hydroxylysyl amino groups are free to react with FDNB, may act as a suitable 'seeding template' for the growth of hydroxyapatite crystals. The fact that apatite crystals are deposited on collagen fibres predominantly at the cross-banded regions (Robinson & Watson, 1955), where it is thought that the content of basic and acidic amino acids is high, supports this concept.

SUMMARY

1. A variety of hard and soft connective tissues of bovine and human origin were treated with fluorodinitrobenzene before, during and after treatment with demineralizing agents.

2. It was found that the collagens of soft and demineralized hard tissues could readily be distinguished from each other on the basis of the reactivity of their ϵ-lysyl and hydroxylysyl amino groups with fluorodinitrobenzene. In intact soft-tissue collagen only about two-thirds of these ϵ-amino groups reacted with fluorodinitrobenzene, but the concentration of reactive ϵ-amino groups of hard-tissue collagens increased from very low levels almost to the theoretical value at complete decalcification.

3. The significance of these findings is discussed in relation to calcification *in vivo*.

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Studies in Carotenogenesis

24*. THE CHANGES IN CAROTENOID AND CHLOROPHYLL PIGMENTS IN THE LEAVES OF DECIDUOUS TREES DURING AUTUMN NECROSIS

By T. W. GOODWIN
Department of Biochemistry, The University, Liverpool 3

(*Received* 7 *August* 1957)

It has long been stated that the change in colour of the leaves of deciduous trees in early autumn is due to the preferential destruction of the green chlorophylls, which unmasks the much more slowly disappearing yellow carotenoids. Early work indicated that the carotenoids also undergo qualitative as well as quantitative changes. From 70 to 80 % of the total carotenoids in all green leaves are unesterified xanthophylls (Goodwin, 1952), which are hypophasic when partitioned between light petroleum and 90 % (v/v) aqueous methanol (Goodwin, 1955). Tswett (1911) found that the carotenoids from autumn leaves were almost entirely epiphasic, but that the major fraction could be separated from β-carotene; he named these pigments 'autumn xanthophylls'; Palmer (1922), considering their partition properties, suggested the name 'autumn carotenes'. Kuhn & Brockmann (1932) found that these autumn pigments were not hydrocarbons but esterified xanthophylls, which behaved like carotenes in the partition test. Karrer & Walker (1934), however, considered that they were unidentified oxidation and degradation products of lutein. On the other hand, Strain (1938) stated that the major pigment in yellow-shed leaves was zeaxanthin (3:3′-dihydroxy-β-carotene). Kuhn & Brockmann (1932) agreed with Willstätter & Stoll (1918) that the total amount of carotenoids did not alter in autumn leaves; however, Karrer & Walker (1934) and Nagel (1939) found that both the carotenes and xanthophylls disappeared, but that the carotenes were destroyed more quickly than xanthophylls. The carotenoids are reported to undergo these same autumnal changes in leaves of tropical evergreens (Egle, 1944). The *aurea* varieties of *Acer*

* Part 23: Goodwin, Land & Sissins (1956).

laetum and *Buxus japonica* during the period from late May to late October tended to increase their carotene levels whilst maintaining their xanthophyll levels constant; in *Ulmus montana* var. *aurea*, however, both levels remained constant during the same period, except that in October a sharp decline, much more marked in the carotene fraction, had set in (Bauer, 1956).

Because of the various inconsistencies in the literature and because most of the work had been done in the early days of investigations on carotenoids, it was decided to use the newer techniques and knowledge now available to follow the changes in carotenoid content of the leaves of three deciduous trees. Those chosen were the common sycamore (*Acer pseudoplatanus*), the oak (*Quercus robur*) and the cultivated black plum (*Prunus nigra*). The first two were chosen because sycamore leaves turn yellow much earlier than oak leaves, and the last because it contains, in addition to the chloroplast pigments, a cytoplasmic anthocyanin. In addition qualitative and quantitative changes in the chlorophylls and anthocyanin levels present were also investigated. Willstätter & Stoll (1918), in their classical experiments, considered that chlorophylls *a* and *b* disappeared at the same rate; on the basis of recent work with more refined techniques there are indications that chlorophyll *a* is destroyed somewhat faster than chlorophyll *b* (Rudolph, 1934; Nagel, 1939; Egle, 1944; Seybold, 1943; Wolf, 1956). There appears to be little known about the changes in anthocyanin levels during the autumn.

EXPERIMENTAL

Materials. The same tree of each of the three genera examined was used throughout. *Acer pseudoplatanus* (sycamore) and *Quercus robur* (oak) were growing in a small copse in north-west Cheshire and *Prunus nigra* (black plum) in a nearby garden. Their leaves were collected during 1956, always from the same region of the tree, between 8 and 9 a.m., and transported to the Laboratory in a polythene container. Extraction of the pigments began within 30–45 min. of collecting the leaves.

Extraction of chlorophylls and carotenoids. The leaves (between 0·5 and 2·0 g.) were ground in a mortar under acetone (25 ml.) with acid-washed silver sand, and the mass was filtered through a sintered-glass filter (G 1). The residue was returned to the mortar and re-extracted with acetone; this process was repeated (usually three times) until the final acetone extract was colourless. The acetone extracts were combined in a separating funnel and transferred to diethyl ether by adding approximately an equal volume of that solvent, followed by water added dropwise until two layers formed. The lower layer was run off, the ether layer was washed free from acetone with water (3 × 20 ml.), and dried by standing for 30 min. over anhydrous Na_2SO_4, filtered and made up to a known volume (usually 100 ml.). This was then divided into four portions: (*A*) 50 ml. was taken to dryness under N_2 and dissolved in a small volume of light petroleum (b.p. 40–60°) for column chromatography; (*B*) 25 ml. was taken for determination of total carotenoids; (*C*) 20 ml. was taken for paper chromatography; (*D*) 4–5 ml. was required for the direct spectrophotometric determination of chlorophylls.

Extraction of anthocyanin. With *P. nigra* a small amount of anthocyanin pigment was removed in the first acetone extraction; this was ignored. The major portion, representing at least 95 % of the total, was present in the material remaining after treatment with acetone. The most suitable solvent for extraction was found to be ethanol containing 1 % (v/v) HCl. The extraction was carried out in the same way as for the carotenoids. The residue, already containing silver sand, was transferred from the sintered-glass filter to the mortar and ground under the acid ethanol (25 ml.). The mass was filtered through the sintered-glass filter and the residue returned to the mortar for re-extraction; three extractions were usually sufficient for complete removal of the pigment. The extracts were combined and made up to a known volume for spectrophotometric analysis.

Separation of the chloroplast pigments by column chromatography. The adsorbent found most suitable for the separation of the major chloroplast pigments was (confectioner's) icing sugar (Strain, 1940; Zechmeister & Cholnoky, 1943; Lederer & Lederer, 1953; Goodwin, 1955). The icing sugar was dried in a vacuum desiccator overnight before use. A column (2 cm. × 12·5 cm.) was prepared in the dry state (Zechmeister & Cholnoky, 1943) and light petroleum (the fraction boiling between 40° and 60° was used throughout this investigation) drawn through under slight suction. The pigment extract from 0·125–0·5 g. of fresh leaves was placed on the column in 5–10 ml. of light petroleum and the chromatogram developed with the same solvent until all the β-carotene, which is not adsorbed on icing sugar, had run through the column. This fraction was then made up to a volume suitable for spectrophotometric measurements. The remaining pigments were then separated in one of two ways: (*a*) by a 'liquid chromatogram' in which the developing solvent (light petroleum) was made increasingly polar by addition of acetone and the fractions were collected consecutively in the eluate as they moved off the column; (*b*) by developing the column with light petroleum containing 0·5–1·0 % (v/v) of acetone until discrete zones were formed on the column; about 200–250 ml. of eluent was usually required. The residual solvent was removed from the column by suction, the column extruded and the zones were separated by cutting the column at the appropriate points. The pigment from each zone was then eluted by shaking with diethyl ether (25 ml.) and filtering through a sintered-glass funnel. The fractions were taken to dryness under N_2 and dissolved in appropriate volumes of light petroleum for spectrophotometric examination.

Separation of chloroplast pigments by paper chromatography. The pigments were separated by ascending paper chromatography with Whatman no. 1 paper and the solvent system of Loeffler (1955), which is light petroleum–ethyl ether–ethanol (30:10:0·5, by vol.). It was found necessary to have the chromatography vessel lined with Whatman no. 1 paper dipping in the solvent and for the solvent to reach the top of the paper before inserting the chromatogram. In this way the space was kept saturated with

solvent vapour. The portion of the pigment extract retained for paper chromatography (20 ml.) was taken to dryness and dissolved in ethanol (1 ml.); 0·05 ml. of this was applied to the paper in small portions, each addition being allowed to dry at room temperature before application of the next portion. Drying by heating destroys the pigments. After separation, the paper was dried in air and the spots were cut out, eluted with ethyl ether and examined spectrophotometrically in that solvent. Chromatograms were always run in duplicate.

Quantitative determination of carotenoids. Total carotenoids were determined by evaporating a known volume of the crude ether extract of the leaves to dryness and removing the chlorophylls by saponifying the extract according to the method of Goodwin & Morton (1946). This destroys the chlorophylls and leaves the carotenoids in the unsaponifiable fraction. The unsaponifiable matter is dissolved in a known volume of light petroleum and the total carotenoid content obtained from measuring E (446 mμ), assuming $E^{1\%}_{1\,cm.}$ for the crude mixture to be that of β-carotene (2500; Goodwin, 1955).

The concentration of the various components obtained after chromatography was obtained similarly by measuring $E_{max.}$ and using the appropriate $E^{1\%}_{1\,cm.}$ value: β-carotene, 2500 at 449 mμ; lutein, 2500 at 445 mμ; neoxanthin, 2270 at 435 mμ; violaxanthin, 2550 at 441 mμ; lutein-5:6-epoxide, 2400 at 441 mμ (Goodwin, 1955).

Quantitative determination of chlorophylls. The concentrations of chlorophyll *a* and chlorophyll *b* were determined in the crude ether extract of the leaves by differential spectrophotometry, with the equations developed by Smith & Benitez (1955): $C_a = (0{\cdot}0101E_{662}) - (0{\cdot}00101E_{644})$ and $C_b = (0{\cdot}0164E_{644}) - (0{\cdot}00257E_{662})$, where C_a and C_b are the concentrations of chlorophyll *a* and chlorophyll *b* in g./l. and E_{644} and E_{662} are the extinction measurements in a 1 cm. cell, at 644 and 662 mμ respectively.

Direct spectroscopic measurements were also made on the chlorophyll *a* and chlorophyll *b* fractions obtained by column and paper chromatography; the spectroscopic characteristics reported for these pigments by Smith & Benitez (1955) were used. The ratio of chlorophyll *a*:*b* obtained by direct spectrophotometry was always similar to that found by using the chromatographic methods, except in the later samples when the small amounts of chlorophylls remaining in the leaves had obviously been altered so that their absorption spectra had changed and the equations were no longer applicable.

Determination of the anthocyanin in P. nigra *leaves.* The anthocyanin ran as a single spot on paper chromatography with butan-1-ol–acetic acid–water (4:1:5, by vol.) and it is almost certainly cyanidin-3′-glucoside (see Results). Its concentration was determined by measuring E at 538 mμ, assuming the $E^{1\%}_{1\,cm.}$ of cyanidin-3-glucoside to be 700 (Geissman, 1955).

Dry-weight determinations. These were carried out by drying the leaves overnight at 80° in an air oven.

Isolation of chloroplasts. The leaves (2 g.) were thoroughly ground up at 5° with acid-washed silver sand under 0·5 M-sucrose (50 ml.) (Granick, 1938). The cell debris was centrifuged off at 500 *g* and the chloroplasts were collected by centrifuging at 1000 *g*. Fractions were collected half-hourly for 1·5 hr.

Spectrophotometric measurements. These were carried out with a Unicam spectrophotometer model SP. 600.

RESULTS

Nature of the pigments

Chlorophylls. It has been accepted for many years that only chlorophyll *a* and chlorophyll *b* are found in the green tissues of higher plants (Smith & Benitez, 1955); these were the only pigments found in the green leaves now under examination. In yellow autumn leaves the small amounts remaining had chromatographic properties very similar to those of authentic chlorophylls *a* and *b*, but differed from these slightly in their absorption maxima. These 'changed chlorophylls' had their absorption maxima (light petroleum) in the red at 652 mμ for chlorophyll *a* and at 635 mμ for chlorophyll *b* instead of at 662 mμ and 644 mμ respectively.

In yellow leaves, traces of pale-grey zones occasionally appeared just below the 'changed' chlorophylls *a* and *b*. These are phaeophytins *a* and *b* (Smith & Benitez, 1955). Because of their sporadic occurrence, it would be premature to conclude at this stage that they were intermediates in the degradation of chlorophylls *in vivo*; they can easily arise from the parent compounds during manipulation, especially in the presence of traces of acid.

Carotenoids. It has long been accepted that β-carotene is the major carotene of green leaves and that it is accompanied by varying but smaller amounts of α-carotene and traces of *cis*-isomers of α- and β-carotene (Goodwin, 1952). It has only recently been shown that the xanthophyll mixture is also comparatively simple, the earlier reports of complex mixtures being due to changes brought about during chromatography by traces of free HCl liberated from ethylene dichloride used as eluent (Strain, 1938, 1948). Some 90–95% of the total xanthophylls of lucerne (alfalfa) (Bickoff, Livingston, Bailey & Thompson, 1954) and maize (corn) (Moster, Quackenbush & Porter, 1952) are represented by lutein (3:3′-dihydroxy-α-carotene), violaxanthin (5:6:5′:6′-di-epoxyzeaxanthin) and neoxanthin (structure unknown). Small amounts of cryptoxanthin (3-hydroxy-β-carotene, zeaxanthin and flavoxanthin (5:8-epoxylutein) are also occasionally encountered. These pigments had been repeatedly noted in earlier work but additional artifacts were also described.

In the three types of green leaves examined here β-carotene, lutein, violaxanthin and neoxanthin were the major components. Table 1 illustrates a typical chromatogram obtained with icing sugar as adsorbent. Violaxanthin was not separable from chlorophyll *a* with this adsorbent and its presence was established by removing the chlorophyll *a* zone, saponifying the extract to destroy the chlorophyll *a*

and rechromatographing the yellow unsaponifiable extract on icing sugar. This yielded a single band corresponding in position and absorption spectrum to violaxanthin.

In autumn leaves, the extracts of plum and oak leaves contained β-carotene (traces), lutein, violaxanthin but no neoxanthin. A new pigment was found in small amounts adsorbed just below violaxanthin and chlorophyll *a*; as this pigment is hypophasic to 90% aqueous methanol and has an absorption spectrum very similar to violaxanthin but a lower adsorptive affinity, it is probably lutein 5:6-epoxide (Goodwin, 1955).

In addition to lutein, violaxanthin and lutein epoxide, already noted in plum and oak leaves, two further pigments were observed in autumn leaves of the sycamore. These were adsorbed between β-carotene and lutein. They were epiphasic in the phase tests, indicating no free hydroxyl groups, but on saponification they became hypophasic and were identified by their absorption spectra and by mixed chromatography with authentic specimens as violaxanthin and lutein respectively. These new pigments were obviously the 'autumn xanthophylls' of Tswett (1911), and were correctly identified as xanthophyll esters by Kuhn & Brockmann (1932).

Paper chromatography with Loeffler's (1955) solvent system is effective for separating the major pigments in leaves, and Fig. 1 shows the different chromatograms obtained with the extracts of green and yellow sycamore leaves.

Anthocyanin. The anthocyanin present in *P. nigra* has not previously been described but all other species examined (*P. persica*, *P. divaricata* var. *pissardii*, *P. lannesiana* var. *donarium*, *P. nipponica*, *P. avium*, *P. pissardii*) contain a cyanidin-3-monoside, most probably cyanidin-3-glucoside (chrysanthemin) (Lawrence, Price, Robinson & Robinson, 1939; Hayashi & Abe, 1953; Reznik, 1956). The pigment in *P. nigra* is also almost certainly a cyanidin monoside, it has the same absorption spectrum as the pigment from *P. pissardii* and cannot be separated from it on paper chromatography with butanol–acetic acid–water.

Table 1. *A typical chromatogram of the major pigments of green leaves*

Adsorbent: icing sugar; column size: 2 cm. × 12·5 cm.; pigments from 0·125 to 0·5 g. of fresh leaves; developer: light petroleum containing 0·5–1·0% of acetone. Zones are in order of increasing adsorptive affinity.

Zone no.	Description	Pigment
1	Orange, passing straight through the column	β-Carotene*
2	Yellow	Lutein
3	Light green	Chlorophyll *a*† violaxanthin
4	Dark green	Chlorophyll *b*
5	Lemon yellow	Neoxanthin

* Possible traces of α-carotene are not separated from β-carotene by this adsorbent.

† These two pigments cannot be fully separated on this adsorbent.

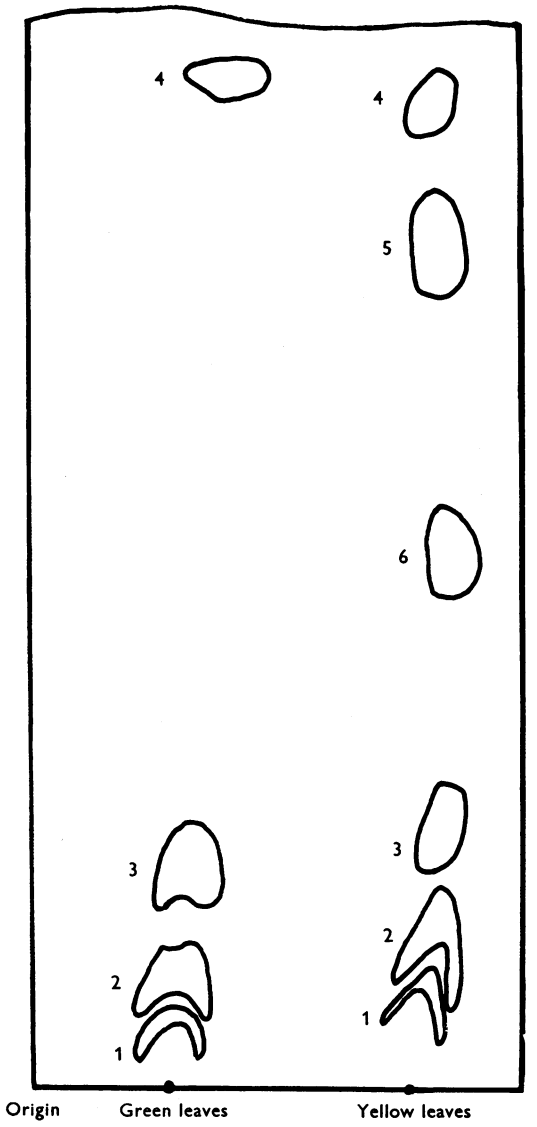

Fig. 1. Paper chromatograms of pigments extracted from green and yellow sycamore leaves. 1, Chlorophyll *b*; 2, chlorophyll *a* + violaxanthin; 3, lutein; 4, β-carotene; 5, lutein esters; 6, violaxanthin esters. Ascending chromatogram was run at room temperature on Whatman no. 1 paper with light petroleum–ethyl ether–ethanol (30:10:0·5, by vol.) as solvent system (Loeffler, 1955). Only traces of β-carotene were present in the extract from yellow leaves.

Quantitative observations

Oak leaves. The variations in percentage dry weight, total chlorophyll and total carotenoid are given in Fig. 2. From this it is obvious that both the chlorophylls and carotenoid decreased precipitately from the middle of September to the middle of October. There is no indication in these leaves that the chlorophylls are destroyed before the carotenoids. The values recorded here for the total carotenoids of the green leaves (11–15 mg./100 g. dry wt.) are within the range previously recorded for *Quercus* spp. by Kemmerer, Fudge & Fraps (1942). The relative changes in the carotenoid components are recorded in Fig. 3. During the period when the sharp drop in the total carotenoid level is occurring (see Fig. 2), the major changes are (i) the rapid and complete disappearance of neoxanthin, (ii) the appearance of lutein-5:6-epoxide for the first time, (iii) the relatively more rapid disappearance of β-carotene compared with that of lutein, which results in the latter being the major component in late leaves, and (iv) the comparative constancy of the violaxanthin fraction. β-Carotene-5:6-epoxide could not be detected in significant amounts; if it is an intermediate in the breakdown of β-carotene, it is presumably rapidly destroyed as soon as formed. The relative amounts of the constituent carotenoids in the green oak leaves fall within the normal values for green leaves (Goodwin, 1952).

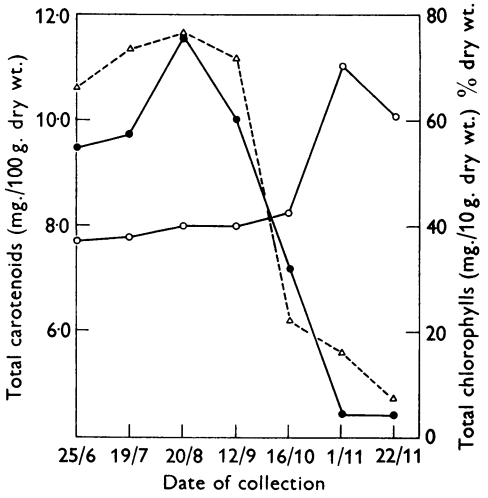

Fig. 2. Variations in dry weight, total chlorophyll and total carotenoid values for oak leaves during summer and autumn. ●, Total chlorophylls; △, total carotenoids; ○, dry weight. Samples collected on 1 and 22 November were shaken off the branches. Chlorophylls in these samples were 'changed chlorophylls'. In this and other figures the dates are represented, for example, as 1/11 (meaning 1 November).

The chlorophyll *a*:*b* ratios during necrosis are given in Table 2. Until the chlorophylls have almost completely disappeared from the leaf there is only a slight tendency of chlorophyll *a* to be destroyed more quickly than chlorophyll *b*. In the very late stages, when the traces of pigments remaining are no longer true chlorophylls *a* and *b*, the chlorophyll *a* derivative disappears much more rapidly than the chlorophyll *b* derivative.

Plum leaves. The variations in percentage dry weight, total chlorophyll, total carotenoid and total anthocyanin are given in Fig. 4. Here again there is a sharp drop in chlorophyll levels during early October, but it is very much less marked than with the oak (Fig. 2) or sycamore (see later). The drop in carotenoids is much more gradual, occurring throughout the period under observation; it did

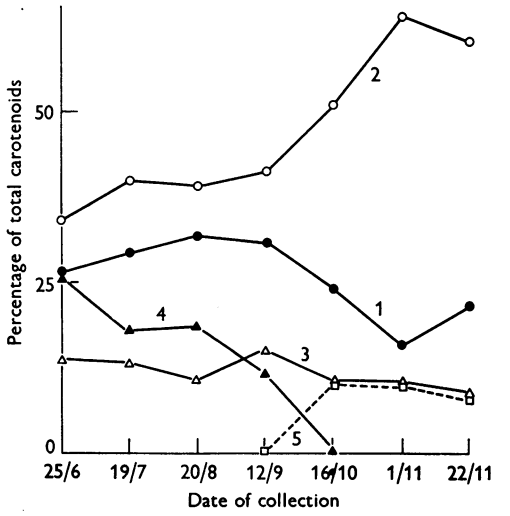

Fig. 3. Changes in the relative amounts of carotenoids in oak leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein 5:6-epoxide.

Table 2. *Chlorophyll* a:*chlorophyll* b *ratio in oak leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectrophotometry (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
25 June	2·60	16 October	2·35
19 July	2·72	1 November*	1·67
20 August	2·81	22 November*	1·08
12 September	2·43		

* The chlorophylls in these two specimens were 'changed chlorophylls' (see text) and therefore the ratios could not be determined spectrophotometrically; they were determined chromatographically.

not parallel the disappearance of chlorophylls but had begun before the autumnal drop in chlorophylls. The final 'autumn' levels of chlorophylls and carotenoids reached were much higher than in the oak (Fig. 2) or sycamore (see later). The increase in percentage dry weight in early autumn is also much less marked than in oak and sycamore leaves. The anthocyanin level does not show any downward trend during the period studied. The chlorophyll *a*:*b* ratio (Table 3) remained constant until the latter part of October, when it dropped significantly. The relative amounts of the constituent carotenoids, which in early summer are within the normal levels for green leaves, alter with the onset of autumn in the same way as observed with oak leaves (Fig. 5). When the carotenoids begin to disappear the β-carotene and neoxanthin are preferentially destroyed; this is especially marked with the latter. Lutein is much more stable and lutein 5:6-epoxide makes its appearance.

Sycamore leaves. The general variations in percentage dry weight, total chlorophyll and total carotenoid are given in Fig. 6. Here the fall in chlorophylls and carotenoids is as marked as in oak leaves, and more marked than in plum leaves. The chlorophylls tend to disappear before the carotenoids and these changes begin much earlier than in the other two leaves studied; they are well advanced at the beginning of August. Dry-weight percentages rise very sharply as the leaves begin to necrose. Table 4 shows that, as in the other two cases, the chlorophyll *a*:*b* ratio drops during necrosis but the fall is marked only in the later stages. When the relative changes in the constituent carotenoids are considered, sycamore leaves are similar to the other leaves examined in that β-carotene and neoxanthin are the first pigments to disappear. However, by contrast, new pigments appear which have been identified as esters of lutein and violaxanthin. Fig. 7 shows how the relative amounts of these increase during necrosis, so that at the time the leaves fall from the trees they are the only carotenoids present. When the esters first appear they consist of almost equal amounts of lutein and violaxanthin esters; as the leaves become older, the lutein esters tend to

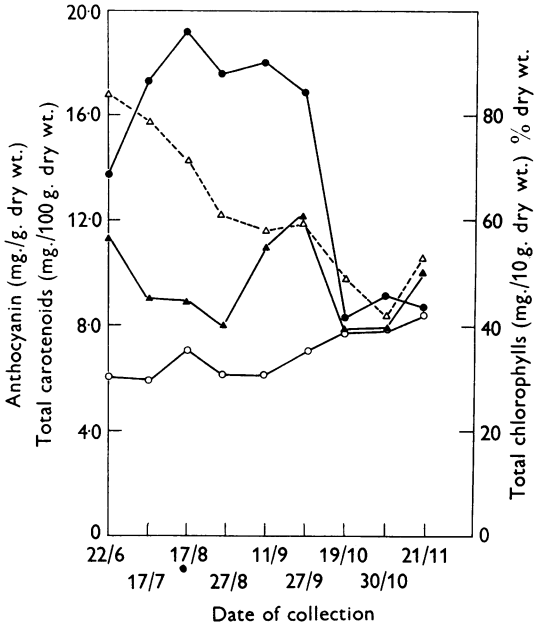

Fig. 4. Variations in dry weight, total chlorophyll, total carotenoid and anthocyanin values for plum (*Prunus nigra*) leaves during summer and autumn. ●, Total chlorophyll; △, total carotenoids; ○, dry weight; ▲ anthocyanin.

Table 3. *Chlorophyll* a:*chlorophyll* b *ratio in plum leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectroscopy (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
22 June	2·39	27 September	2·18
17 July	2·58	19 October	2·33
17 August	1·90	30 October*	1·83
27 August	2·66	21 November*	1·70
11 September	2·70		

* 'Changed' chlorophylls; see footnote to Table 2.

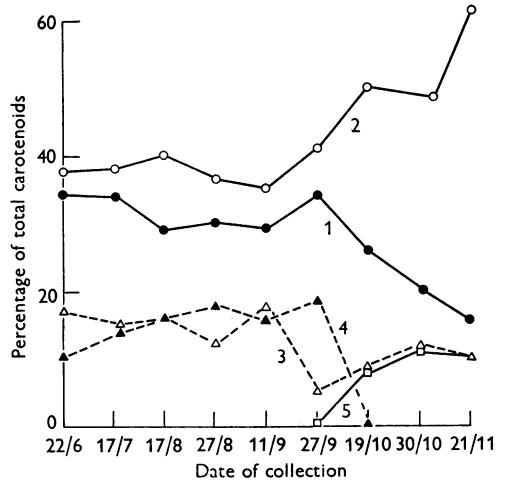

Fig. 5. Changes in the relative amounts of carotenoids in plum leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein 5:6-epoxide.

disappear more quickly than violaxanthin esters, so that in fallen leaves the very small amount of residual carotenoid appears to be almost entirely violaxanthin esters (Table 5), but traces of the lutein esters may also be present.

Stability of pigments in harvested autumn leaves

Leaves from the three trees examined were collected during early October, stored in the Laboratory at room temperature and examined periodically. It was found that the pigment changes in these leaves were similar to those observed with leaves left on the tree; the results are therefore not recorded here.

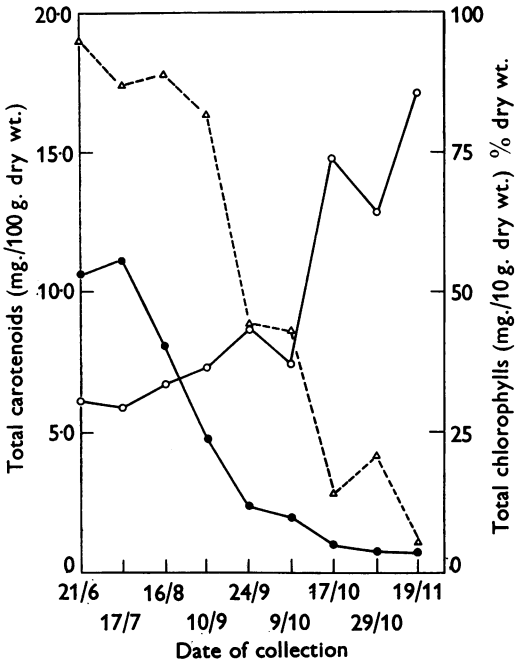

Fig. 6. Variations in dry weight, total chlorophyll and total carotenoid for sycamore leaves during summer and autumn. ●, Total chlorophyll; ○, dry weight; △, total carotenoids.

Table 4. *Chlorophyll* a:*chlorophyll* b *ratio in sycamore leaves during summer and autumn*

Ratios were determined in an ethereal solution by differential spectroscopy (see Experimental section) and were checked by paper and column chromatography.

Date	Ratio *a*:*b*	Date	Ratio *a*:*b*
21 June	3·38	9 October	1·88
17 July	2·77	17 October*	1·53
16 August	3·03	29 October*	1·51
10 September	2·44	19 November*	1·36
24 September	2·40		

* 'Changed' chlorophylls; see footnote to Table 2.

Location of xanthophyll esters in sycamore leaves

As only minute traces, if any, of esterified xanthophylls exist in the chloroplasts of the higher plants (Goodwin, 1952), it was of interest to know in which region of the cell the newly appearing xanthophyll esters occurred in necrosing sycamore leaves.

Chloroplasts were prepared according to the method of Granick (1938), and these and the supernatant were examined for carotenoids. In Table 6, it will be seen that with yellowing sycamore leaves the early fraction which would contain predominantly unbroken chloroplasts contains relatively little xanthophyll esters (less than 10%) and this might well be due to contamination with particles

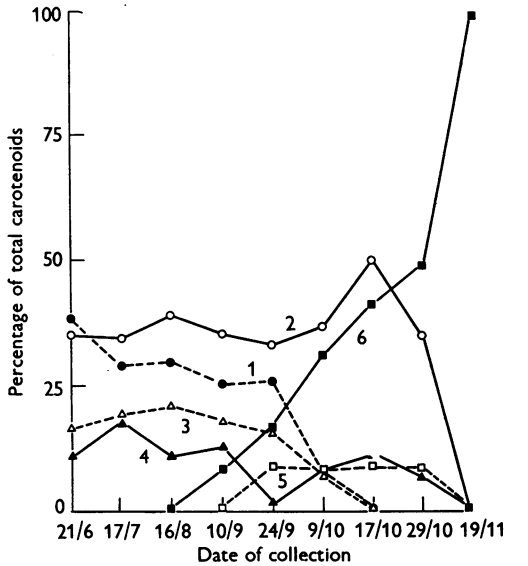

Fig. 7. Changes in dry weight, total chlorophyll and total carotenoid for sycamore leaves during summer and autumn. 1, β-Carotene; 2, lutein; 3, violaxanthin; 4, neoxanthin; 5, lutein epoxide; 6, xanthophyll esters.

Table 5. *Relative amounts of violaxanthin and lutein esters in necrosing sycamore leaves*

The period covered is that from the first appearance of xanthophyll esters to the time the leaves were falling from the trees.

Date	Percentage of total esters present: Lutein esters	Violaxanthin esters
10 September	59	41
24 September	45	55
9 October	45	55
17 October	51	49
29 October	36	64
19 November	Trace	Almost all

Table 6. *Relative distribution of free xanthophylls and xanthophyll esters in cytoplasm and chloroplasts of yellowing sycamore leaves*

Chloroplasts were obtained by the method of Granick (1938). After removal of cell debris by centrifuging at 500 *g* for 30 min., the preparation was centrifuged for 1·5 hr. at 1000 *g*, the centrifugates being collected every 15 min.

Fraction	Percentage of total pigment: Free xanthophylls	Percentage of total pigment: Xanthophyll esters
A. Centrifugate obtained during first 15 min. centrifuging at 1000 *g*	92, 91*	8, 9
B. Centrifugate obtained during final 15 min. centrifuging at 1000 *g*	42	58
C. Supernatant	36	64

* Duplicate determinations on the same preparation.

Table 7. *Relative distribution of free xanthophylls and xanthophyll esters in cytoplasm and chloroplasts of brown sycamore leaves*

Chloroplasts were obtained by the method of Granick (1938). After removal of cell debris by centrifuging at 500 *g* for 30 min., all the chloroplasts were removed by centrifuging at 1000 *g* for 30 min. No further material was obtained on further centrifuging.

Fraction	Percentage of total pigment: Free xanthophylls	Percentage of total pigment: Xanthophyll esters
A. Centrifugate obtained during first 30 min. centrifuging at 1000 *g*	51	49
B. Supernatant	Trace	100

from disintegrating chloroplasts. The last fraction obtained from centrifuging was very little different from the supernatant. This indicates that this fraction probably contained very few intact chloroplasts but consisted mainly of fragments of chloroplasts. If brown leaves are used the results are even clearer (Table 7). No lutein exists in the supernatant but it is the major pigment in the chloroplasts. If the supernatant is spun at 12 000 *g* the pigments remain unsedimented. In normal green leaves there are no detectable amounts of carotenoids in the supernatant. The most reasonable explanation of these observations is that esterification of the esters occurs only when the chloroplasts are disintegrating, that is when the pigments are exposed to the cytoplasm, which probably contains an esterase.

DISCUSSION

The present study indicates that the metabolism of carotenoids in autumn leaves cannot be considered as one process common to all deciduous trees. The previously accepted general picture was that the chlorophylls first disappeared, thus unmasking the carotenoid pigments which then gradually disappeared in the late autumn; as they disappeared the carotenoids were converted into complex oxidation products, according to Karrer & Walker (1934), or into xanthophyll esters (Kuhn & Brockman, 1932). The preferential loss of chlorophyll is well demonstrated in the sycamore (Fig. 6), but in the oak (Fig. 2) the carotenoids and chlorophylls disappear simultaneously, and in the black plum (Fig. 4) the carotenoids are preferentially destroyed. *P. nigra* differs from the other two in that the quantitative disappearance of both groups of pigments is very much less; whereas in the oak and the sycamore the falling leaves contain only traces of pigments, falling plum leaves still contain at least 50 % of their original pigments. It will be interesting to see whether future work will indicate that this is a characteristic of leaves containing anthocyanins in their cytoplasm.

The qualitative changes in the carotenoids were rather less marked than was expected and some were common to all three species. In all the onset of necrosis was characterized by the preferential destruction of β-carotene [confirming the observations of Karrer & Walker (1934) and Nagel (1939)] and neoxanthin and by the appearance of lutein-5:6-epoxide. This is probably the first stage in the oxidative degradation of lutein, because in isolated tomato leaves β-carotene epoxides are the first breakdown products of β-carotene (Glover & Redfearn, 1953). The failure to detect more than traces of β-carotene epoxides in autumn leaves is probably due to their extreme lability, which

would also account for the rapid disappearance of β-carotene. The lability of β-carotene in leaves compared with that of lutein, which appears to be a general phenomenon, is most striking; apart from the fact that it disappears rapidly from autumn leaves, it also rapidly disappears from isolated tomato leaves kept in darkness in the absence of carbon dioxide, although the xanthophyll levels remain constant; on illumination β-carotene returns to its normal level (Glover & Redfearn, 1953). Furthermore, on illuminating etiolated maize seedlings β-carotene is rapidly synthesized whereas the xanthophyll levels alter only slightly (Kay & Phinney, 1956; Goodwin, 1956, unpublished work).

There was no indication in any of the autumn leaves examined of significant amounts of unknown oxidation products reported by Karrer & Walker (1934), although they included sycamore leaves in their study, nor of zeaxanthin which Strain (1938) found in fallen peach, poplar and catalpa leaves. The xanthophyll esters of Kuhn & Brockmann (1932) were encountered only in the sycamore; Kuhn & Brockmann found them to be widely distributed, although they did not examine the oak or black plum.

It is plain from these observations that no simple generalization can be made for the fate of xanthophylls in autumnal leaves, because significant differences were found to exist in the three trees examined in this study.

SUMMARY

1. The changes in the constituent chlorophylls and carotenoids of the leaves of three trees [the black plum (*Prunus nigra*), the oak (*Quercus robur*) and the sycamore (*Acer pseudoplatanus*)] have been followed from June to November. In *P. nigra* the anthocyanin was also examined.

2. In both the oak and the sycamore, the chlorophyll and carotenoid levels fell almost to zero, but whereas in the oak the levels fell simultaneously, in the sycamore the drop in chlorophylls preceded that in carotenoids. In the black plum the carotenoids tended to disappear first, but both carotenoid and chlorophyll levels fell only to about 50 % of their starting values.

3. In all cases β-carotene and neoxanthin disappear more rapidly than do the other carotenoids, and lutein 5:6-epoxide appears in small amounts. In the sycamore but not in the other two trees, both lutein and violaxanthin esters appear in relatively large amounts in the later stages. These esters exist mainly in the cytoplasm and not in the chloroplasts.

4. In all cases the ratio chlorophyll *a*:*b* falls slightly during the late autumn.

5. There was no loss of anthocyanin in *P. nigra* leaves.

Thanks are due to Miss B. M. Eales for skilled technical assistance, to Dr E. C. Bate-Smith for information concerning the anthocyanins present in *Prunus* spp. and to Mr J. Duncan (University of Liverpool Botanic Gardens) for providing leaves of *P. pissardii*.

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