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CHLOROPHYLLIDE *b*

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SUMMARY Chlorophylls *a,b* and chlorophyllides *a,b* were isolated from pea chloroplasts as pheophorbides *a,b* following the administration of [^{14}C]δ-aminolevulinic acid. Relative pool sizes suggest that chlorophyllide *b* precedes chlorophyll *b* and does not arise from the latter by the action of chlorophyllase.

The kinetics of chl b formation have been formulated primarily by A.A. Shlyk and colleagues (1) as arising from chl a which is itself heterogeneous, consisting of two or more pools. Previously, this same group had demonstrated the origin of chl a from chlide a (2). There is no spectroscopic evidence of chlide b formation on illumination of the protochlide holochrome of etiolated plants (3) and its occasional observation in greened leaves is thought to arise from chlorophyllase hydrolysis of the phytylated compound, chl b (4). Kinetic study of the relation between chl a and chl b formation shows an initial zero slope of the latter, in contrast to the positive initial slope of chl a (5), as would be expected in a sequential product relation (see *Discussion*). None of these findings is inconsistent with the existence of a small pool of chlide b which is subsequently esterified to chl b.

In this communication evidence is presented both for the existence of chlide b as well as for its preceding chl b in the biosynthetic sequence.

Abbreviations. Chl, chlorophyll; chlide, chlorophyllide; protochlide, protochlorophyllide; fbd, pheophorbide; ftn, pheophytin; δ-ALA, delta-aminolevulinic acid.

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Materials. [^{14}C]-δALA was purchased from New England Nuclear. [^{14}C]-chl *a* was made by growing *Anacystis* sp. in $^{14}CO_2$. Like other blue-green algae, this organism is devoid of chl *b*. Following extraction it was purified twice by chromatography on sugar and a third time by a reverse phase column on HPLC (6). *p*-hydroxymercurobenzoate was purchased from Sigma. Fbds were prepared by standard procedures (see *Methods* below).

Methods. Chloroplasts were prepared from 9-14-day-old peas by grinding one minute at 4°C in a mortar and pestle, using a bit of washed sand and Ridley's homogenizing medium (7). The brei was squeezed quickly through 8 layers of cheese cloth and the expressed juice filtered through 4 layers of 80 μm nylon mesh. The resulting solution, microscopically free of cells, was centrifuged 2m at 1500*g* and the precipitated chloroplasts suspended in Ridley's medium.

In a standard protocol 2 mL of chloroplast suspension was placed in boiling water for 2m and used as control. To this was added 20 μL of [^{14}C]δALA (ca. 2 μCi) as substrate, followed by incubation for 30m in ambient light with occasional shaking. Samples were treated identically, except for the denaturation. At the end of the incubation period p-hydroxymercuribenzoate was added (final conc., 5×10^{-4}M) to suppress chlorophyllase activity (8). After one minute this was followed by 4 vols. of isopropanol for delipidation. Membrane ghosts and other precipitated proteins were removed by centrifugation for 5m at 1000*g*.

To the aq. isopropanol there was added an equal vol. of freshly distilled ethyl ether and the ethereal solution washed fourfold with distilled water. This solution was then supplemented with mixed fbds *a,b* and these, together with the chlides extracted as fbds by 20% HCl (twice, 10 mL each). The fbds were then transferred into $CHCl_3$ by dilution of the HCl. The $CHCl_3$, in turn, was removed by flash evaporation and the crude fbds banded onto Whatman 3 MM paper for chromatography in hexane, acetone (7,3) (9). The well-separated bands were eluted in two stages. In the first, the bands were cut out and stood in small beakers containing acetone. The pigment was driven up the paper and collected at the other end which had been cut to a point. The collected pigment was then readily eluted with a small volume of acetone into a small vessel. The solvent in the latter was flash-evaporated, a few drops of acetone added, and the solution transferred quantitatively to a planchet for counting in a Nuclear Chicago Automatic Planchet counter. Counts were made for 100m, allowing a background accumulation of approximately 1000 counts. At a 2σ (approx. 95%) confidence level, the background count was 9.97 ± 0.63 cpm. Counting efficiency was 13.19%. Using highly labeled fbd *a* derived from the [^{14}C]chl *a* the level of activity in the fbd *b* was less than 4% and presumed to be due to partial oxidation of the fbd *a*, possibly as 13^2-hydroxyfbd *a*.

Results. Table 1 shows typical data of a kinetic experiment, illustrating both the enzymatic nature of the experiment and the accumulation of products with time. Table 2 depicts the

TABLE 1

Incorporation of [^{14}C]-δALA Into Chlides *a*,*b** and Chls *a*,*b** by Pea Chloroplasts

No.	Sample time (m)	fbd (dpm)** *a*	fbd (dpm)** *b*	ftn (dpm)** *a*	ftn (dpm)** *b*
1a	control	11.1 ± 7.1	2.5 ± 6.9	22.0 ± 7.3	15.8 ± 7.2
b	30	4190 ± 36	272 ± 11	166 ± 10	23.1 ± 7.5
2a	10	953	108	208	76.9
b	30	1940	423	246	94.6
c	60	7060	507	854	246

* Isolated as fbds.
** Counting errors at 2σ (approx. 95%) confidence level.

amount of chlorophyllase activity during the same time periods using two different methodologies, a colorimetric procedure (10) and a radioactivity iteration. The data show both methods to yield identical results.

Discussion. Table 2 demonstrates that the activity found in fbd *b* cannot be due to chlorophyllase action on

TABLE 2

Chlorophyllase Activity in Pea Chloroplasts Using a) [^{14}C]Chlide *a* as Substrate and b) Spectrophotometry.

a) time (m)	dpm chlide *a*	dpm chl *a*	%
10	284	48,600	0.8
30	215	37,900	0.6
60	369	27,000	1.1
b)	μg		
10	6.1	706	0.9
20	5.5	701	0.8
60	9.5	723	1.1

chl *b*. The numerology also forces a similar conclusion. Consider, as an example, Sample 1b in Table 1 where the fbd *b* pool is 272 dpm and the ftn *b* is, correspondingly, 23.1 dpm. The sum of the two (295 dpm) implies that if the chlide *b* pool were to have originated from the ftn *b* initially, it would have required approximately 92% hydrolysis of the chl *b* to produce the chlide *b*. From the data of Table 2 this would be virtually 2 orders of magnitude more than occurred. Further, the residual chl *b* in the ethereal solution, following extraction of the fbd *b* with 20% HCl, was always two-to-three times the amount of carrier fbd *b*. The data not only suggest that chlide *b* does not arise by chlorophyllase action on chl *b* but that any equilibrium reaction between them must be a slow one.

The kinetics of Table 1 show that this relationship persists in pea chloroplasts at least through the first hour and that, in all probability, chl *b* parallels chl *a* in arising from its chlide, at least in part. That chlide *b* may be a substrate for chlorophyll synthetase has recently been shown by Benz and Rüdiger (11). This does not exclude the possibility that chl *b* may arise directly from one of the chl *a* pools.

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