in acetone or benzene, but although quite dark red as a solid, it gave a pale yellow color to *ca.* 50% aqueous acetone, with a very low absorptivity, *ca.* 2.0 at 433 nm in that solvent. The phytol content was negligible, *ca.* 1.0%, but the percentages for N and Mg, respectively, were 4.9 and 2.6. The ratio of the three components is 4:1.25:0.03.

It seems clear from the foregoing data that the pigmented fractions retain their N:Mg ratio and for short exposure times, also their phytol, as in chlorophyll.

The Supernatants. Fractionation of the supernatants has presented certain problems. Chlorophyll dissolved in pure solvent in concentrations 5–10 μM are seemingly bleached to a virtually colorless state. At concentrations around 50 μM, the color after irradiation is a light tan, and this is due to red pigment of low tinctorial power, devoid of phytol, but representing at least 10–20% of the original chlorophyll. When the supernatants were evaporated to dryness, the residue coating the inside of the evaporating flask tended to cake. This mixture was incompletely extracted with petroleum ether, but the cake was effectively penetrated by water, leaving residual pigment adhering to the flask. Acetone cannot be used to loosen the cake until the last, or the purpose of the fractionation is defeated, as there will then be pigment in the aqueous phase. The phytol which has not been destroyed during irradiation and which is not accounted for in the red precipitate should be present in the colorless petroleum ether extract of the supernatant residue. In fact, it is distributed between this extract and the turbid aqueous extract. (The final acetone extract of pigment is devoid of phytol.)

The phytol and nitrogen analyses in the petroleum ether extracts for these three experiments, in μmol of constituent present, are as follows: phytol, 1.78, 4.26, 5.02; and nitrogen, 4.57, 7.93, 7.85. The N–phytol ratios are 2.8, 1.85, and 1.56, a clear indication that the massive chlorophyll ring has been fragmented.

The nitrogen distribution, in μmol, can be summarized as follows: original N, 100.8, 109.2, 93.2; red precipitate, 50.7, 35.1, 33.8; petroleum ether extract, 4.6, 7.9, 7.9; aqueous extract, 25.0, 48.6, 38.0; and recovery, 80%, 84%, 85%.

A start has been made as to the relevance of these findings to the breakdown of chlorophyll in the leaf (Park *et al.*, 1973). The pigmented breakdown products retain their 4:1 nitrogen–magnesium relationship. It is now evident that the low tinctorial power of these pigments will make them difficult to detect in the presence of numerous other colored compounds to be found in a plant extract. In a large scale extraction of leaves with acetone, the extracted cake may be left in the Buchner overnight, outside, or in a hood, to allow residual solvent to evaporate. The surface layer which is originally straw-colored often turns red. This may well be an artifact, but it is to be expected, on the basis of work by Barrett (1967) and Fuhrhop and Mauzerall (1971) with magnesium octaethyl porphine that chlorophyll degradation in the leaf will begin with an oxidative attack either on the pyrrole rings at α positions, or on the methine bridges. In the final breakdown, the ring to which phytol is attached should be the most readily identifiable.

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On Chlorophyll Breakdown in Senescent Leaves

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The phytol content of green leaves coincides (±6% on the average) with that calculated on the basis of the chlorophyll present. During senescence, 95% of the chlorophyll may disappear, leaving no trace of colored breakdown products as the leaf turns yellow, brown, or red. In all cases studied, the phytol ester linkage is highly stable during yellowing. The phytol is often recoverable in amounts equivalent to those found in green leaves. The bulk of the nitrogen in petroleum ether extracts from green leaves is associated with the chlorophyll; *i.e.*, the N–phytol mole ratio is close to 4.0. In yellow leaves, this ratio varies from 0.79 to 0.14, higher in fresher leaves, lower in many older ones. The bulk of the chlorophyll nitrogen is now in water or alcohol-soluble form.

During senescence, green leaves lose nearly all their chlorophyll. The resulting color change, whether yellow, red, or brown, depends upon the plant species and many other factors. In most cases the leaves turn bright yellow with little or no change in moisture content. At this stage, they may have lost 95% or more of their original endowment in chlorophyll, leaving no clue with regard either to end products or to the mechanism of degradation.

The instability of the isocyclic ring in extracted chlorophyll is well known, but there is no evidence for allomerization at any stage in the yellowing process. Apart from some fall in the ratio of chlorophylls $a:b$, no change in the green pigments can normally be detected.

Both light and oxygen are required for the bleaching of chlorophyll solutions, and work in this laboratory has proceeded on the assumption that both are normally required in the disappearance of chlorophyll *in vivo*. Light is not, of course, invariably a factor. The center of a pile of newly mown grass will turn bright yellow on a warm day. Here, however, metabolic processes are abnormally disrupted.

The marked decrease in stability to light of porphyrins when complexed with magnesium is well-documented (Barrett, 1967; Fuhrhop and Mauzerall, 1971), and chloro-

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Table I. Chlorophyll and Phytol Contents of Photosynthetic Tissues in μmol per g of Dry Weight

Plant		Moisture, %	Chlorophyll	Phytol		Recovery, %
				Free	Total	
Ginkgo	Green	79.2	4.97	1.33	5.25	105.7
	Yellow	60.8	0.04	0	5.41	
Poison hemlock	Green	80.2	9.46	0.58	10.55	111.6
	Yellow	83.6	0.56	0.27	8.31	
Boston ivy	Green	59.3	3.39	0	3.78	111.5
	Yellow	69.1	0.03	0	4.65	
Olive	Green	58.0	3.12	0	3.30	105.9
	Yellow	56.2	0.39	0	0.73	
Loquat	Green	52.5	4.21	0	3.81	90.5
	Bronze	54.0	0.08	0	2.43	
Manroot	Green	85.7	11.16	0	10.60	94.9
	Yellow	83.2	0.42	0	5.67	
Poplar	Green	44.1	3.40	Trace	4.24	124.9
	Yellow	56.0	0.05	Trace	4.16	
Virginia Creeper	Green	66.7	6.89	Trace	7.30	106.0
	Red	74.5	0.44	Trace	2.94	
Wild oat	Green	65.6	4.91	0	5.57	113.4
	Yellow	23.5	0.01	Trace	4.49	
Live oak	Green	62.1	4.83	0	5.03	104.2
	Brown	35.7	0.03	0.43	2.23	
Bracken	Green	72.2	5.95	0	6.29	105.2
	Brown	11.6	0.005	0	1.88	
California redwood	Green	60.8	3.90	0	3.86	99.0
	Brown	14.3	0.05	0	1.31	

phyll is also very much less stable to light than is pheophytin (Jen and Mackinney, 1970a).

Irradiated chlorophyll solutions will yield, in addition to red-pigmented intermediates, a colorless blue-fluorescing petroleum ether extract, and a colorless and more strongly fluorescing aqueous extract. The former contains a phytyl ester, and the latter contains most of the nitrogen not accounted for by the red intermediate. Some nitrogen remains in the petroleum ether phase, the nitrogen–phytol mole ratio has fallen from 4:1 in chlorophyll to between 1 and 2:1 (Morris *et al.*, 1973).

It is the purpose of this paper to examine to what extent these findings *in vitro* are paralleled in the senescent leaf.

MATERIALS AND METHODS

Solvents, reagent chemicals, and preparation of the chlorophyll standards have already been described (Jen and Mackinney, 1970a,b). Leaves, both green and yellow, were picked at the same time wherever practicable. In some cases, *e.g.*, ginkgo and poplar, yellow leaves were only available in the late autumn. A few petals, roots, and fruit were also examined to determine whether findings were independent of the presence of chlorophyll.

Moisture contents were determined by drying samples of plant tissue, 5 to 10 g, for 15 hr in a vacuum oven at 70°. Pigments were extracted from *ca.* 30 g of fresh leaves which were blended with 300–500 ml of acetone to which $NaHCO_3$ was added to neutralize acidity and prevent pheophytin formation. The residue was reextracted, and the combined filtrates were partitioned against 150 ml of petroleum ether. The upper layer was washed to remove most of the acetone, dried with Na_2SO_4, and made to volume. Aliquots were taken for determination of chlorophyll, phytol, and nitrogen.

For practical reasons (see later discussion), chlorophyll determinations were made in petroleum ether containing 5% acetone. The curves for the two chlorophylls intersect near 651 nm in this solvent, and the absorptivity at this wavelength was found to be 34 (±1.5) l./(g cm).

Table II. Chlorophyll and Phytol Contents of Nonphotosynthetic Tissues in μmol per g of Dry Weight

Plant	Moisture, %	Chlorophyll	Phytol	
			Free	Total
California poppy petals	83.5	0.06	0	0.33
Red rose petals	84.7	0.002	0	0
Potato tuber	79.1	Trace	0	0
Carrot root	86.8	Trace	0	0
Tomato fruit, red	93.1	0.015	0	Trace

RESULTS AND DISCUSSION

In Table I, results are listed for 12 species of plants. The free phytol is nowhere a significant proportion of the total. Within the limits of experimental error, the phytol in green leaves is almost entirely associated with the chlorophyll. As shown in Table II, the phytol content of nonphotosynthetic tissues appears to be negligible.

It is therefore a fair assumption that the phytol to be found in yellow leaves after saponification was originally part of the chlorophyll molecule in the leaf when green. There is, on the average, a recovery of phytol from green leaves which is 6% in excess of that calculated from the chlorophyll content. A deficit would be more readily explicable, and one possibility for these results is that chlorophyll turnover in the leaf maintains a low level of the phytol ester to be found in yellow leaves. Whether this could also explain the excess nitrogen recorded in Table III is more doubtful. There is considerable variation between calculated and found amounts of nitrogen in the petroleum ether extracts, but the average N-phytol ratio for all ten (green) leaves is 4.24 ± 0.44, compared with a theoretical ratio of 4 for chlorophyll itself. At least 90% of the nitrogen found in the extract must be attributed to chlorophyll. The N-phytol ratio for green leaves is calculated directly. For yellow leaves, the phytol and the nitrogen attributable to the residual chlorophyll are deducted first. Thus for ginkgo, 0.04 mol of phytol (actually 0.0355) is subtracted from 5.41 (Table I) and 0.14 mol of N is subtracted from 1.58 (Table III) to give a mol ratio of 0.266.

All nitrogen analyses were run in duplicate and were in agreement within 10% except for two, the oak and redwood. These proved more difficult to digest, and excess digestion mixture may have given erroneous Nessler values. The duplicates in these two were obviously questionable.

This excess nitrogen may or may not be significant when we consider the figures for yellow leaves. If ring IV remains more or less intact after rupture of the chlorophyll molecule, a N-phytol ratio of unity is to be expected. This value is approached only in two cases, the poison hemlock and the common manroot or wild cucumber vine. Whereas we can account for 80 to 90% of chlorophyll nitrogen *in vitro*, the situation in yellow leaves is quite different. Much of the chlorophyll nitrogen has either been translocated from the leaf or it is no longer transferrable to the petroleum ether fraction. The highest N-phytol yet observed for the yellow leaf extract is 0.79, so in most cases it must be inferred that much of ring IV nitrogen is also lost, even though the phytyl ester linkage remains intact in the fragment.

Possible errors in the accuracy of the chlorophyll determination do not affect this conclusion with respect to data on the yellow leaves. The chlorophyll absorption spectrum is sensitive to minor changes in solvent and particularly so to traces of acid. The crude acetone extract must therefore be transferred to petroleum ether, which is then washed with water to remove acids extracted from the plant tissue. Some acetone must be retained in the petroleum ether phase or the chlorophyll will not remain in solution. We have chosen to make dilutions for the measurements with petroleum ether containing 5% acetone; the maximum for chlorophyll *a* shifts from *ca.* 661–662 nm for 1 to 2% acetone to 664–665 nm for 10% acetone in petroleum ether. This could affect our choice of intersect for the two chlorophylls, and the coefficient at 651 nm has an uncertainty of ±1.5.

However, the overall data for green leaves are too consistent for serious error from this source.

Two striking instances of rapid chlorophyll disappearance provided abundant source material. Within a couple of weeks in the Fall months, the chlorophyll content of ginkgo leaves falls from *ca.* 1.35 mg per g to 0.05, with no appreciable change in moisture content. Hemlock leaves give a similar result in the early summer. In both cases, the leaf becomes a bright yellow.

An abundance of source material is a prerequisite if one is to trace the fate of chlorophyll nitrogen. This is admittedly a difficult undertaking, as the chlorophyll nitrogen is not 2% of the total nitrogen in ginkgo or hemlock (Lu, 1970), and one must concur with Seybold (1943), who surmised that there was a rapid and extensive cleavage of the chlorophyll molecule with the formation of colorless low-molecular weight compounds.

On this basis, Lu attempted a systematic fractionation of nonprotein nitrogenous constituents of green and yellow leaves. If substances such as hematinic acid or methylethylmaleimide were to be found in yellow leaves, it would be strong presumptive evidence that it had been derived from chlorophyll. Although Lu concluded that it was possibly unrealistic to expect that relatively small fractions of such material would not be masked by more abundant "contaminants," two imide fractions were found of special interest: one from the neutral fraction of yellow ginkgo leaves (*m/e* 173) and the second from the acidic fraction of both yellow hemlock and ginkgo with a strong blue fluorescence. The latter chromatographed with a blue-fluorescing compound obtained by photodecomposition of chlorophyll *in vitro* (Jen, 1969).

The discovery that phytol can be demonstrated in yellow leaves in quantities comparable with those in green leaves only after saponification simplifies the problem considerably. There must exist a phytol-containing fragment (or fragments) in the yellow leaf, derivable from ring IV, and it seems probable that some of its nitrogen has been retained in imide form. In any event the phytol, bound in its ester linkage, is as useful a marker as if ring IV had been uniquely labeled with an isotope.

Table III. Nitrogen–Phytol Ratios in Petroleum Ether Extracts

Plant		Nitrogen, μmol per g		N–phytol mol ratio
		Found	Calculated	
Ginkgo	Green	22.2	20.4	4.23
	Yellow	1.58	0.14	0.27
Poison hemlock	Green	35.7	37.5	3.38
	Yellow	8.00	2.24	0.74
Boston ivy	Green	15.6	13.4	4.13
	Yellow	1.39	0.11	0.28
Olive	Green	14.3	12.4	4.33
	Yellow	1.23	1.56	
Loquat	Green	20.1	16.7	5.27
	Bronze	0.87	0.31	0.24
Manroot	Green	40.7	44.8	3.84
	Yellow	5.82	1.68	0.79
Wild oat	Green	25.9	19.5	4.65
	Yellow	0.65	0.05	0.13
Live oak	Green	20.1	19.1	4.00
	Brown	0.84	0.12	0.33
Bracken	Green	23.3	23.6	3.70
	Brown	0.90	0.01	0.48
California redwood	Green	19.1	15.5	4.95
	Brown	0.99	0.19	0.64

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