The voluminous mass was then strongly pressed, the alcohol running out almost uncoloured. The residue was dried and extracted with 3 litres of acetone and 5 litres of light petroleum, and the acetone removed by washing with water. The solution was then washed 5 times with alcohol (80 %) which did not remove the colouring matter from the light petroleum. The petroleum solution was then concentrated *in vacuo* at 50° to a volume of 200 ml., the solution separated by filtration from several resinous products and saponified by adding 500 ml. of ether and the same volume of 5 % methyl alcoholic KOH, keeping the liquid for 2 days under an atmosphere of nitrogen. After saponification 1·5 litres of ether and then 3 litres of water were added, and the solution was washed free from methyl alcohol and alkali and then evaporated *in vacuo*. The residue was dissolved in 1 litre of light petroleum, and the xanthophylls were extracted from this solution by agitating several times with a total volume of 600 ml. of alcohol (90 %). The light petroleum solution, containing the hydrocarbons and cryptoxanthin, was dried with sodium sulphate and kept in an ice-box for 2 days. After this time the colourless material which had separated was filtered off.

The alcoholic solution, containing the xanthophylls, was mixed with 1·5 litres of light petroleum and the xanthophylls were driven into the latter by pouring in very carefully a large quantity of water. The aqueous layer was extracted with a further 1·5 litres of light petroleum, the latter washed with water, dried with sodium sulphate and kept in the ice-box for several days in the course of which colourless material separated out.

Chromatographic analysis.

(*a*) *Hydrocarbons.* The concentrated solution of the hydrocarbons was diluted to a volume of 3 litres and then adsorbed on to activated aluminium oxide. After total adsorption of the colouring matters, the column was washed with a large quantity of light petroleum. Four distinct zones developed:

	First absorption band in light petroleum (B.P. 80°) $m\mu$
1. Red-brown very sharp zone	484
2. Red, narrow, very sharp zone	506
3. Orange, more diffuse zone	484
4. Yellow, very narrow and diffuse zone	479

The filtrate contained little colouring matter with the same bands as the fourth zone.

The third and fourth zones were eluted with light petroleum containing 1 % of methyl alcohol—the solution was added to the coloured part of the filtrate and then adsorbed a second time on to activated aluminium oxide. By washing with light petroleum two distinct zones were obtained, an orange one of β-carotene in the higher part and a narrow yellow one of α-carotene in the lower part of the column. We did not succeed in obtaining the α-carotene in the pure crystalline state, its quantity being very small. The zone of the β-carotene was eluted and the solution concentrated *in vacuo* to 10 ml. After keeping the concentrate several hours in the ice-box, colourless material separated and was filtered off. The solution was then kept for 2 days in the ice-box after which time β-carotene crystals were observed in addition to many colourless crystals. The precipitate was boiled several times with methyl alcohol to remove the impurities and the carotene was then recrystallised from a mixture of benzene and methyl alcohol (1 : 2); M.P. 180° (uncorr. in evacuated tube). Absorption bands: in CS_2 519,485$m\mu$; in light petroleum (B.P. 80°) 484,452$m\mu$.

The second zone of the chromatogram containing lycopene was eluted and the solution concentrated *in vacuo* to 7 ml. After remaining 2 days in the ice-box, crystals of lycopene had separated along with colourless material. The precipitate was filtered off, washed on the filter with a little cold light petroleum and boiled three times with methyl alcohol. After recrystallisation from a mixture of benzene-methyl alcohol (1:1), the lycopene was pure. Absorption bands: in CS_2 548,508,576$m\mu$; in light petroleum: 506,474$m\mu$.

The first zone of the chromatogram, containing cryptoxanthin, was eluted and the solution evaporated *in vacuo*. The residue was dissolved in 200 ml. of ether to which was added the same volume of a concentrated solution of KOH in methyl alcohol, and the mixture was kept for 3 days, being heated to 35° for 4 hours at the end of this period. The subsequent distribution of the pigment between light petroleum and methyl alcohol (90 %) showed that no pigment had passed into the lower layer, this being the case with 95 % methyl alcohol as described by Kuhn and Grundmann [1933] for cryptoxanthin. A second chromatographic adsorption was then made and yielded a small amount of lycopene. The cryptoxanthin was adsorbed on to the activated aluminium oxide forming a narrow red zone, above which was a broader slightly-coloured zone, which showed the same absorption bands. The middle zone was eluted and the solution evaporated *in vacuo*. On keeping the residue in the ice-box, it solidified. It was then boiled 3 times with methyl alcohol, which dissolved a large part of the colourless material, and the residue was dissolved in 5 ml. of hot benzene. On adding 10 ml. of methyl alcohol to the hot solution a large part of the colourless material was precipitated and was filtered off. The filtrate was evaporated, dissolved in hot benzene and the same volume of methyl alcohol added. After being kept in the ice-box for 2 days, 50 mg. of cryptoxanthin crystallised out. The crystals were boiled with methyl alcohol and twice crystallised from benzene-methyl alcohol (1 : 1). 35 mg. of pure cryptoxanthin were obtained; M.P. 166–167° (uncorr. in evacuated tube). Absorption bands, in CS_2 518,484,455$m\mu$; in chloroform 495,465$m\mu$.

(*b*) *Xanthophylls.* The fraction containing the xanthophylls was adsorbed from solution in 3 litres of light petroleum on to calcium carbonate. On washing with the solvent a large uniform yellow zone developed, which filled the greater part of the column and was limited above by a small deep yellow zone. This latter was eluted with methyl alcohol, the liquid evaporated *in vacuo* and the residue

boiled several times with light petroleum. The xanthophyll was then crystallised from a little methyl alcohol. About 1 mg. of yellow brown needles was obtained. Absorption bands in alcohol, 476,445$m\mu$; in CS_2: 502,472$m\mu$. In ethereal solution a strong blue colour developed with 25 % HCl, whilst acid of 19·5 % gave no reaction. We believe therefore, that this xanthophyll is identical with violaxanthin.

The middle zone was eluted with methyl alcohol and concentrated to a small volume. On adding the same volume of light petroleum, zeaxanthin crystallised out; this was purified by recrystallisation from methyl alcohol. It forms long yellow leaflets in the shape of swallow-tails; M.P. 210° (uncorr. in evacuated tube). Absorption bands in light petroleum 482,453$m\mu$; in CS_2 519,483$m\mu$; in alcohol 483,452$m\mu$.

Colorimetric determination of the pigment.

In 15 kg. of fresh fruit (1·1 kg. of dry substance) we have found the following quantities:

α-Carotene	5·4	mg.
β-Carotene	15	,,
Lycopene	5	,,
Cryptoxanthin	75	,,
Zeaxanthin	180	,,

The author wishes to express his acknowledgments to Dr Mendonça of the Botanical Institute of the University of Coimbra for supplying the fruits.

II. THE CAROTENOIDS OF ARBUTUS FRUITS (*ARBUTUS UNEDO*).

The arbutus is generally cultivated in the Mediterranean countries, its fruits serving both for food and for the preparation of aromatic drinks.

The exterior of the fruit is covered with red cicatrices, which contain an anthocyanin. This may be extracted with alcohol; on the addition of sodium hydroxide the red solution turns to blue-green; with ferric chloride a deep blue colour develops, which after some time turns to violet and then becomes pale.

The flesh of the fruit is coloured yellow by carotenoids, which after drying may be extracted with light petroleum and ether. When the pigment is shaken with light petroleum and alcohol (90 %) the latter remains uncoloured, indicating the absence of free xanthophylls. After saponification with alcoholic potash, however, the greater part of the pigment passes into the alcoholic layer.

Chromatographic analysis of the pigment has shown the presence of diverse carotenoids. The petroleum layer, containing the hydrocarbons and the xanthophylls with only one hydroxyl group, gives after adsorption five zones. The uppermost zone, possessing, after elution, only undefined absorption bands, consists of oxidation products. From the second distinct zone we were able to isolate cryptoxanthin in the pure crystalline state. The third very narrow zone contained lycopene, which, however, we could not isolate in the crystalline state because of its extremely small quantity and because of the great amount of uncoloured contaminants. The last two zones contained β- and α-carotene, from which we were able to isolate β-carotene in the pure crystalline state, the quantity of the α-isomeride being very low.

The xanthophyll fraction, which represents about 80 % of the total pigment, proved in the chromatographic analysis to be almost pure violaxanthin.

In addition to violaxanthin we isolated a very small quantity of crystallised xanthophyll, which represents a mixture of zeaxanthin with a little lutein.

Violaxanthin in general occurs in appreciable quantities only in certain flowers, whilst its content in fruits is very meagre. It is therefore surprising to note that it forms the greatest part of the pigment of arbutus fruits. Its isolation presents no difficulty and for this reason arbutus fruits are a convenient source of violaxanthin, although they contain it only in relatively small quantities.

Kuhn and Winterstein [1931] record for violaxanthin a M.P. of 199–199·5° (corr.), while Karrer and Morf [1931] found it 8° higher. Our purest preparations had M.P. 203° (corr. in an evacuated tube).

EXPERIMENTAL.

25 kg. of the ripe fruits were crushed by hand and kept for 1 day in 25 litres of acetone (70 %). The mass which had an acid reaction was previously neutralised with potassium carbonate. The acetone, which contained the greatest part of the anthocyanin, was pressed out and the residue (5 kg.) kept in alcohol (95 %) for 1 day, the rest of the anthocyanin and a little carotenoid passing into solution. The pressed and dried residue (4 kg.) was then extracted in several portions with a total of 10 litres of light petroleum and then with 10 litres of ether. The solutions were concentrated *in vacuo* at 40–50° to 200 ml., and filtered from a colourless precipitate; the filtrate was then agitated twice with alcohol (80 %) and twice with alcohol (90 %). At this stage the colouring matter remained in the upper layer. The solution was then evaporated *in vacuo*, the residue dissolved in 200 ml. of ether and this solution saponified by adding the same volume of 5 % methyl alcoholic KOH and keeping under an atmosphere of nitrogen for 1 day. After adding 200 ml. of light petroleum 50 ml. of water, were added, the saponified xanthophylls passing into the alcoholic layer. The upper layer was again shaken four times with 70 ml. of methyl alcohol (80 %), the various methyl alcoholic solutions being shaken once with 50 ml. of light petroleum.

The ether-light petroleum solution was carefully washed with water to avoid emulsification until no longer alkaline and then evaporated *in vacuo*. The residue was dissolved in 2 litres of light petroleum and this solution served for chromatographic analysis.

The chromatographic analysis by means of activated aluminium oxide showed the following zones after washing with 3 litres of light petroleum.

	Absorption bands in light petroleum ($m\mu$)	
1. Narrow red zone	—	Oxidation products
2. Broad sharp yellow-red zone	480,447	Cryptoxanthin
3. Narrow sharp violet-red zone	504,472	Lycopene
4. Broad orange zone	483,452	β-Carotene
5. Yellow zone not distinct	473,442	α-Carotene

The filtrate was coloured yellow with a little α-carotene.

The colorimetric determination of the fractions gave the following result (25 kg. of fresh fruits).

1. Cryptoxanthin	102 mg.
2. Lycopene	5 ,,
3. β-Carotene	60 ,,
4. α-Carotene	12 ,,
(Violaxanthin ...	... *ca.* 800 ,,)

Cryptoxanthin. The zone containing cryptoxanthin was eluted with light petroleum containing 1 % of methyl alcohol and saponified a second time with concentrated methyl alcoholic KOH for 3 days, at the end of which it was heated for 4 hours to 40°. It was then washed with water to remove alcohol and alkali, the solution dried over sodium sulphate and the chromatographic analysis made a second time by means of activated aluminium oxide, a small quantity of lycopene being separated. After elution the solution was evaporated. The resinous residue crystallised in the course of 1 day when kept in the ice-box. It was boiled in small portions with a total of 150 ml. of methyl alcohol and dissolved in 3–4 ml. of benzene and to the hot solution were added 15 ml. of methyl alcohol. After some time, cryptoxanthin crystallised in characteristic six-sided leaflets. It was recrystallised twice from a benzene-methyl alcohol mixture (1 : 1) and then had M.P. 169° (uncorr. in evacuated tube). Absorption bands[1]: in light petroleum 483,452 $m\mu$; in CS_2 518,458 $m\mu$.

β-Carotene. The zones containing the carotenes, after elution, were united, washed with water, dried and separated a second time. The upper uniform zone contained β-carotene. It was eluted, agitated five times with methyl alcohol (90 %), evaporated and kept in the ice-box. After some time, carotene crystallised in addition to colourless matter. The carotene was boiled several times with methyl alcohol, to remove the impurities and then recrystallised from a mixture of benzene and methyl alcohol (1 : 1). 5 mg. of pure product were obtained. M.P. 177° (uncorr. in evacuated tube). Absorption bands in CS_2 520,485 $m\mu$; in light petroleum (B.P. 80°) 483,452 $m\mu$.

Xanthophylls. The alkaline methyl alcoholic solutions containing the xanthophylls were diluted with water and extracted with ether. The ethereal solution was evaporated and the residue dissolved in a little chloroform. To this solution, containing about 800 mg. of xanthophyll, light petroleum was added until a permanent opacity was produced, and the mixture was then kept in the ice-box. After 1 day 450 mg. of xanthophyll had crystallised out. The part which remained in solution could be extracted with methyl alcohol (90 %). On diluting with water an oily precipitate was formed, which could not be crystallised.

The crystalline xanthophyll—giving a deep blue colour with strong hydrochloric acid—was dissolved in 150 ml. of benzene and the solution diluted with 450 ml. of light petroleum and adsorbed on a column of calcium carbonate. A broad uniform yellow zone developed below which appeared a narrow orange-yellow zone. The upper zone was eluted with methyl alcohol and the solution concentrated *in vacuo* to 150 ml. and kept in the ice-box. After 1 day yellow crystals had separated, which were filtered off. To the filtrate water was added until a permanent opacity resulted. After being kept for a further day in the ice-box, a second fraction of crystals resulted. After recrystallisation from CS_2 the two fractions gave identical preparations of pure violaxanthin. M.P. 203° (corr. in evacuated tube). The xanthophyll gave the colour reactions with acetic acid, picric acid *etc.* as described by Kuhn and Winterstein. Absorption bands in CS_2 501,469 $m\mu$; in chloroform 482,451 $m\mu$; in alcohol 472,442 $m\mu$.

The lower zone was eluted with ether and concentrated to a small volume. After the addition of 5 ml. methyl alcohol 20 mg. of xanthophyll crystallised. It was recrystallised from methyl alcohol, depositing 12 mg. of long oblique leaflets, probably consisting of a mixture of zeaxanthin with a little lutein, in which the former predominates; M.P. 201–202° (uncorr. in evacuated tube). The product darkened slightly at 196°. Absorption bands in CS_2 514,479 $m\mu$;

[1] All spectroscopic measurements were made with a Hilger prism spectroscope and a copper sulphate-ammonia filter.

in chloroform 490,459$m\mu$. The reaction with strong hydrochloric acid was negative.

The author wishes to express his acknowledgments to Prof. E. P. Basto, Director of the Chemical Institute of the University of Coimbra for the use of a spectroscope.

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