and 2 were confirmed in a much more extensive experiment (Roth and Losty, 1968).

The Effect of Increasing Irradiation Dose and Irradiation at Different Temperatures. Samples were irradiated with 0 (nonirradiated/control), 2.0–2.3, 4.0–4.6, or 6.0–6.9 Mrad at 0 to 4, −30 ± 10, or −80 ± 10°, and were stored frozen until delivered to the laboratory. These samples were not blanched. The results are shown in Table I.

Three major conclusions may be drawn from the data in Table I. There is a marked decrease in total proteolytic activity with increasing radiation dose. With respect to destruction of proteolytic activity, irradiation at 0 to 4° is considerably more effective than irradiation at −30 or −80° (±10°). Irradiation alone (without blanching) does not destroy all proteolytic activity and is, in actuality, less effective than blanching.

It has also been determined (not illustrated) that storage of these irradiated samples at −16° or room temperature (21–25°) for up to 4 weeks does not significantly change the total proteolytic activity.

DISCUSSION

The methods presented in this report make it possible to determine the optimum conditions for the destruction of the total proteolytic activity of meat samples by blanching alone or blanching and γ irradiation. It is clear that of the two methods blanching is more effective but only when carried out at temperatures of 70°. Even 4.5–5.2 Mrad of γ radiation will destroy only about 75% of the proteolytic activity of beef samples. A combination 4.5–5.2 Mrad γ radiation and blanching at 70° for 5 min may be expected to destroy 95 to 100% of the total proteolytic activity of meat. Such samples would probably undergo little further enzymic change even when stored at room temperature for long periods of time.

Although it is possible that there is some latent proteolytic activity in the meat samples tested, this seems unlikely for several reasons. First of all, the method of preparing the samples for assay by homogenization in water would probably release any lysosomal bound cathepsins. Secondly, assays on samples, both irradiated and blanched, at intervals after up to 8 weeks of storage at room temperature indicated that no significant changes in proteolytic activity occurred.

By use of isolated cathepsins as well as crude tissue homogenates, it may be possible to define more precisely conditions under which complete destruction of proteolytic enzyme action may be expected.

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On the Photodecomposition of Chlorophyll *in vitro*

Marvin M. Morris, Yoong Park, and Gordon Mackinney*

Chlorophyll solutions can be irradiated to yield variable amounts of reddish intermediates and a virtually colorless supernatant. Precipitated after brief irradiation, the red intermediates retain a nitrogen–phytol–magnesium ratio of 4:1:1. After longer exposures, the nitrogen–magnesium ratio remains unchanged but the phytol is lost. The supernatant is evaporated to dryness and the residue is extracted successively with petroleum ether, water, and acetone. The petroleum ether extract is colorless with a weak blue fluorescence. It contains a phytyl ester and 10 to 15% of the original nitrogen. The water extract exhibits a whitish turbidity and a strong blue fluorescence. It contains a substantial fraction of the original nitrogen and magnesium. The original supernatant has negligible absorbance in the visible, but some reddish precipitate is formed on concentration. This is soluble in acetone and is devoid of phytol.

The decomposition of chlorophyll in solution in the presence of light and oxygen can be arrested and red intermediates can be precipitated, provided the solution contains a high percentage of a nonpolar solvent such as petroleum ether (Jen and Mackinney, 1970a). Under the most favorable conditions, yields of red intermediate have never exceeded 75%, and in view of a considerable uptake of oxygen, it is doubtful if 50% of the original chlorophyll has been accounted for.

Department of Nutritional Sciences, University of California, Berkeley, California 94720.

This warrants further examination of both the precipitates and the supernatant solutions, and this paper is concerned primarily with the fate of the phytol, the nitrogen, and the magnesium as a result of the photooxidation of the chlorophyll.

MATERIALS AND METHODS

Solvents and Chlorophylls. The necessary solvents and procedures for preparing the pure chlorophylls have already been described (Jen and Mackinney, 1970b).

Phytol Determination. Phytol was detected by gas–liquid chromatography (glc) in a Varian Aerograph, Model

200, with 2.5% of free fatty acid phase (FFAP) on 80/100 mesh high performance Chromosorb W, acid-washed, and dimethyl(di) chlorosilane (DMCS)-treated column under the following conditions: temperatures, injector 241°; column, 212°; detector, 238°; helium flow rate, 100 ml per min. Under these conditions, phytol had a retention time of 4.6 min.

The phytol standard was obtained from Calbiochem, San Diego, Calif., lot 901453. Quantitative results were obtained by measuring peak areas (height × width at half-height), which were then compared with a standard curve made concurrently (within 1 hr of the time that unknowns were analyzed). Failure to do so would result in a perceptible diminution of the measured peak areas. This happened when the phytol in leaf extracts was injected. The column efficiency could be restored by replacing the column inlet glass wool plug and heating the column to 270°, thereby removing contaminants.

For the standards, 40 μl containing 30 to 100 μg of phytol in petroleum ether were injected. At these levels, the uncertainty is of the order of 1.0% for duplicate runs. Below 5 μg per 40 μl, it rises to 50% and it is possible only to verify the presence of phytol.

Saponification. Chlorophyll, the red precipitates, and even the colorless supernatants must be saponified to liberate the phytol from its ester linkage. The saponification of chlorophyll proceeds rapidly whether the solution is heated or not, provided it is a single phase solution, and the completeness of the step is shown by failure of the green pigment to transfer from an alkaline methanolic phase to an added epiphase of petroleum ether. The liberated phytol can then be extracted with petroleum ether and assayed by glc.

The red precipitates are much less soluble in methanol and are quite insoluble in petroleum ether. No simple test is available to determine the completeness of the saponification, either with the red precipitates or with the supernatants. Except for estimates of free phytol, made prior to saponification, samples were digested in 20% KOH in methanol for 10 min at 60° and then cooled. Acetone was added to facilitate subsequent extraction of the phytol by petroleum ether. The petroleum ether extract was then washed with water, dried with anhydrous Na_2SO_4, and reduced to a small volume, and duplicates of 40 μl were then injected.

Under such conditions, phytol recovery from chlorophyll preparations varied from 95 to 99% of theoretical.

Nitrogen Determinations. The method described by Minari and Zilversmit (1963) was used for nitrogen determinations. The sample was digested by micro-Kjeldahl, diluted with water, and then Nesslerized. A yellow-orange color was developed, the absorbance of the solution was measured at 420 nm, and the nitrogen content was determined by comparison with a standard curve for maleimide nitrogen. In the range 5–30 μg per 6 ml, the absorbance is a linear function of the concentration.

Magnesium. Magnesium was determined, after perchloric acid digestion of the sample, by atomic absorption spectrophotometry.

Irradiation of Solutions. When several milligrams of end product are desired, solar radiation provides the simplest light source. Solutions were irradiated for various times in plain glass-stoppered volumetric flasks immersed in a water bath at temperatures between 25 and 28°.

Preparation of Chlorophyll Solutions. These were of two kinds, in benzene and in petroleum ether containing 5% benzene. The pure chlorophyll was first dissolved in benzene, and then made to volume with additional solvent. Concentrations within the range 20–100 mg/l. were most practical, avoiding unduly prolonged irradiation.

Fractionation of End Products. In the mixed solvent system, chlorophyll *a* gives rise to a finely divided reddish sediment after a 2–5 hr exposure. The solution is centrifuged, yielding a friable powder and a pale pinkish supernatant. In pure benzene, chlorophyll *a* yields no precipitate after 5 days until after the solution has been concentrated. With chlorophyll *b*, a red sediment is deposited by the fifth day.

These precipitates or sediments are deep red in color, and are completely insoluble in petroleum ether with which they are washed. They are also rinsed with water and both washings are retained.

The supernatants are taken to dryness in a flash evaporator, and the residue is extracted successively with petroleum ether, water, and finally acetone. All three show a blue fluorescence, with that of the water extract being far the strongest. The petroleum ether extract is however completely colorless; the water extract exhibits a milky white turbidity which clears on the addition of methanol; the final acetone extract dissolves residual pigment. The pigment is transferred to diethyl ether and washed with water. The latter exhibits some fluorescence and is combined with the water extract, and the pigment is added to the red precipitate fraction.

RESULTS AND DISCUSSION

Conclusions are based on nine individual exposures of solutions of chlorophyll *a* and two of chlorophyll *b* to sunlight. Three are discussed in detail. Analysis of the chlorophyll *a* used for two experiments had the following percentage composition. *Anal.* Calcd: C, 73.90; H, 8.12; N, 6.27; Mg, 2.72; phytol, 33.1. Found: C, 73.15; H, 8.09; N, 6.13; Mg, 2.72; phytol, 31.4. The C, H, and N analyses were run by courtesy of the Department of Chemistry. The specific absorptivity in benzene was 84.4 at 667 nm and 114.8 at 433 nm. The chlorophyll *b* had a specific absorptivity in benzene of 53.2 at 650 nm and 142 at 465 nm.

Red Intermediates. The red intermediates vary in composition, depending upon the solvent system and the time of irradiation.

The red precipitate from chlorophyll *a* in petroleum ether containing 5% benzene after 5 hr irradiation had the following percentage composition. *Anal.* Found: C, 62.74; H, 7.46; N, 5.47; Mg, 2.26; phytol, 24.3.

This friable powder lost 9% of its weight when dried over P_2O_5, regaining it when exposed to air. A probable formula of $C_{54}H_{76}N_4MgO_{14}$ may therefore be expressed as $C_{54}H_{66}N_4MgO_9$ (5 H_2O) with some degree of assurance. The N–Mg–phytol ratio is 4:0.95:0.82, indicative of no change from the original chlorophyll. The absorptivity in benzene at 430 nm is *ca.* 12, showing a tremendous loss of tinctorial power compared with either chlorophyll or any common porphyrin.

No precipitate was obtained when the chlorophyll *a* was irradiated in pure benzene for 5 days until the volume of the solution had been reduced by two-thirds. A tacky reddish sludge was then removed by centrifuging. The original deep blue color of chlorophyll *a* changed to lavender, deep red, and finally a light tan after the 5-day irradiation. Before concentration, the solution had an absorbance of 0.12 at 430 nm in a 1-cm cell. In these circumstances, the reddish color of the sludge appears somewhat anomalous.

This fraction could only be handled in methanol solution, aliquots of which were taken for nitrogen and phytol determinations. Analyses of aliquots, in duplicate, gave 35.1 μmol of N and 3.54 μmol of phytol; *i.e.*, a ratio of 4:0.4, which shows the marked effect of prolonged irradiation on phytol recovery.

The reddish sediment obtained from chlorophyll *b* in pure benzene was friable but hygroscopic. It was insoluble

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

Yoong Park, Marvin M. Morris, and Gordon Mackinney*

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-

Department of Nutritional Sciences, University of California, Berkeley, California 94720.