Reports

Chlorophyll *a* Appearance in the Dark in Higher Plants: Analytical Notes

Abstract. *The increase in the ratio [chlorophyll* a]/[*chlorophyll* b] *which occurs in expanding bean leaves in the dark, is a result of chlorophyll* a *formation with a concomitant loss in chlorophyll* b. *The analytical methods for assay of these pigments were examined closely and found to be adequate for this purpose when proper safeguards are taken.*

The established pathway to chlorophyll *a* in angiosperms is via the action of light on protochlorophyll, or on a closely related precursor (*1, 2*). On less secure grounds, chlorophyll *b* is often thought to arise from chlorophyll *a,* probably by an oxidation process (but see *1, 3*). Sometime after chlorophyll *a* has begun to accumulate in the light in an etiolated leaf, chlorophyll *b* appears; gradually a minimal ratio of chlorophyll *a* to chlorophyll *b* is established. Fluctuations in this ratio, diurnal or otherwise, have been noted in the past and currently these are receiving attention in the study of chlorophyll metabolism (see *4*). There is a question, however, whether these observed fluctuations are not the result of the procedures used (*5*).

While following the rise of a soluble leaf protein during greening of bean leaves, the ratio [chlorophyll *a*]/[chlorophyll *b*] was observed to increase during alternate dark intervals and return to the minimal value after reillumination (*6*). This increase of the ratio in the dark was most pronounced when the leaf was growing rapidly, and became insignificant as maximal growth was achieved. A strict correlation between the increase in the *a/b* ratio with gain in either fresh or dry weight of leaf during dark periods, however, was not demonstrated (*7*). For rapidly growing leaves the increase in the *a/b* ratio after 1 to 2 days in the dark may amount to several hundred percent of the minimal value found in the previous light period. Accordingly, we have attempted to examine critically whether a real change in the ratio of the concentrations of the two chlorophylls takes place during dark growth, and if so, to determine whether there is a net formation of chlorophyll *a* (see, for example, *8*).

For these experiments, seedlings of bush bean (Stringless Green Pod, W. Atlee Burpee Co.) were grown in watered beds of vermiculite at 26° ± 1°C in the dark until they were 18 to 20 cm high (10 to 14 days) before beginning light treatments of 1000 ft-ca with tungsten lamps. On occasions, alternating 12-hour periods of light and darkness were begun immediately after planting, but the same effects were noted during dark growth intervals. Better experimental control was obtained with the former protocol, because the leaves normally were out of the cotyledons before illumination was started.

Extracts containing the pigments in 80-percent acetone were prepared under green light in an ice bath from dry leaf powders. The latter were obtained by pulverizing fresh leaves in liquid nitrogen and lyophilizing the ground material in the dark at an external temperature of about 0°C. The powders were extracted six to eight times with 80-percent acetone; the residues, however, appeared devoid of green pigments after the third or fourth extraction. Sufficient powder was used to provide an optical density of between 0.5 and 0.8 at the maximum in the red region with a light path of 1 cm after the combined extracts were brought to 25 ml. Clarity of the extracts was maintained by filtering, with intermittent gentle suction, through a fine pore, sintered glass filter in ice. Clarification by centrifugation was not adequate; scattering was apparent in some extracts at 750 mμ even after 10,000*g* for 1 hour. The amount of chlorophylls *a* and *b* were determined spectrophotometrically by simultaneous equations in the 80-percent acetone (*9*) and again after transferring an aliquot of the pigments to diethyl ether (*10*). The wavelengths for these determinations were the maxima obtained for the chromatographically separated pigments. These maxima were all approximately 1 to 1.5 mμ shorter in our spectrophotometer (Cary, model 14) than those with the instruments used by the aforementioned authors (*9, 10*); we used the same extinction coefficients, however, that they did. The *a/b* ratios were essentially the same by both methods, although the yield of each pigment was 2 to 5 percent lower in ether. Copigmentation association of chlorophyll pigments apparently does not exist in ether (*11*); therefore, its effect, if present in the acetone solutions, was not significant. Two to five determinations were made on each sample; a precision of within 2 percent was maintained.

The pheophytins of chlorophyll *a* and chlorophyll *b,* which sometimes arise in plant material or during extraction thereof, are the chief pigments known to interfere in the method of simultaneous equations traditionally used to evaluate the amounts of each chlorophyll from the spectra of extracts. The necessary equations for estimating the pheophytin content by a procedure involving oxalic acid have been developed by Vernon (*9*). In all experiments, the number of moles of each pheophytin formed in acid corresponded, within experimental error, to the moles of each chlorophyll calculated from the control extracts not containing the acid. This correspondence obtained for leaves after either light or dark periods, both before and after maximal growth.

The possibility remained, however, that during a dark interval other pigments might arise which would absorb in the 600 to 700 mμ region of the spectrum and vitiate the assay method. Accordingly, the chlorophylls were separated chromatographically on powdered sugar columns (1 by 15 cm) with spectro-grade *n*-hexane containing 0.4 percent propanol. The pigments were transferred at 0°C, or colder, from the 80-percent acetone extract (above) to an equal volume of hexane,

Table 1. Calculated amounts of chlorophylls *a* and *b* in bean leaf extract (80 percent acetone) versus amounts of the chromatographically resolved components.

Item	Chlorophyll		
	a (mg)	*b* (mg)	*a/b*
Extract	0.176	0.040	4.40
After chromatography	0.168	0.038	4.42

followed by one volume of 100-percent acetone. Less than 2 percent of the green pigments remained untransferred. The hexane layer was washed three times with ice-cold water, chilled to −22°C to remove excess water, and evaporated under a stream of dry nitrogen gas at 0°C. The residue was taken up in a very small volume of the hexane-propanol solvent and applied to the sugar column previously equilibrated with this solvent at 2°C. The separated bands of chlorophyll *a* and chlorophyll *b* were eluted, the solvent was evaporated as above, and the residues taken up in 80-percent acetone for spectral analysis. A second chromatographic resolution was necessary to remove the last traces of yellow pigments which absorbed in the 400- to 500-mμ region; however, the spectra above 500 mμ after the first separation were identical to those of the authentic specimens published by Vernon. Usually about 1 percent of the chlorophyll *a* was observed in the chlorophyll *b* eluate after a single pass through the column. Pheophytins and other greenish pigments invariably were found if any of the operations were conducted at room temperature. Recoveries of chlorophyll *a* and chlorophyll *b* after separation have exceeded 90 percent of the amount calculated to be present in the original 80-percent acetone extract. The totals from a recent experiment on leaves after a dark period are shown in Table 1. Since virtually the same *a/b* ratios were obtained after chromatographic resolution, we conclude that the method of simultaneous equations on spectra of clear extracts of bean leaves is reliable with the procedure here employed.

In order to determine whether the chlorophyll pigments increase or decrease per leaf during dark growth, the following protocol was followed: during a light period, 50 to 150 seedlings were selected on which the two primary leaves were well matched by eye with respect to size and uniformity of pigmentation. By many trials, we have found that the weight and the chlorophyll content of pairs of leaves so matched agreed to within 5 percent of one another. With this number of plants, there is good probability of picking as many heavier mates as lighter ones.

After a given light period, one of each pair of leaves was removed and the seedlings, each with only one leaf, were placed in the dark for 12 or more hours before the second harvest. The lyophilized leaf powders from each harvest were weighed and aliquots were taken for chlorophyll, dry weight, and other analyses. The dilution of the chlorophylls on a dry-weight basis during a dark period corresponded almost exactly with the increase in dry weight of leaf. The amount of chlorophyll *a* per leaf, however, was always found to be greater in growing leaves after a dark period. Within experimental error, a corresponding decrease in chlorophyll *b* was observed. The results of a few paired experiments with growing leaves and one from fully expanded leaves (experiment 6) are shown in Table 2. These represent only a selection from numerous experiments required for a further purpose, but all results were equally consistent with the conclusion that no change in total chlorophyll per leaf takes place in the dark despite marked changes in the amounts of both *a* and *b*. As noted in Table 2 (experiments 1 and 5), a 22- to 23-percent increase in chlorophyll *a* and a 75-percent decrease in chlorophyll *b* occurred during the dark interval in leaves which were about 30-percent green initially. In other experiments, leaves less green than this lost all measurable chlorophyll *b* after 2 days or more in the dark. Thus, the proportionate change in specific chlorophylls during leaf growth is substantial and apparently of physiological importance. On occasions, with single plants, one matched leaf was enclosed in a black box in which fresh air was circulated by a pump while the other leaf remained in the light. After 12 or more hours both leaves were extracted; the results showed an increase in *a/b* ratio for the leaf in the box similar to that found when one leaf was excised prior to a dark period. Restoration of the minimal *a/b* ratio in the light after a dark period is not immediate; several hours were required, depending on the intensity and on the disparity of the ratios. No transient decrease of chlorophyll *a* was noted when the light was restored.

The results of these studies suggest that chlorophyll *a* arises from chlorophyll *b* during dark growth of bean leaves (a similar statement was made recently by Wieckowski, see *4*). The question whether chlorophyll *b* arose initially from chlorophyll *a* during a previous light period must be left open. Convincing evidence is lacking that chlorophyll *a* is the principal precursor of chlorophyll *b* even in the light. On the other hand, the accepted precursor of chlorophyll *a*, during greening, may be questioned. Our previous observations do not support the hypothesis that the photochemical mechanism producing the first chlorophyll *a* in etiolated leaves serves also for the high rate of chlorophyll formation in greening leaves (*6*). In subsequent experiments, we have found no indication of this photochemical reaction by difference

Table 2. Content of chlorophylls (Chl) *a* and *b* in the same number of paired leaves of bean seedlings before and after a dark period.

Expt.	Time (hr) light (L), dark (D)*	Dry leaf aliquot† (g)	Chl (mg/aliquot)			Chl *a/b*
			a	*b*	*a* + *b*	
1	12L	1.00	3.70	1.10	4.80	3.36
	12L,48D	3.00	4.57	0.28	4.85	16.32
2	12L	1.00	3.59	1.14	4.73	3.15
	12L,48D‡	2.22	4.21	0.52	4.73	8.10
3	24L	1.00	4.25	1.24	5.49	3.43
	24L,12D	1.48	4.62	0.85	5.47	5.44
4	24L	1.00	3.46	1.15	4.61	3.01
	24L,24D§	1.56	3.94	0.67	4.61	5.88
5	24L	1.00	3.99	1.16	5.15	3.44
	24L,60D	3.18	4.87	0.29	5.16	16.79
6	4(12L,12D)12L	1.00	11.75	3.73	15.48	3.16
	4(12L,12D)12L,24D	0.84	11.85	3.70	15.55	3.20

*White light, 1000 ft-ca; indicated treatment was begun after dark-grown plants were about 18 to 20 cm high; then one leaf per pair was removed for analysis prior to final dark period. †Aliquots were identical in a given experiment. ‡Temperature during final dark period was about 21° instead of the usual 26°C. §Leaves were not completely out of cotyledons before illumination.

spectra with the opal glass method (*12*) before and after illumination of bean leaves, which are more than 10 percent green, after periods of growth in the dark. Thus, after the leaf gains photosynthetic power, the existence of another pathway to chlorophyll *a*, perhaps via chlorophyll *b*, is not ruled out (*13*).

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Quantitative Molecular Approach to the Permeability Changes of Excitation

Abstract. *Functional relationships, available from only a few monolayer studies, can be applied to a relatively simple model of the excitable membrane to give permeability-potential curves quite similar to the conductance-potential curves obtained experimentally in voltage-clamped giant axons. Contrary to the usual view in terms of "carrier systems," the present model considers the permeability to sodium and potassium to be reduced by the increase in the surface pressure induced by large lipophilic cations and anions in the outer layer of the lipoidal bimolecular leaflet constituting the living membrane; hence, the increase in permeability during depolarization, for example, is due to a decrease in the amount of the organic anions in this layer, whereas the decrease in sodium permeability during inactivation is caused by a rise in content of organic cations. The present proposal has the advantage that it is in keeping with known phenomena observed in simple physico-chemical systems as well as in excitable systems; moreover, the current actually transferred by the postulated lipophilic ions can be negligible compared to that transferred by the inorganic cations they control. The latter situation, as well as the steepness of the permeability-potential relationships obtained, have been pointed out to be critical requirements of a satisfactory molecular hypothesis.*

Recent monolayer studies provide a basis for the labile permeability properties of living membranes. Shanes and Gershfeld (*1*) called attention to the correlation between the increase and decrease of membrane permeability in veratrum alkaloids and local anesthetics, respectively, and the ability of these drugs to induce a corresponding decrease or increase in the surface pressure of monolayers of a fatty acid. It was suggested in consequence of this, as proposed earlier (*2*) on the basis of Skou's monolayer work (*3*), that surface pressure may govern ion permeability by determining the proximity of adjacent membrane molecules in the regions of ion passage, a greater proximity due to a higher surface pressure reducing ion entry and vice versa. Archer and La Mer (*4*) and La Mer and Barnes (*5*) have actually shown the dependence of the rate of penetration of molecules through monolayers on surface pressure. They have demonstrated, moreover, that in the case of fatty acids the presence of small amounts of calcium, which changes the state (that is, the relative solidity and packing) from the liquid condensed to the solid condensed, has two effects: a substantial decrease in the penetrability to water molecules for the same surface pressure, and an increase in the range of surface pressure over which a change in penetrability may be induced. These offer a basis for the well-known dependence of membrane ion permeability on calcium in the medium (*2*) and for the enhancement by this ion of the permeability changes during excitation (*6*). It must be stressed that surface pressure of itself in the absence of proper alignment or strong interactions between neighboring molecules may not affect monolayer penetration by water vapor and other gases (*7*). Moreover, the presence of small amounts of weakly interacting molecules may also greatly increase penetrability (*4*, *8*).

It has been pointed out qualitatively, on the basis of Guastalla's demonstration (*9*) of the enhanced accumulation of lipophilic ions at the oil-water interface because of an electric field, that the redistribution of such ions within the living excitable membrane, brought about by changes in transmembrane potential, offers a possible basis for the well-known dependence of membrane permeability on the potential (*10*). A mathematical approach to this, based on the application of known monolayer properties to a model in which the concentration of lipophilic ions varies continuously with distance through the membrane, has given permeability-potential curves resembling those found experimentally (*11*).

A somewhat simpler set of relations giving an even better fit with the experimental permeability-potential curves has now been obtained by considering the possibly more realistic situation wherein, by virtue of the bimolecular structure of the lipoidal part of the membrane, large lipophilic ions (of the order of one membrane layer thickness in length) are regarded as residing either in one or the other of these layers, their polar group being only at either the inner or outer membrane-water interface. In the insert of Fig. 1 the situation is depicted for an organic anion. Equation 1 given in Fig. 1 represents the application of Boltzmann's principle to this type of distribution Z is the valence of the anion; E, the transmembrane potential; F, Faraday's constant; R, the universal gas constant; and T, the absolute temperature. The possibility of a work function for the transfer of the anion between I and M of the membrane is neglected and C_I, the content of the anion in the inner layer, I, is regarded as constant by virtue of an intracellular source of the anion with which it is in equilibrium. Variations in C_M, the content of the organic anion in M, are considered responsible for the permeability changes of the membrane. Thus, an increase in C_M will increase the packing of the membrane molecules in M and hence its surface pressure, S. The interaction