Changes in Chlorophylls and Carotene Contents of Green and Bleached Lima Bean Seeds During Development and Maturation[1]

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Abstract. Green seeds from lima bean (*Phaseolus lunatus* L.) cvs. G 2 and Green Fordhook 861, contain chlorophylls a and b in a ratio of about 2:1. Chlorophyll content per seed increases during development, reaches a max about 5 weeks after pod formation, then declines sharply during maturation. Bleached seeds have less chlorophyll, especially chlorophyll a, and carotene than non-bleached seeds.

In developing and mature lima bean seeds max chlorophyllase activity appears during the stage which corresponds with max chlorophyll content rather than the stage of most rapid loss of chlorophyll. The enzyme is particulate, located in the chloroplast membranes, and has an optimum pH of 8.5 ± 0.2.

The low carotene content of seeds of green-seeded commercial cultivars might contribute to their sensitivity to light. Several cultivars that were obtained from other countries. (Plant Introductions) contain 10 to 20 times as much carotene as the commercial cultivars tested. These "Introductions" provide genetic material for increasing the carotene content of lima bean seeds. High carotenes would improve nutritional value of beans and might reduce or prevent bleaching.

There are many physiological and biochemical differences between bleached and non-bleached lima bean seeds (1, 5, 11). Some of these differences include low seed vigor (expressed as low % emergence of seedlings), reduced ability of the embryonic axes to synthesize proteins and polysaccharides but not lipids during germination, and reduced green color.

Although bleaching in seeds of lima beans, peas, and a few other species has been recognized for years, neither the factors that underlie this susceptibility nor the processes involved in chlorophyll destruction have been investigated. Bleaching in other plants has been attributed to 2 phenomena. The first is photooxidative and is well known in mutants of the purple bacterium *Rhodopseudomonas spheroides* (6, 14), the green alga *Chlorella* (6), corn (2, 8, 13) and sunflower (17)–all of which differ from the wild types in failure to synthesize colored carotenes. Bleaching of these mutants, which lose chlorophyll under light intensities normally favorable for its synthesis, has been attributed to lack of protection by yellow carotenoids against photodestruction of chlorophylls (6, 8, 13, 14, 17). The second phenomenon, which is light-independent, is attributed to chlorophyllase–a hydrolytic enzyme, localized in the chloroplast, and capable of cleaving chlorophylls a and b into their respective porphyrins and phytols. In ripening apples chlorophyll is hydrolyzed by chlorophyllase during the climacteric and post-climacteric stages (12). The activity of this enzyme during the period when chlorophyll is broken down increases to about 300% of that during the pre-climacteric stage.

Materials and Methods

Materials. Seeds of 'G 2' (Ben Fish and Co.[3], California, 1969 crop) and 'Green Fordhook 861' (Charter Seed Co., California, 1969 crop) were used in following changes in chlorophylls, carotenes, and chlorophyllase activity of seeds during development and maturation. Seeds were planted at the Beltsville Farm of the U.S. Department of Agriculture on June 16, 1970. Flowers were tagged at pod formation and seed samples were taken after 2½, 5, 7½ and 15 weeks. These 4 stages, in order, are characterized by (a) formation of the seed with distinct embryonic axis and cotyledons, (b) rapid increase in seed size due to synthesis of food reserves, (c) decline in seed moisture and a small increase in dry wt, and (d) dry seed in storage. Healthy fully-expanded leaves from the same plants were assayed for chlorophylls and carotenes for comparison with seeds. Chlorophyll from lima bean leaves also was used as a substrate for chlorophyllase activity of seeds.

Seeds of commercial cultivars (Table 4) were produced in California during the summer of 1969 and obtained from Bolgiano and Co., Washington, D. C. Seeds from cultivars which were introduced from other countries and referred to as "Plant Introductions" (Table 5) were kindly supplied by Dr. Sam Dietz, W-6 Plant Introduction Station, Pullman, Washington.

Extraction of pigments. Chlorophylls a and b were extracted, separated by column chromatography, and measured by the method of Sweeney and Martin (15). Total carotene was determined by the procedure of Moore and Ely (10) as modified by Wall and Kelley (16). Crystalline β-carotene from carrots (Sigma Chemicals Co., St. Louis, Mo.) was used as a standard. Five g of seeds were used per extraction. Extraction and separation of pigments were carried out under dim light and at 10°C. Moisture contents were determined at each stage of development (3).

Preparing and assaying chlorophyllase. In preliminary experiments, relative distribution of chlorophyllase activity among subcellular fractions was determined in 4 fractions produced by different centrifugal speeds. Hundred-g samples of fresh beans (5 weeks after pod formation) were washed several times in cold distilled water, ground in a mortar in 100 ml of 5 x 10^{-3}M Tris buffer, pH 8.2, and centrifuged for 10 min at 1000 *g*. The precipitate was ground once more in 50 ml of Tris buffer, centrifuged as before, and the 2 supernatants combined. The precipitate is referred to as "cell-wall fraction." The supernatant was centrifuged for 20 min at 10,000 *g* and the resulting pellet is referred to as 'mitochondrial fraction'. The supernatant was then centrifuged for 70 min at 105,000 *g* in a Spinco ultracentrifuge. The precipitate which appeared dark green and contained most of the chloroplast fragments and the ribosomes is referred to as 'ribosomal plus chloroplast fragments fraction'. The high-speed supernatant is referred to as the 'particle-free fraction'. Each of the 3 particulate fractions was suspended in cold acetone to remove the pigments, centrifuged, then

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resuspended in a small volume of 5 10^{-3}M Tris buffer, pH 8.2, before it was used in reactions for determining chlorophyllase activity. Pigment was not removed from the particle-free fraction before assaying for chlorophyllase activity. All steps were carried out at 4°C.

In subsequent experiments, which were designed to determine changes in chlorophyllase activity during seed development and maturation and which might contribute to seed bleaching, only one fraction which precipitated between 1,000 *g* and 105,000 *g* was used. This fraction, which contained almost 97% of the total activity and was equivalent to the mitochondrial and ribosomal fractions in Table 2, was taken to represent chlorophyllase activity in the sample.

Chlorophyllase was assayed and chlorophyllides were separated from non-used chlorophylls following the method described by Rhodes and Wooltorton (12). The reaction mixture contained 1.5 ml of the enzyme preparation which precipitated between 1,000 *g* and 105,000 *g* and 1.5 ml of crude chlorophyll freshly prepared from fully-expanded lima bean leaves and suspended in acetone to give 1.5 mg chlorophyll in 3 ml of final reaction. The reaction mixture had a final pH of 8.2. The reactions proceeded for 2 to 3 hr in the dark in stoppered 25-ml Erlenmeyer flasks covered with aluminum foil and shaken in a water-bath at 25°C. Reactions containing boiled enzymes were conducted simultaneously as controls. The rate of conversion of chlorophylls into chlorophyllides reflected the activity of chlorophyllase.

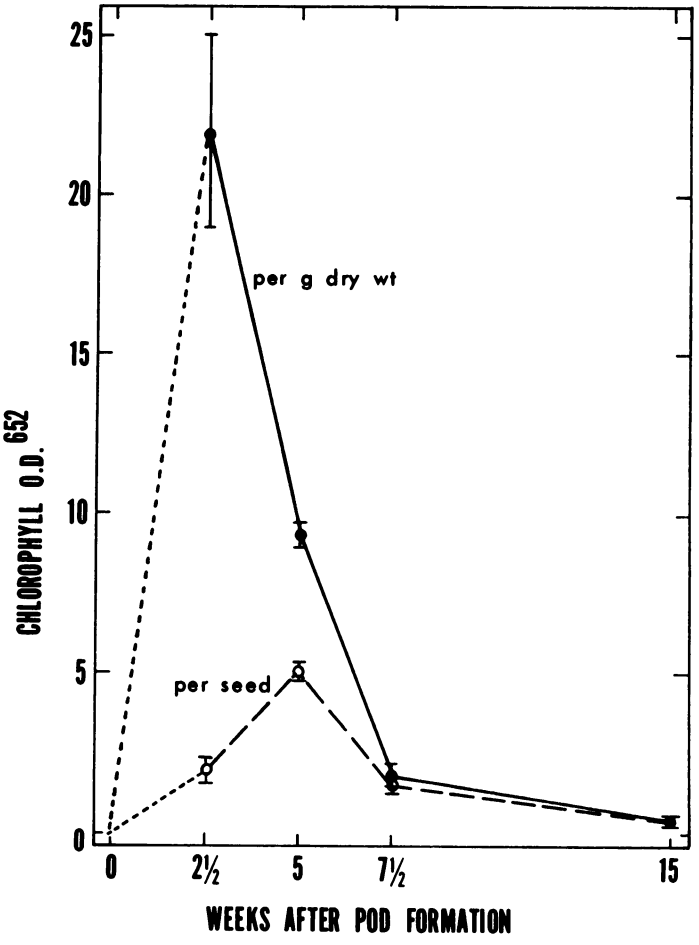

Fig. 1. Chlorophyll content of developing and mature dry lima bean seeds. Vertical bars represent standard errors of the means.

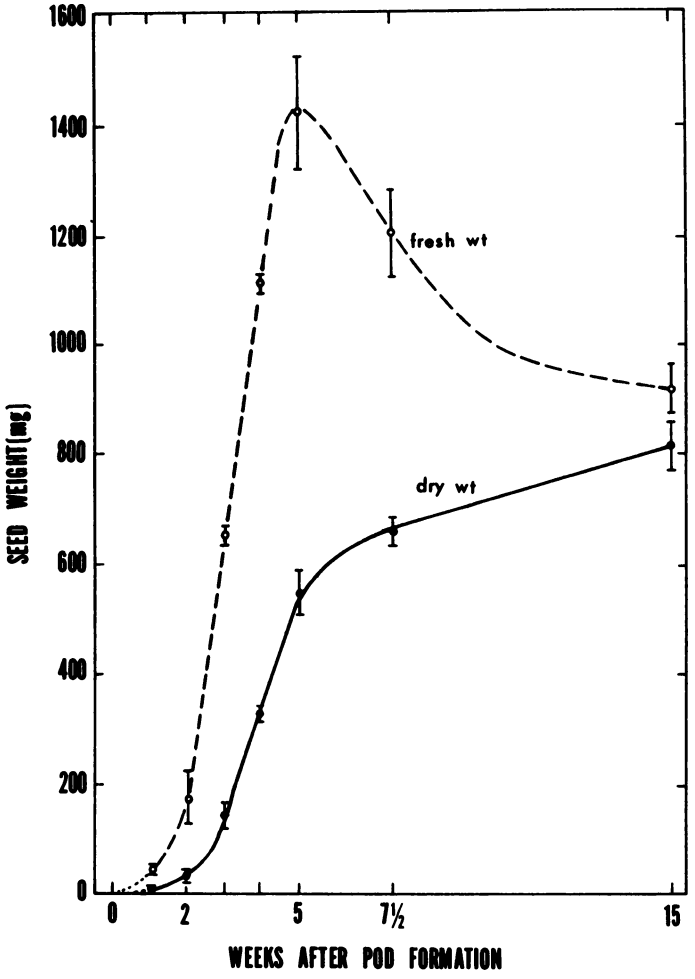

Fig. 2. Changes in fresh and dry wt of lima bean seeds during development and maturation. Vertical bars represent standard errors of the means.

Results

Data on total chlorophyll of developing and mature seeds of 'Green Fordhook 861' (Fig. 1) indicate that chlorophyll content per g dry wt is highest in the developing seed and declines with maturation. On the other hand, clorophyll per seed is maximum 5 weeks after pod formation–the stage at which the seed reaches maximum fresh wt, the prime state for use as fresh beans (Fig. 2). Loss of chlorophyll per seed is most rapid between 4 and 7½ weeks after pod formation. During this period seed moisture decreases rapidly whereas dry wt increases slightly. Loss of chlorophyll from dry seeds, stored in light after harvest, takes place at a much slower rate.

Quantitative measurements were made on chlorophylls a, b and carotenes in seeds at 2 stages; (a) 5 weeks after pod formation when pigment per seed was found to be maximum and (b) dry seeds after harvest (Table 1). Dry seeds, a mixture of bleached and non-bleached, were hand sorted into green and bleached (1) and each color-type was analyzed separately to determine the relative loss of each kind of pigment when seeds bleach. Fully-expanded green leaves from the same plants were also analyzed to show their relative contents of these pigments in comparison to seeds.

Leaves contained approximately 600 times as much chlorophyll and 100 times as much carotene per unit dry wt as developing seeds 5 weeks after pod formation (Table 1). As seeds matured and lost moisture, chlorophyll and carotene contents declined sharply. Because chlorophyll a is destroyed more rapidly than b the a/b ratio, which in green seeds (both

Table 1. Chlorophylls and carotene contents of leaves and seeds of lima bean plants of 'G 2' and 'Green Fordhook 861'.

Cultivar	Tissue and maturity stage	Chlorophylls			Chlorophyll a/b ratio	carotenes	Carotenes/ chlorophylls
		a	b	a + b			
		μg/g dry wt[x]				μg/g dry wt[x]	
G 2	Leaves; fully-expanded; green	5814 ± 190	1909 ± 86	7723 ± 272	3.1	486 ± 34	0.063
	Seeds; mature green	93.3 ± 5.7	44.0 ± 2.8	137.3 ± 24.4	2.1	5.0 ± 1.1	0.036
	Seeds; dry; non-bleached	3.73 ± 0.7	1.55 ± 0.3	5.28 ± 1.3	2.4	0.27 ± 0.02	0.051
	Seeds; dry; bleached	0.08 ± 0.02	0.10 ± 0.19	0.18 ± 0.04	0.8	0.06 ± 0.001	0.333
Green Fordhook 861	Leaves; fully-expanded; green	6181 ± 114	2360 ± 45	8541 ± 42	2.6	526 ± 32	0.062
	Seeds; mature green	86.6 ± 2.9	37.0 ± 1.6	123.6 ± 4.4	2.3	3.6 ± 0.3	0.029
	Seeds; dry; non-bleached	2.05 ± 0.28	1.13 ± 0.05	3.18 ± 0.03	1.8	0.23 ± 0.01	0.072
	Seeds; dry; bleached	Trace	Trace	Trace	---	0.062 ± 0.01	-----

[x]Each figure represents the mean and standard error of the mean of 3 to 5 experiments, one replicate in each.

fresh and dry) was about 2:1, fell to less than 1.0 in sunbleached seeds. Likewise, carotene content of dry mature seeds fell, though more slowly than chlorophyll a or b.

Table 2. Distribution of chlorophyllase activity among sub-cellular fractions of lima bean seeds of 'Green Fordhook 861'.

Fractions	Chlorophyllase activity O. D.^{663}/100 g dry wt[x]	% of total activity
Cell wall	48 ± 5	2.7
Mitochondria	342 ± 40	19.0
Ribosomes + chloroplasts fragments	1415 ± 43	78.3
Particle-free	none	-

[x] Each figure represents the mean and standard error of the mean of 3 experiments, one replicate in each. Reaction time is 2 hr.

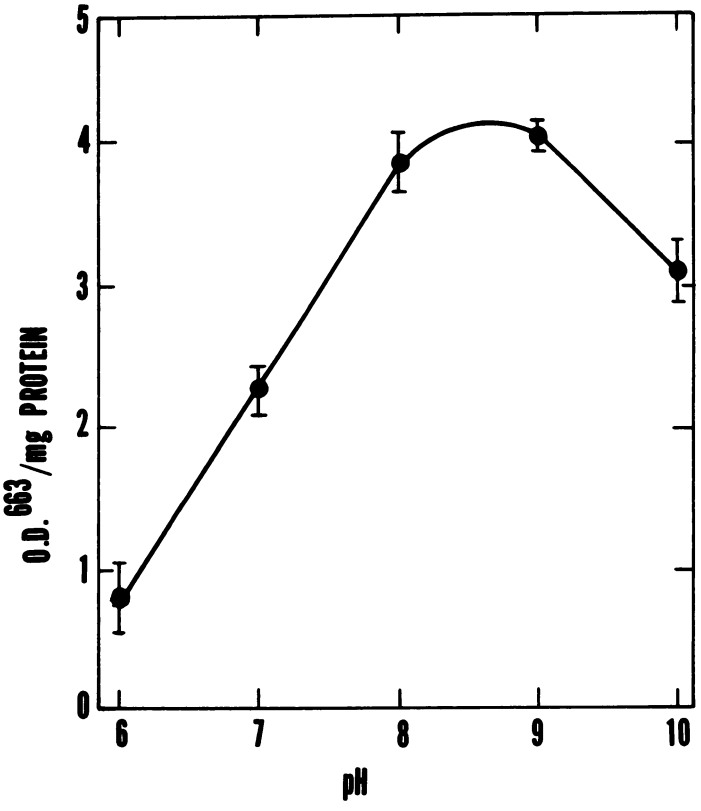

Fig. 3. Dependence of chlorophyllase activity on pH. Vertical bars represent standard errors of the means.

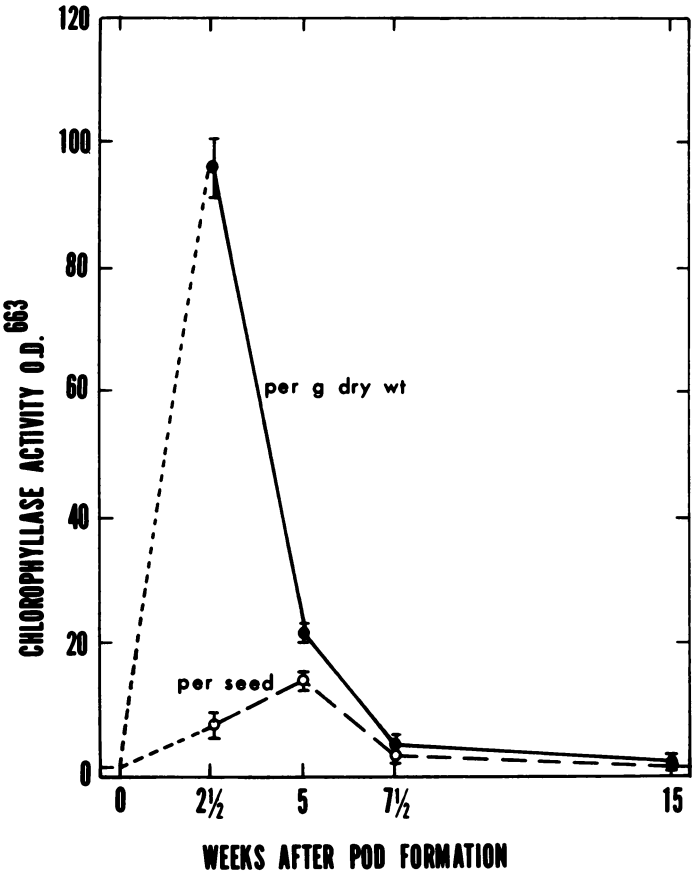

Fig. 4. Chlorophyllase activity of developing and mature dry lima bean seeds of 'Green Fordhook 861'. Vertical bars represent standard errors of the means.

Table 2 indicates that chlorophyllase is a particulate enzyme since the major activity appeared in the ribosomal fraction which contained most of the chloroplast fragments. The optimum pH is about 8.5 (Fig. 3). Furthermore, the activity of chlorophyllase, whether expressed per g dry wt or per seed, was highest during the stage when chlorophyll content was high and was low during the later stage when bleaching was most rapid (Figs. 1, 4).

Quantitative determination of carotenes in dry seeds from 10 commercial cultivars which produce green or genetically white seeds (Table 4) indicated that seeds from these cultivars are low in carotenes. Similar determinations were made on seeds from more than 100 "Plant Introductions." Several of these "Introductions" were found to contain 10 to 20 times as much carotene per unit dry wt as some commercial cultivars (Table 5

Table 3. Chlorophyllase activity in mature green and dry lima bean seeds of 'G 2' and 'Green Fordhook 861'.

Cultivar	Weeks after pod formation	Maturity stage and seed color	Chlorophyllase activity O.D.^{663}/100 g dry wt[x]
G 2	5	mature green	133.5 ± 10.8
	15	dry, non-bleached	62.7 ± 8.3
	15	dry, bleached	45.9 ± 3.4
Green Fordhook 861	5	mature green	434.1 ± 3.3
	15	dry, non-bleached	34.2 ± 1.1
	15	dry, bleached	33.8 ± 1.4

[x]See Table 2.

Table 4. Total carotenes in dry lima bean seeds of commercial cultivars.

Cultivar	Plant growth type	Color of cotyledons[x]	Total carotenes μg/10 g dry seed[y]
G 2	bush	green	2.43 ± 0.30
Fordhook 242	bush	white	1.68 ± 0.28
Green Fordhook 861	bush	green	1.41 ± 0.06
Enormous	bush	white	1.20 ± 0.17
Burpee's Big 6	pole	white	1.15 ± 0.06
Baby Fordhook	bush	white	0.82 ± 0.07
King of the Garden	pole	white	0.81 ± 0.09
Carolina	pole	white	0.53 ± 0.09
Jackson Wonder	bush	white	0.40 ± 0.05
Henderson's	bush	white	0.20 ± 0.02
Fordhook Pole	pole	white	0.17 ± 0.04

[x]White refers to the lack of chlorophyll genetically and does not refer to green seeds that became bleached.
[y]See Table 2.

Table 5. Total carotenes in dry lima bean seeds of "Plant Introductions."

P. I. No.	Original source	Place and year increased	Total carotenes μg/10 g dry seed[x]
257377	Colombia	Hawaii; 1968	18.60 ± 0.26
260417	Bolivia	Bolivia; 1960	12.17 ± 0.79
257412	Argentina	Argentina; 1960	9.58 ± 1.03
249041	Nigeria	Hawaii; 1968	8.37 ± 0.23
256386	El Salvador	Hawaii; 1968	6.97 ± 0.39
241775	Peru	Hawaii; 1968	4.72 ± 0.32
256860	Peru	Hawaii; 1968	4.25 ± 0.14
256911-B	Peru	Hawaii; 1968	4.24 ± 0.55
260415	Bolivia	Bolivia; 1960	4.04 ± 0.06
256873	Peru	Hawaii; 1968	3.86 ± 0.73
256911-A	Peru	Hawaii; 1968	2.63 ± 0.06

[x]See Table 1.

vs 4). No attempt was made to measure chlorophyll in these "Introductions" because the extracts from the mature dry seeds lacked the green color. Whether seeds of these "Introductions" contain chlorophyll during early development is presently under investigation.

Carotene was assayed in seed of 'G 2' and 'Green Fordhook 861' produced in 2 or 3 different locations (Table 6).

Table 6. Total carotenes in dry lima bean seeds of 'G 2' and 'Green Fordhook 861' produced in different locations.

Cultivar	Color of cotyledons	Source	Total carotenes μg/10 g dry seed[x]	
			Exp. 1	Exp. 2
G 2	green	Chili	3.72	3.60
	bleached	Chili	0.27	0.28
	green	California	4.80	5.06
	bleached	California	0.13	0.07
	green	Maryland	2.90	3.19
	bleached	Maryland	0.52	0.63
Green Fordhook 861	green	California	1.40	1.54
	bleached	California	0.15	0.15
	green	Maryland	2.16	2.20
	bleached	Maryland	0.42	0.31

[x]Each figure represents one experiment.

Comparison of location differences with genetic differences suggests that seeds of the same cultivar, produced in different locations, do not differ appreciably in carotene.

Discussion

The evidence shows that when lima bean seeds bleach, their chlorophyll and carotene contents decrease. Based on these data, the possibility that chlorophyll in lima bean seeds is destroyed by chlorophyllase, though not ruled out, is weakened by (a) chlorophyllase activity in seeds is rather low during the stage of rapid bleaching, and high during earlier stages; and (b) chlorophyllase activity in dry bleached seeds is not higher than in non-bleached seeds of the same cultivar. This contrasts to the process of chlorophyll destruction in mature apple fruits where chlorophyllase activity correlated directly with decreased chlorophyll. It is still possible, however, that the breakdown of chlorophylls by chlorophyllase becomes more prominent in a mature tissue, even when the total enzyme activity appears relatively low, in comparison to that found in the same tissue during earlier stages of development. The breakdown of chloroplasts in the mature cotyledons of the seed and the structural changes in the membranes of the chloroplast due to dehydration may make chlorophylls more accessible to chlorophyllase and ultimately increase their breakdown (4,7). Rupture of chloroplasts in pea (4) cotyledons by large starch grains that are formed late in seed development is well documented. In such event, chlorophylls and chlorophyllase, both bound to the chloroplast membranes, would have more access to each other in a disintegrated chloroplast than they would in an intact one.

The excessive bleaching in the field, where pods are exposed to sunlight, suggests the possible role of photooxidation in the loss of chlorophylls. Chlorophyll in mutants of brown bacteria, algae, corn, and sunflower is destroyed by light intensities which stimulate photosynthesis. The sensitivity of these mutants to light has been attributed to the lack of chlorophyll protection by colored carotenes particularly in the blue region of the visible spectrum where carotenes absorb maximally. This protection seems to depend on the concn and type of carotenoid (9). The low carotene content of lima bean seeds supports, but does not confirm, such a hypothesis.

Should carotenes serve, among other things, as protective molecules against photooxidation of chlorophyll, then bleaching could be reduced or even inhibited by increasing the carotene content of green-seeded commercial cultivars. For such a program the PI's which contain 10 times as much carotene as some of the green-seeded cultivars, would provide valuable genetic material. The production of green seeds with high carotene content would provide material for testing the hypothesis that carotenes protect chlorophylls from photooxidation.

Carotenes, as precursors of vitamin A, are important in food. The nutritional implications of high-carotene cultivars of lima beans might overweigh other benefits.

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