follows that the action of BHDB in inhibiting the effect of thyroxine on the oxygen consumption of mice and its opposite action in the case of triiodothyronine (MacLagan *et al.* 1952) cannot have been due to changes in the total excretion rate. They must therefore reflect alterations in the activity of the circulating thyroid hormone, probably consequent upon inhibition of deiodination processes.

SUMMARY

1. Details are given for a method of preparation from 3:5-diiodo-L-thyronine of triiodothyronine labelled in the 3' position with ^{131}I . Radioactive triiodothyronine appears to be much more stable in solution at pH 8–9 than labelled thyroxine.

2. The labelled compound was injected into rats, with or without simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), and the radioactivity of the excreta was measured.

3. Administration of BHDB caused a marked reduction in the output of urinary iodide in intact rats after a single injection of labelled triiodothyronine at a number of different dose levels. This is interpreted as being due to interference with the deiodination of this compound.

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Studies in Carotenogenesis

13. THE CAROTENOIDS OF THE FLOWER PETALS OF *CALENDULA OFFICINALIS*

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The presence of β - and γ -carotenes, lycopene, violaxanthin and rubixanthin in *Calendula officinalis* (pot marigold) has already been reported in the early investigations of Zechmeister & Chohnoky (1932) and Kuhn & Grundmann (1934); lycopene was absent from the very yellow varieties (Zechmeister & Chohnoky, 1932). Preliminary tests on the petals of a variety of *C. officinalis* producing very rich dark orange flowers showed that they contained a complex mixture of carotenoids including some which had not previously been reported. The investigation was therefore continued in greater detail.

EXPERIMENTAL

Materials. Fully opened flowers were gathered from a garden in north-west Cheshire. The seeds were obtained from Messrs Elsoms, Ltd., Spalding.

Extraction of pigments. The flower petals were removed from the calyx, placed in a Waring Blendor, covered with acetone and blended for 2 min. Most of the acetone extract was decanted, and the remainder removed from the residue by filtration through a sintered glass funnel. The residue was then returned to the Blendor and extracted again with acetone. This procedure was repeated until all the pigments had been extracted. The acetone extracts were combined and an equal volume of ether was added, followed by water

(slowly) until two layers formed. The carotenoids passed quantitatively into the ether layer and the water-soluble pigments (flavones, etc.) remained in the lower layer, which was discarded. The ether layer was washed twice with small quantities of water to remove acetone, dried (Na_2SO_4), filtered, and the solvent removed *in vacuo* at room temperature. The residue was dissolved in light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) and subjected to chromatographic analysis.

Chromatography and identification of pigments

The pigments were separated by column chromatography on either activated alumina (grade '0', P. Spence and Co., Widnes), deactivated alumina made by treating grade '0' with methanol (Goodwin & Srisukh, 1949), or by an appropriate mixture of the two.

The well authenticated carotenoids were identified (a) by

their relative positions on the column when chromatographed under the standardized conditions used in this laboratory, and (b) by the shape and location of their absorption spectra (Goodwin, 1952*a, b*, 1953, and unpublished observations). These two criteria can generally give unambiguous identification; cases where any possible ambiguity exists are emphasized in discussing the results obtained.

Two main chromatographic separations of the carotenoids of *C. officinalis* were carried out; the first on deactivated alumina (separation 1) and the second on a mixture of 3 parts activated and 2 parts deactivated alumina (separation 2). The resolution obtained on the first chromatogram is given in Table 1. Fraction 1 which ran straight through the column of weakened alumina and consisted of a mixture of hydrocarbons was resolved on the more active alumina, resulting in the separation of the components listed in Table 2.

Table 1. *First separation of C. officinalis carotenoids*

Adsorbent, deactivated alumina; developer light petroleum containing varying amounts of ether; zones numbered in order of increasing adsorptive power.

Zone	Colour	Absorption maxima in light petroleum (m μ .)	Concn. (% v/v) of ether required to remove zone from column	Provisional identification
1	Yellow-brown	—	0 (Moved straight down column)	Mixture of carotenes
2	Lemon-yellow	418, 438, 467	2.5–5	?
3	Lemon	~415, 434, 463	5	? <i>cis</i> Isomer of 2
4	Lemon	401, 420, 446, 466	10	Mixture of 3 and 5
5	Lemon	~401, 420, 446	12	Flavochrome
6	Brown-orange	459, ~482	15	Rubixanthin-like
7	Lemon	410, 422, 448	15–20	Mutachrome
8	Lemon	400, 422, 424	25	Mixture containing mostly 7
9	Brown	423, 446, 472	50	Lutein
10	Lemon	400, 422, 440	50–60	Flavoxanthin +
11	Lemon	400, 422, 446	75	chrysanthemaxanthin
12	Brown	402, 422, 443	Ether	—
13	Khaki (small)	Indeterminate band	Ether + 1% ethanol	Oxidation product
14	Khaki (small)	420, 441, 460	Ether + 5% ethanol	? Neoxanthin

Table 2. *Resolution of fraction 1 (Table 1) into its component polyenes*

Adsorbent, 3:2 mixture of activated and deactivated alumina; developer, light petroleum containing varying amounts of ether; zones lettered in order of increasing adsorptive power.

Zone	Description	Absorption spectrum maxima in light petroleum (m μ .)	Concn. (% v/v) of ether required to remove zone from column	Provisional identification
1A	Colourless; non-fluorescent in ultraviolet light	Indeterminate	0	Possible traces of phytoene
1B	Colourless; vivid green fluorescence in ultraviolet light. Tendency to separate into two zones	330, 348, 367	1	Mixture of phytofluene isomers
1C	Brownish yellow	~425, 449, 474	2–3	β -Carotene
1D	Lemon-yellow	380, 400, 422, 451	5	Mixture of ζ -carotene and pigment X (Goodwin & Osman, 1953)
1E	Brownish	~410, 432, 456, 483	10	Mixture
1F	Pink	438, 462, 487	10–15	Mixture
1G	Yellow	439, 467, 485	15–20	?
1H	Pink	439, 468	20	? Lycopene isomer
1J	Bright pink	443, 469, 495	25–30	Neolycopene A
1K	Brick red	445, 471, 499	30	Lycopene

Table 3. *Resolution of mixed fractions IE, IF, IG (Table 2) into their components*

Adsorbent, fully activated alumina; developer, light petroleum containing varying amounts of ether; zones lettered in order of increasing adsorptive power.

Zone	Description	Absorption spectrum maxima in light petroleum (m μ .)	Concn. (% v/v) of ether required to remove zone from column	Provisional identification
EA	Pinkish-brown	430, 458, 487	35	γ -Carotene
1EB	Pink	443, 469, 495	50-60	Neolycopene A
1EC	Bright pink	446, 471, 500	60-70	Lycopene

Examination of the fractions

Fractions 1A-1M. The following were identified after purification by rechromatography: β -carotene (1C), neolycopene A (1J) and lycopene (1K). Fraction (1B) was rechromatographed on active alumina using light petroleum containing 10% (v/v) ether as developer and separated into two colourless zones which were detected by their green fluorescence in ultraviolet light. Both the zones had the same absorption spectrum ($\lambda_{\max}$ 330, 348, 367 m μ .), characteristic of phytofluene. The lower zone represents phytofluene which is a *cis*-polyene (Petracek & Zechmeister, 1952) and the upper zone *trans*-phytofluene.

Fraction 1D was chromatographed on fully activated alumina and on elution with light petroleum separated into two zones: 1DA, the less strongly adsorbed pigment with absorption maxima at 405, 424, 451 m μ . and 1DB ($\lambda_{\max}$ 381, 401, 425 m μ .); 1DA is the pigment X described by Goodwin & Osman (1953) and 1DB is the ζ -carotene of Porter & Zscheile (1946).

Fractions 1E, 1F and 1G, which originally were not well separated, were combined and rechromatographed on activated alumina (see Table 3). The main pigment is γ -carotene, accompanied by small amounts of lycopene and neolycopene.

Fraction 1H was rechromatographed on strong alumina but was essentially homogeneous, only traces of neolycopene being present. It is probably prolycopene I (Zechmeister & Pinckard, 1947). Fraction 1J was rechromatographed and separated into lycopene and neolycopene, whilst fraction 1K was almost entirely lycopene.

Fractions 2-8 (*xanthophylls containing one polar grouping*). It appears from their adsorptive power that fractions 2-8 are carotenoids containing one oxygen atom as a hydroxyl, epoxy or keto group. This is confirmed by the fact that none of these pigments is hypophasic in the partition test with 90% (v/v) aqueous methanol.

Fraction 2 from its chromatographic behaviour and the shape and position of its absorption spectrum, is a pigment not previously described; fraction 3 appears to be a *cis* isomer of fraction 2 because its spectrum has the same shape but the maxima have been shifted slightly to a lower wavelength. Similarly, fraction 5, characterized by a spectrum very similar to that of ζ -carotene, but adsorbed above instead of below lycopene, is flavochrome (Karrer & Jucker, 1950; Goodwin, 1952c).

Fraction 6 has the adsorption properties and the position of its main absorption band very similar to those of rubixanthin (Kuhn & Grundmann, 1934); its subsidiary bands were, however merely inflexions and not true bands, whilst those of rubixanthin are well marked and indistinguishable from that of the parent γ -carotene (Mackinney, 1935; Goodwin, unpublished observations).

Fraction 7 has properties similar to those of flavochrome but with its absorption bands at slightly higher wavelengths and with a slightly greater adsorptive power than flavochrome; it is probably mutachrome.

Fraction 8 was a mixture containing mostly fraction 7 (mutachrome), together with small amounts of a pigment with at least one absorption band at a lower wavelength than the lowest of mutachrome (402 m μ). Persistent efforts to purify this fraction were not successful, but the spectrum of the best fraction obtained strongly suggested that the pure pigment would probably be aurochrome.

Fractions 9-14 (*xanthophylls containing more than one oxygen grouping*). Fraction 9 was identified in the usual way as lutein; Fractions 10-12 spread on the column and do not separate cleanly. They have the characteristic adsorption properties and absorption spectrum of flavoxanthin and chrysanthemaxanthin. These pigments are extremely difficult to separate (Karrer & Jucker, 1950) and from the spread of these fractions one can only conclude that both pigments are probably present. Fraction 13 is a small band with an indeterminate spectrum and appears to be a carotenoid degradation product, whilst the final zone (14), although not pure, exhibited a spectrum which suggests that it might contain small amounts of neoxanthin.

Quantitative determination of pigments. The amounts of each pigment present were determined by measuring the E (at $\lambda_{\max}$) of each fraction dissolved in a known volume of light petroleum and comparing it with the known $E_{1\%}^{1\text{cm}}$ values (at $\lambda_{\max}$) of the pure pigment (Goodwin, 1952a-c). For unknown pigments $E_{1\%}^{1\text{cm}}$ ($\lambda_{\max}$) was assumed to be 2500. All carotenoids which have been obtained crystalline have $E_{1\%}^{1\text{cm}}$ values varying between 1900 and 3100, most of the values being around 2500.

Spectroscopic measurements. These were carried out either on a Beckman Model DU quartz spectrophotometer or on the corresponding Unicam (SP. 500) instrument.

RESULTS

Polyene hydrocarbons

The following polyene hydrocarbons have been identified in the petals of *C. officinalis*: phytofluene, β -carotene, γ -carotene, pigment X, ζ -carotene, and lycopene. The phytofluene fraction (1B, Table 2) was resolved into two polyenes with very similar absorption spectra. The lower adsorbed fraction is phytofluene whilst that adsorbed above this fraction is *trans*-phytofluene. The recent work of Petracek & Zechmeister (1952) has shown that the normal naturally occurring phytofluene is probably a *cis* form; and it is very difficult to decide whether

trans-phytofluene occurs naturally or is an artifact, for it can be formed from *cis*-phytofluene by very gentle treatment (Petracek & Zechmeister, 1952). Similarly, about 30% of the lycopene isolated was in the form of a *cis* isomer, neolycopene A, and again this is so easily produced from the all-*trans* form that it is probably an artifact. On the other hand, Fraction 1G which is probably polycopene I, (a poly-*cis*-lycopene) cannot be an artifact, for it is not possible to detect poly-*cis*-lycopenes in heat- or iodine-isomerized lycopene solutions (Zechmeister & Petracek, 1952).

This appears to be the first occasion on which pigment X has been observed naturally; it was first found in cultures of *Phycomyces blakesleeanus* grown on media containing diphenylamine: it is not produced by this organism on normal media (Goodwin & Osman, 1953). It is separable from ζ -carotene only with difficulty; its absorption spectrum is recorded in Fig. 1.

Xanthophylls

The following xanthophylls have been identified: flavochrome, mutatochrome, lutein, and probably both flavoxanthin and chrysanthemaxanthin. Traces of aurochrome and neoxanthin are probably also present. Fraction 2 (Table 3) cannot be identified with any known pigment; its absorption spectrum is recorded in Fig. 1. Fraction 6, from the position of the absorption maximum of its main

Fig. 1. Absorption spectra in light petroleum of A, pigment X, —; B, unknown pigment (fraction 2, Table 1), ---; C, rubixanthin-like pigment, ●—.

band and from its adsorptive power would appear to be rubixanthin (3-hydroxy- γ -carotene) which has previously been reported in the petals of *C. officinalis* (Kuhn & Grundmann, 1934). The essentially single-banded spectrum of fraction 6, however (Fig. 1), suggests a ketocarotenoid rather than a hydroxy- γ -carotene; the latter, as stated previously, has a well-defined three-banded spectrum, indistinguishable from that of γ -carotene. In this variety of *C. officinalis* it does appear that not rubixanthin but a closely related compound is produced.

Quantitative experiments

A quantitative separation of the pigments into xanthophylls and carotenes (including traces of the colourless polyenes) showed that each group represented about one-half of the total pigments present. The relative amounts of the components in these two main fractions are given in Tables 4 and 5. Although

Table 4. Relative amounts of hydrocarbon polyenes in *C. officinalis*

Polyene hydrocarbons represent 45.3% of the total polyenes present.

Polyene	Wt. (as % of total polyene hydrocarbons present)
Phytofluene*	0.4
β -Carotene	13.8
Pigment X	2.2
ζ -Carotene	7.8
γ -Carotene	14.1
Unknown (zone 1G, Table 2)	2.6
Lycopene†	59.1

* A trace of *trans*-phytofluene was also present, but may be an artifact.

† The lycopene fraction includes 30.1% neolycopene A; this may also be an artifact.

Table 5. Relative amounts of xanthophylls in *C. officinalis*

Xanthophylls represent 54.7% of the total polyenes present.

Xanthophylls	Wt. (as % of total xanthophylls present)
Unknown (zone 2, Table 1)	5.2
(?) <i>cis</i> isomer of unknown	1.9
Flavochrome	2.8
Rubixanthin-like	7.4
Mutatochrome	17.8
Aurochrome*	7.4
Lutein	5.2
Flavoxanthin†	24.5
Chrysanthemaxanthin†	26.3
Oxidation product	0.4
? Neoxanthin	0.9

* Never completely separated from mutatochrome; this represents an upper limit.

† Separation not complete; these values are only approximate.

there may be some variations in the relative amounts during the development of the petals, it is obvious that lycopene is the main carotene, and that flavoxanthin and chrysanthemaxanthin the main xanthophylls.

DISCUSSION

The examination of *C. officinalis* petals confirms two generalizations which have been made with regard to carotenoid distribution in petals (Goodwin, 1952c); (a) that 5:8-epoxides (flavochrome, mutatochrome, aurochrome, flavoxanthin and chrysanthemaxanthin) are widely distributed in petals in relatively large amounts and (b) that lutein, although present is not, as in green leaves, the major xanthophyll. Furthermore, the investigation adds emphasis to the recent observations made on berries (Goodwin, 1953, and unpublished observations) that when examined with the aid of present knowledge concerning separation and identification of trace carotenoids, the pigment mixture is found to be much more complex than was originally thought. This is mainly due to the fact that the pioneer investigators were mainly interested in isolating the major pigments in order to examine them chemically. The biological significance of these complex mixtures is at present quite obscure.

The present investigation confirms the original observation of Zechmeister & Cholnoky (1932) and Kuhn & Grundmann (1934) on the major pigments present, except that violaxanthin was not detected and although a 'rubixanthin-like' pigment was observed, rubixanthin itself was not detected. These differences may be varietal.

SUMMARY

1. The following hydrocarbon polyenes have been found in the petals of a 'dark' variety of

Calendula officinalis (pot marigold): phytofluene, β -carotene, γ -carotene, ζ -carotene and lycopene. Pigment X closely associated with ζ -carotene, was also present. A poly-*cis*-lycopene was also present; it was probably prolycopene I.

2. The xanthophylls present were flavochrome, mutatochrome, lutein, flavoxanthin and chrysanthemaxanthin; traces of neoxanthin and aurochrome are also probably present. Two pigments were unidentified; pigment 6 was similar to, but not identical with rubixanthin.

3. The relative distribution of these pigments in the petals is recorded.

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Purification and some Properties of Cephalosporin N, a New Penicillin

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A species of *Cephalosporium* isolated by Brotzu (1948) from a sewage outfall off Sardinia can produce at least six different antibiotics (Burton & Abraham, 1951; Crawford *et al.* 1952). One of these substances, named cephalosporin N, has been reported to be a new type of penicillin, distinguished clearly from

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the penicillins previously encountered by its hydrophilic character and by an activity of the same order of magnitude against a number of Gram-positive and Gram-negative bacteria (Abraham, Newton, Crawford, Burton & Hale, 1953). An account of some of the biological properties of the substance has been given by Heatley & Florey (1953). This paper describes a process whereby cephalosporin N