THE EFFECTIVENESS OF THE SPECTRUM IN CHLOROPHYLL FORMATION

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PROBLEM

Light is essential for chlorophyll formation in higher plants, and it is likely that this process involves the building up of a light-sensitive precursor in the dark, and the subsequent formation of chlorophyll upon radiation. That such a precursor exists has been demonstrated many times since Pringsheim first recognized it in 1874. He observed an absorption band at 620 to 640 mμ in an extract of etiolated leaves, corresponding to a substance which he named etiolin. Monteverde, in 1893, obtained essentially the same result from a spectroscopic examination of extracts of wheat, maize, and sunflower. He called this red-absorbing pigment protochlorophyll, the name by which it is most commonly known today. Lubimenko (1928) believed protochlorophyll to be a breakdown product of the true chlorophyll precursor, chlorophyllogen, because he was unable to identify protochlorophyll in living plants. Subsequently the protochlorophyll band was observed in living, etiolated leaves of *Zea* plants (Scharfnagel, 1931), establishing it as a natural component of etiolated plants. However, it is not possible to observe directly the conversion of protochlorophyll into chlorophyll in the living plant (Noack and Kiessling, 1929) because the appearance of the strong absorption band of chlorophyll at 620 to 640 mμ would mask the protochlorophyll band in the same place.

Protochlorophyll has not been isolated in pure state, but chemical studies have been conducted on impure extracts of the inner seed coats of the pumpkin seed, which contain a greenish substance with the protochlorophyll absorption bands (Noack and Kiessling, 1929, 1930). This work reveals the presence of a magnesium-rich molecule with porphyrin-like properties. It remained for Fischer, Mittenzwei, and Oestreicher (1939) working on the same experimental material, to identify the molecule as the phyllin of vinylpheoporphyrin a_5 phytyl ester, in other words differing from chlorophyll a only in lacking two hydrogens in the 7, 8 position.

Nevertheless, the main question concerning the pumpkin seed protochlorophyll still remains unanswered: is this substance the true precursor of chlorophyll, or is it a breakdown product of chlorophyll? Both lines of thought can be found in the literature.

The basic fact remains that in etiolated plants there is a substance which

performs the function of precursor to chlorophyll. It would seem highly desirable to obtain an action or effectiveness spectrum of this pigment. The action spectrum is the reciprocal of the amount of incident light necessary to produce a constant physiological effect at various wavelengths, and when properly investigated is closely related to the absorption spectrum of the pigment. In the absence of isolation and synthesis, information about the chemical nature of a pigment can be gleaned from an effectiveness spectrum with the assurance that the substance studied is functioning in the organism. This technique has been used successfully by Hecht (1921, 1922) in the study of photosensitive pigments of *Mya arenaria* and of the human eye, and by Warburg (1927) in identifying the respiratory enzyme in yeast.

PREVIOUS WORK

Attempts to investigate the relative effectiveness of different regions of the spectrum in chlorophyll formation have been made, but because the correct way of studying effectiveness spectra was not properly understood, the results obtained cannot be quantitatively related to the absorption spectrum of protochlorophyll (Sayre, 1928; Rudolph, 1933; Strott, 1938; and Seybold and Egle, 1938).

In each investigation the same inadequacies can be found. Relatively large regions of the spectrum were compared in effectiveness with the result that the data have only rough quantitative significance. This is borne out by the fact that in no case were quantitative curves given, but only verbal expressions of the relative effectiveness of the regions studied.

Virtually every experimenter determined the amount of chlorophyll formed after different exposure times, with the exception of Sayre who studied the time of exposure for the first appearance of chlorophyll. Filter combinations of known transmissions were used to investigate this function for three different wavelength regions, and the light source was placed at varying distances from the experimental plants to correct for the differences in energy of the lamp arising from the use of filters. By plotting the amount of chlorophyll formed against the duration of exposure for the different wavelengths it becomes possible to evaluate the relative effectiveness of the various colors by reading off how much chlorophyll is formed for a given time of exposure. Critical examination of curves obtained in this manner shows that the relative effectiveness differs depending on what time of exposure one chooses. Clearly in order for this evaluation to be meaningful and quantitative it must be true regardless of a particular choice of experimental conditions.

The method of studying effectiveness spectra employed by the previous workers is valid only if the effect (concentration of chlorophyll) is linearly proportional to energy, which is not true in this case nor in any other known physiological process. A saturation curve is typically found for such functions

(*e.g.* Smith, 1938). This being the case, the only other approach to the problem is to study the amount of light necessary to produce a constant physiological effect.

As has been pointed out in the papers of Hecht (1921, 1922, 1924, 1940) the above becomes clear when one examines the meaning of the ratio of absorbed light, I_{abs}, to incident light, I_o. The variation of this ratio with wavelength is essentially a description of the absorbing power of a colored substance or, in other words, of its absorption spectrum. In order to obtain a relative evaluation of this ratio at different wavelengths, one must hold either the numerator (I_{abs}) or the denominator (I_o) fixed and vary the other in a known way. The amount of light absorbed bears a relation to the effect produced; if this relation were linear, one could study the ratio of absorbed to incident light *versus* wavelength by determining the amount of chlorophyll formed (which would be directly proportional to I_{abs}) for a constant incident energy. Since this linear relation does not obtain, the only other method is to vary the I_o for a constant physiological effect. This is in conformity with the photochemical law that a constant absorption (constant number of quanta) corresponds to a constant effect.

Two additional objections can be raised concerning previous work on protochlorophyll effectiveness spectra. One has to do with controlling the duration as well as the intensity of the light exposure. In all the previous work mentioned above, time of exposure was considered equivalent to varying the total energy of the incident light; that is, the assumption was made that intensity times time was constant. It is known and indeed discussed by the same authors that once chlorophyll is formed, it is subject to back reactions which take place in the dark. Thus for a 5 hour exposure one will not get five times as much chlorophyll formed as in a 1 hour exposure, but something less than five. There has been more light but also more time for the back reaction to take place. Since this "dark" destruction of chlorophyll cannot be evaluated, the proper procedure is to eliminate it as a variable by keeping the duration of the exposure constant and varying the intensity.

The fourth objection to the previous work has to do with the problem of screening by other pigments present in the plant. Protochlorophyll is small in concentration as compared with the carotenoids present in etiolated plants, so that in the blue end of the spectrum where the carotenoids absorb heavily one would expect distorted results due to the filtering action of the carotenoids. By introducing an amount of energy of blue light equal to that of red an equivalence is achieved that may be more apparent than real because only a small and indeterminate amount of the incident blue light actually reaches the protochlorophyll molecules. Unless the screening can be evaluated, a meaningful spectrum cannot be attained.

In the experiments to be reported here, it has been possible to show that the

carotenoids do not screen the light from the protochlorophyll molecules. Consequently, a quantitative effectiveness spectrum is described, meeting successfully the shortcomings of the earlier work and providing insight into the chemical nature of protochlorophyll.

Apparatus and Methods

The apparatus is designed to insure uniform and diffuse illumination for each seedling from all sides. In Fig. 1 a diagram of the setup is shown and consists of the following features: The seedlings are arranged around the rim of a beaker 8 cm. in diameter. The beaker is placed on a glass-topped table, and over this is put a

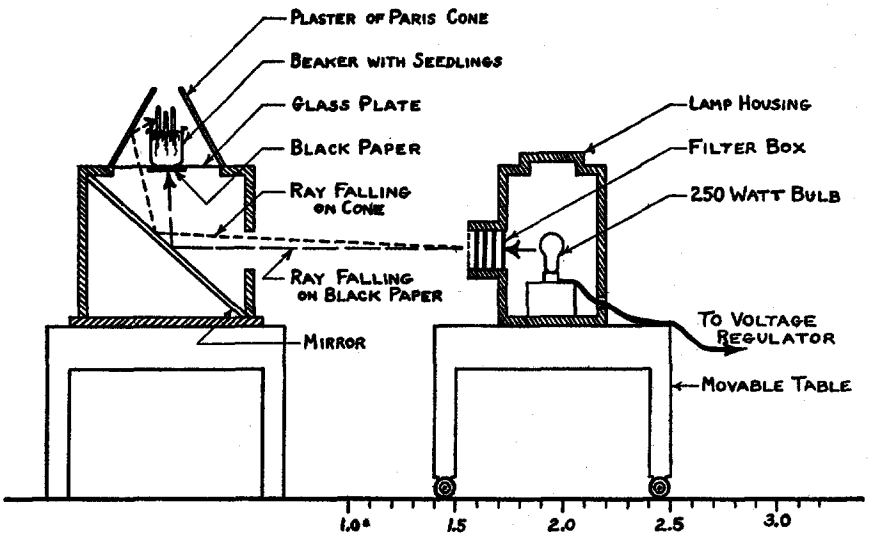

FIG. 1. Diagram of the apparatus used for the light exposure of etiolated *Avena* seedlings.

45° hollow truncated plaster of Paris cone. Below the glass top is a mirror, set at 45°, which reflects upward the light coming from the lamp and filter housing. The glass plate is blackened in the region directly under the beaker so as to exclude any direct light from falling on the plants. This apparatus is similar to that used by Grundfest in his study of the spectral sensibility of the sunfish (Grundfest, 1932).

The housing for the lamp and filters is placed on a movable table so that by varying its distance from the rest of the setup, a variation in the incident energy is achieved. The brightness of the cone surface for different distances of the lamp is obtained with a Macbeth illuminometer. The inverse square law was found to hold, and a calibration curve was constructed relating numbers on the floor and relative energy incident on the plants.

The light source is a 250 watt projection bulb operating through a voltage regulator on 115 volts A.C. This was found to have a color temperature of 2470°K when meas-

ured with an Eastman Kodak color temperature meter, and gave an illumination of 23.66 foot-candles to 0.95 foot-candles depending on its distance from the cone. With the filters in the light beam, the actual illumination used during the investigation is considerably less than this, the filters transmitting only from 1.5 to 60 per cent of the light, the majority of the combinations transmitting 2 or 5 per cent.

Combinations of Corning filters are chosen so that each set transmits only a small spectral region (details in Table V). The transmission properties of each combination

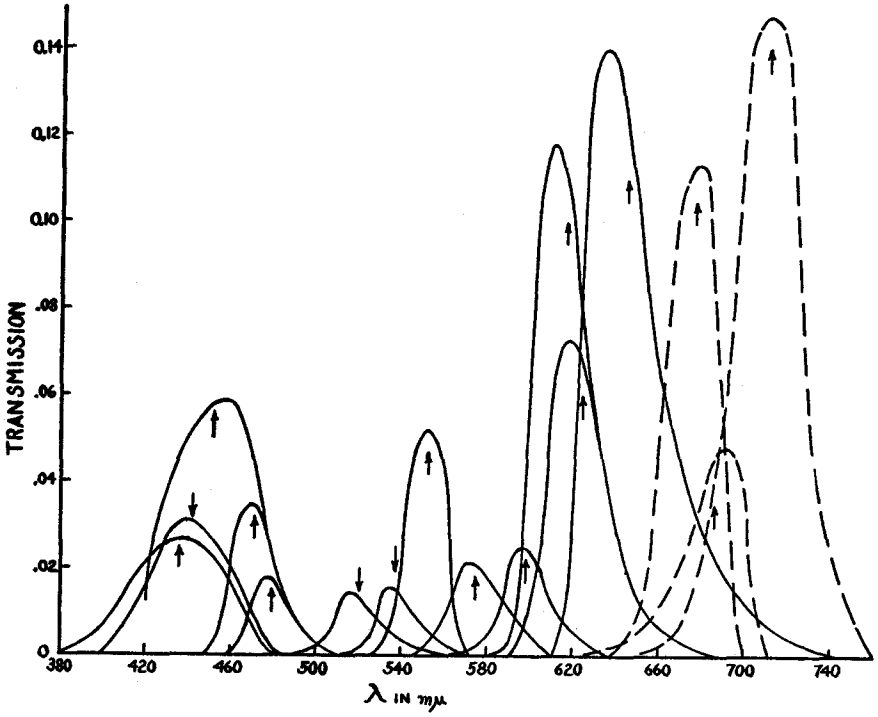

FIG. 2. Relative energy distribution of various Corning filter combinations used with lamp at 2470° K color temperature. Dotted curves are reduced to one-fifth their height (details in Table V).

were measured on the Shlaer photoelectric spectrophotometer (Shlaer, 1938). The visible spectrum was broken up into sixteen overlapping sections each transmitting a range of no more than 40 mμ, with approximately 75 per cent of the transmitted energy falling within a range of 20 mμ. By multiplying the filter transmission values for each wavelength by the relative energy content of the lamp of color temperature 2470°K for the same wavelengths (Smithsonian tables), one obtains curves representing the relative energy distribution for each filter combination. These are plotted in Fig. 2. The center of gravity of each curve is determined by plotting these curves on squared paper and counting squares, and the wavelength corresponding to this

point is used as the dominant wavelength. These are indicated in the figure by arrows on the abscissa. In this way, sixteen points covering the spectrum from 436 mμ to 710 mμ are found, differing from one another by 6 mμ to 41 mμ but averaging a difference of about 20 mμ. In the region of the peaks of absorption of protochlorophyll an attempt is made to get points close together in order to define the maxima with the greatest possible accuracy.

Suitable filter combinations not being available for the investigation of small regions in the far red end of the spectrum, it is possible to study these areas by using cut-off filters. These are filters which transmit sharply and maximally energy beyond a certain point so that at one wavelength the transmission might be zero, 10 mμ from that it might transmit 45 per cent of the light, and 75 per cent of the light might be transmitted at the next 10 mμ increment. By choosing two such cut-off filters which begin to transmit a few millimicra apart, one can evaluate the difference in transmission of the two by plotting the transmission curves on graph paper and counting squares in the area between the two curves. Plotting this difference against wavelength gives a curve of energy distribution similar in shape to that of the other filter combinations. When the effectiveness of these filters in forming chlorophyll is desired, one must determine the effectiveness of each cut-off separately, and subtract the one value from the other. The remainder now become the effectiveness of the difference in relative energy of the two cut-off filters. In Fig. 2 the curves with center of gravity at 676, 686, and 710 mμ were obtained in this manner.

It is fortunate that these points in the red are attainable because of two conditions found there. The first is that there is a great deal of available energy due to the high transmission of the filters and to the energy distribution of the lamp. The second is that these wavelengths are relatively ineffective in chlorophyll formation. So much energy can be introduced that despite the low effectiveness one can work at a level of chlorophyll formation where the precision in the determination of concentration is best. In some of the regions in the green portion of the spectrum, where the effectiveness is also low, the available energy is limited and this cuts down the precision. The maximum amount of chlorophyll formed under these conditions is so small as to be at the limit of detectability.

The areas under the curves in Fig. 2 are determined by plotting the curves on graph paper and counting squares so that the total relative energy of each combination is known. With this information and the calibration curve of brightness *versus* distance of the lamp from the cone surface, it is possible to evaluate the relative energy of a given filter combination when the lamp is placed at any distance from the experimental plants. In actual practice the calibration curve is plotted as the logarithm of relative energy against the distance in meters on the floor. An arbitrary assignment of the yellow-green filter combination is made to this curve, and the ratio of the area of energy distribution of this particular combination to all the others is calculated. The logarithm of this ratio then becomes the distance either up or down the ordinate (which is in log units) that one must shift the yellow-green calibration curve to be able to read off a value of log relative energy for a particular distance for some other dominant wavelength.

Seedlings of *Avena byzantium* var. *sativa* of three ages, 72, 96, and 120 hours after germination are used as experimental material. These were obtained from the

Bureau of Plant Industry, U. S. Department of Agriculture, and are gratefully acknowledged. Plants of 48 hours are too young to use for pigment studies since the primary leaf is practically undeveloped at this time. The seeds are hulled, soaked for 1 hour in distilled water, and about 40 seeds are placed around the rim of beakers and held in place by a layer of moistened filter paper resting on the bottom of the beaker (Kaiser and Albaum (1939)). Distilled water (350 cc.) is placed in the beakers to insure a high relative humidity. The beakers are kept in the dark in an incubator operating at a temperature of 28°C. (± 0.1) until the seedlings reach the proper age for experimentation. The beakers are then placed on the glass-topped table of the apparatus in Fig. 1 and exposed to various lighting treatments for 5 hours. During this exposure time the temperature of the dark room was not controlled but did not vary more than 0.5°. Throughout the investigation, the temperature of the room stayed within a 10° range, from 20–30°, and this variation did not affect the chlorophyll formation insofar as could be detected, since similar determinations made at the extremes of this temperature range gave values of chlorophyll concentration within the experimental error.

After exposure to light, the seedlings are placed in boiling water for 1 minute to kill the plants and to minimize the oxidative destruction of carotenoids (Strain, 1938). In all experiments twenty-five seedlings with the longest coleoptiles are selected for extracting. It was found that the longer the coleoptile, the longer the primary leaf, so by selecting seedlings of maximum coleoptile length, one is assured of getting average or above average lengths of primary leaf. For each of the three ages of plants the average primary leaf length for that particular age was determined. For 72 hour plants 15 mm., for 96 hour plants 25 mm., and for 120 hour plants 30 mm. were chosen as the lengths the coleoptile sections were to be cut.

The coleoptiles selected for their long length are cut to this size before extracting, care being taken to obtain a section containing primary leaf throughout. The cuts are made with a razor blade by placing the coleoptile on a white card with parallel lines marked out the proper distance apart. In this manner the maximum length of primary leaf was extracted at each age, which is desirable because of the low concentration of chlorophyll dealt with in these experiments.

When the occasion arose to compare the amount of pigment formed for different ages of plants, a correction was made to account for the differences in the quantity of primary leaf material used at each age. In order to do this, measurements of the total area of primary leaf were made for each aged plant, and the pigment concentrations were expressed for a unit square millimeter area of leaf. The flat surface of the unrolled leaf was measured under a microscope fitted with an optical micrometer device.

The precision of cutting the coleoptiles to the proper length was excellent due to the fact that this operation was conducted under a green Corning safelight. This was chosen because it transmits energy in a region where the human eye is maximally sensitive, but one which is relatively ineffective in chlorophyll formation.

The twenty-five cuttings are ground in a mortar containing Berkshire sand and methyl alcohol. The Berkshire sand was specially cleaned by excess washing in tap water, distilled water, and methyl alcohol to prevent any foreign coloration of the extract. In each extract 8 gm. of sand were used and 20 cc. of Eimer and Amend

C.P. methyl alcohol as the extractive since a comparatively large volume tends to prevent destruction of the pigments (Strain, 1938).

The grinding of the plant sections is timed carefully with a stop-watch. Two minutes of vigorous grinding is done at the beginning of a 1 hour period of extraction at room temperature (20–30° C.) and one-half minute at the end of that time. The primary leaves always appear colorless after this treatment, indicating complete extraction. Whenever light was necessary the green safelight was used.

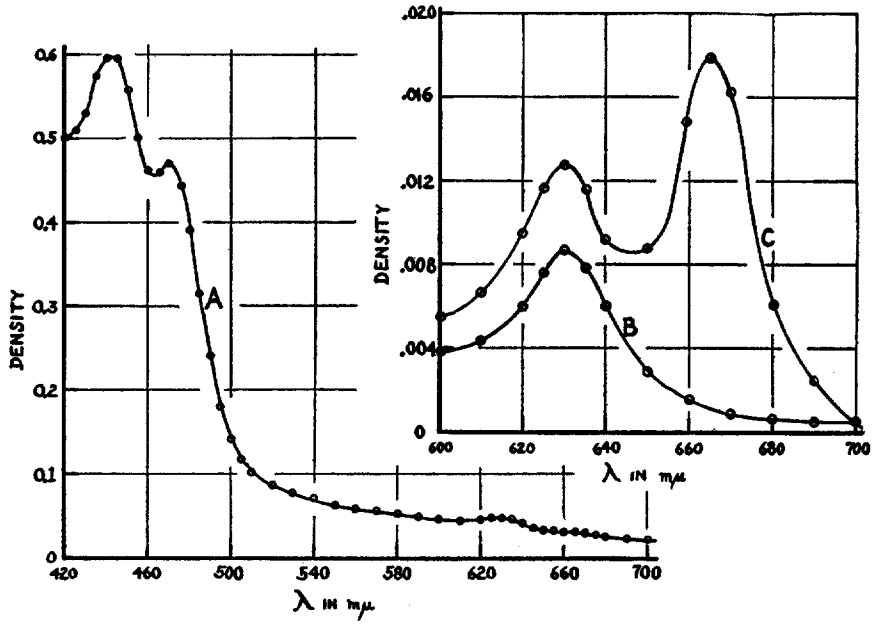

FIG. 3. Absorption spectra of methyl alcohol extracts of total plants: Curve A, in etiolated plants, showing the carotenoid peaks at 440 and 470 mμ; Curve B, red-absorbing portion of curve A on an enlarged scale to show protochlorophyll absorption peak at 630 mμ after correcting for the absorption of the solvent; Curve C, in light-treated plants, showing the chlorophyll peak at 665 mμ.

The extract is filtered with suction, centrifuged for 1 hour at 6°C., and the clear greenish-yellow methyl alcohol solution brought up to constant volume before measuring. This is 15 cc. for 72 hour plant extracts, and 25 cc. for 96 and 120 hour plant extracts, the choice being made in order to work with convenient densities. Here again when it is desired to compare chlorophyll formation for the three aged plants, the chlorophyll values are corrected for this dilution effect.

Immediately afterward the solution is measured spectrophotometrically. It is necessary to time the whole procedure carefully since the pigments are unstable in light and air. Absorption cells 20 mm. in thickness are used with stoppers to prevent evaporation during measurement. The photometric density of the solutions is

measured in the Shlaer photoelectric spectrophotometer, which is especially designed for photosensitive solutions and has a precision of 1/10 of 1 per cent at the region used in these determinations. In this instrument the light passes through two monochromators before being incident on the solution, and the light is of very low intensity so as not to bleach the solution being measured.

Density is equal to $\log(I_o/I_{tr})$, where I_o is the incident light and I_{tr} is the transmitted light. Since density is proportional to concentration as given by Lambert and Beer's law (Hardy and Perrin, 1932) the density of each pigment was used as a measure of its concentration. In Fig. 3 a typical absorption curve is shown for chlorophyll, protochlorophyll, and carotenoid in extracts of total pigment, showing the density of solution at each wavelength.

In order to evaluate the density of each pigment it is necessary to subtract the absorption of the solvent from that of the solution. Having done this the density of the pigment at its characteristic peak is taken as a measure of its concentration. Thus for chlorophyll, the density at 665 mμ is chosen; for protochlorophyll (extract of etiolated plants) the density at 630 mμ is taken; and for carotenoids (extract of etiolated plants) the density at 470 mμ is chosen. The characteristic 440 mμ peak was avoided because the precision of the spectrophotometer is not as good here as at 470 mμ. As the investigation proceeded it was found unnecessary to obtain a whole curve of each extract, measuring the density values at 470, 630, and 665 mμ being adequate, as well as that at 700mμ, the density of the solvent alone.

The Screening of Carotenoids

Before an effectiveness spectrum of protochlorophyll can be studied it is necessary to determine whether the carotenoids act as screens in the blue region of the spectrum where they absorb heavily. To test this, the following experimental plan was devised: first, to evaluate the increase in carotenoid concentration with age; and second, to test the relative effectiveness of blue light and of red light under identical experimental conditions for the three ages of plants. If the concentration of carotenoid increased with age and acted as a screen, one would expect the effectiveness of blue light to drop with increasing age but not that of red. In other words, if the ratio of the effectiveness of blue to red would be found to fall with age, the carotenoids would be acting as screens; if the ratio remained constant, there would be no screening effect.

Measurements on etiolated *Avena* seedlings of three ages (72, 96, and 120 hours after germination) were made to determine the increase in pigment concentration with age. The results are given in Table I. Column 2 gives a sample of the data obtained under the designated experimental conditions, showing the kind of variability between determinations. The data are in density of the primary leaf and equal log (I_o/I_{tr}). In order to make the density values of the pigments at the three ages comparable three corrections were made: one to account for the difference in area of primary leaf used in the extracts; a second to correct for the fact that the extracts were diluted to a

different extent before measuring; and a third to account for the fact that the solutions were viewed through 20 mm. absorption cells. Column 3 thus represents values of pigment density of a primary leaf.

It can be seen that the concentration of carotenoid pigment increases significantly with age, but that the increase in protochlorophyll concentration is small and probably not significant. (That carotenoid pigment increases in the leaf has been reported by Rudolph, 1933.) In the 48 hour period from 72 to 120 hours the density of carotenoid has practically doubled.

Having established that the carotenoid concentration increases significantly with age, the question now becomes: does this increased concentration cause

TABLE I

The Change in Pigment Concentration in Etiolated Seedlings of 72, 96, and 120 Hours after Germination, Expressed As Pigment Density of Primary Leaf

Carotenoid pigment			Protochlorophyll pigment		
Age	Density of pigment	Average photometric density of primary leaf	Age	Density of pigment	Average photometric density of primary leaf
hrs.			*hrs.*		
72	0.1288		72	0.0078	
	0.1244			0.0110	
	0.1972			0.0078	
	0.1544			0.0082	
	0.1816				
	0.1573	0.2160		0.0087	0.0119
96		0.2760	96		0.0114
120		0.4200	120		0.0154

a decreased ratio of effectiveness of blue light to red light in chlorophyll formation? Determinations were next made of the amount of chlorophyll formed by plants when subjected to various amounts of light energy for a constant duration. For the three ages of plants, at least four determinations were made of the concentration of chlorophyll formed after 5 hours' exposure to each of four light energies. This procedure was carried out in a red light with dominant wavelength at 645 mμ as well as in a blue light with dominant wavelength at 442 mμ. The results of the experiment in red light are given in Table II and plotted in Fig. 4, while those for blue light are found in Table III and plotted in Fig. 5. In each table sample data are included to show the kind of variability obtained.

In the tables chlorophyll concentration is expressed as the average chlorophyll photometric density of the primary leaf. This value is plotted against the logarithm of relative energy in the figures.

TABLE II

Chlorophyll Formation for Different Relative Energies after 5 Hours' Exposure to Red Light (Dominant Wavelength 645 mμ) for 72, 96, and 120 Hour Seedlings

Chlorophyll pigment formed in red light			
Age	Log relative energy	Pigment density	Average density of the primary leaf
hrs.			
72	1.37	0.0242	
		0.0305	
		0.0330	
		0.0274	
		0.0288	0.0396
	1.05		0.0192
	0.78		0.0121
	0.54		0.0038
96	1.37		0.0456
	1.05		0.0256
	0.78		0.0144
	0.54		0.0080
120	1.37		0.0460
	1.05		0.0318
	0.78		0.0190
	0.54		0.0098

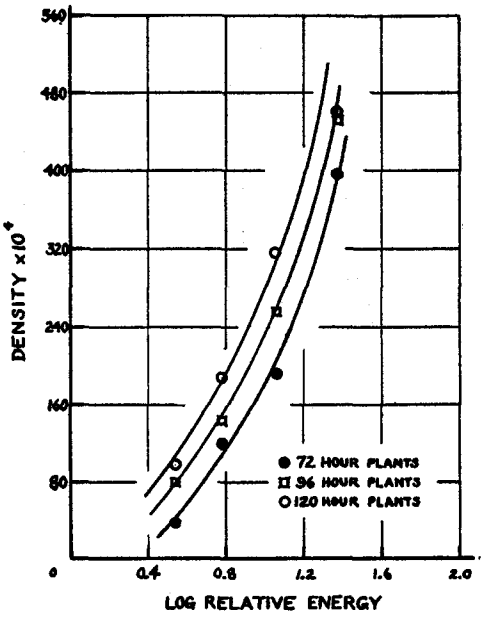

FIG. 4. The chlorophyll density of a primary leaf of three ages of *Avena* seedlings formed after 5 hours' exposure to various energies of red light (dominant wavelength 645 mμ).

In order to evaluate the ratio of effectiveness of blue light to red for each age, values for density of chlorophyll at particular values of log relative energy are read off the corresponding curves in Figs. 4 and 5 and the ratios calculated. These values are listed in Table IV. It will be noted that the ratios turn out to be nearly unity; *i.e.*, that blue light and red light are equally effective in forming chlorophyll. This result was obtained purely by chance. A choice of another red or blue would have given different results as will be

TABLE III

Chlorophyll Formation for Different Relative Energies after 5 Hours' Exposure to Blue Light (Dominant Wavelength 442 mμ) for 72, 96, and 120 Hour Seedlings

	Chlorophyll pigment formed in blue light		
Age	Log relative energy	Pigment density	Average density of the primary leaf
hrs.			
72	1.37	0.0218	
		0.0274	
		0.0366	
		0.0326	
		0.0278	
		0.0292	0.0401
	1.10		0.0222
	0.87		0.0135
	0.40		0.0036
96	1.37		0.0512
	1.10		0.0293
	0.87		0.0184
	0.40		0.0072
120	1.37		0.0494
	1.10		0.0362
	0.87		0.0205
	0.40		0.0062

seen when the effectiveness spectrum is discussed. *The important thing to observe is that the ratio of effectiveness does not change significantly with age.* In spite of a doubling in carotenoid concentration from 72 to 120 hours, the ability of blue light to form chlorophyll is as unimpaired as that of red light for that period. This demonstrates unequivocally that the carotenoids do not act as filters under this particular set of experimental conditions. From a morphological point of view this leads to the unexpected inference that the carotenoids are located behind the protochlorophyll molecules in the plastids.

In Table IV a calculation is made of what would be the expected ratio at

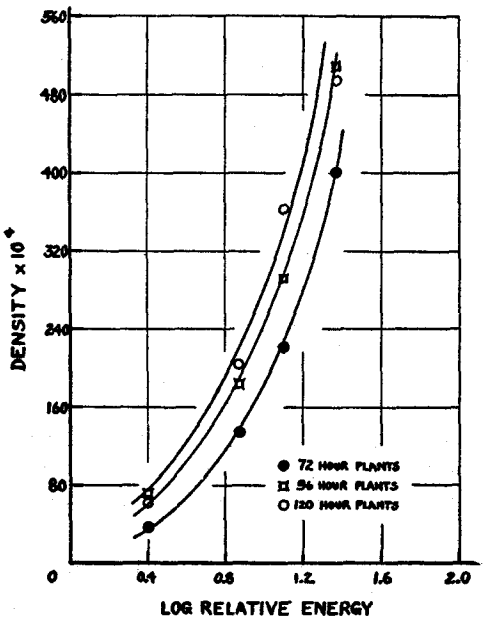

FIG. 5. The chlorophyll density of a primary leaf of three ages of *Avena* seedlings formed after 5 hours' exposure to various relative energies of blue light (dominant wavelength 442 mμ).

TABLE IV

The Ratio of Effectiveness of Blue to Red Light in Forming Chlorophyll at 72, 96, and 120 Hours after Germination

Age	Log relative energy	Ratio of effectiveness of blue to red (experimental)	Ratio of effectiveness of blue to red (expected if carotenoid acted as screens)
hrs.			
72	0.6	1.250	
	0.8	1.050	
	1.0	0.967	
	1.2	1.010	
	1.4	0.995	
Average		1.05	
96	0.6	1.349	1.13
	0.8	1.130	1.08
	1.0	1.080	1.01
	1.2	1.070	1.02
	1.4	1.050	1.02
Average		1.14	1.05
120	0.6	1.01	0.82
	0.8	1.01	0.85
	1.0	1.01	0.86
	1.2	1.02	0.88
	1.4	1.03	0.94
Average		1.02	0.87

the different ages if the carotenoids had acted as filters. This calculation is accomplished in the following way: The assumption is made that there is a random distribution of carotenoid and protochlorophyll molecules in the plastids. One-half the increment in carotenoid density from 72 hours to 96, and from 72 to 120 hours is thus considered the density of a "filter" placed in the light path, because if the carotenoid molecules were layered in front of the protochlorophyll molecules, all the density increment would filter. If layered behind, none of the increment would filter. A random distribution represents a condition in between these two extremes, or one-half the increment. Consequently, the 96 hour curve in Fig. 5 is shifted toward the right 0.03 log unit, and the 120 hour curve shifted 0.102 log unit. As before, the values of density for particular log relative energies are now read off these shifted curves, and the ratio of these values to the corresponding ones for red light are calculated. The ratio of blue to red falls with age significantly as can be seen in Table IV, column 4. This calculation shows that the experimental precision here is adequate to detect the carotenoid screening effect, had it been present.

The Effectiveness Spectrum

A proper effectiveness spectrum must record the reciprocal of the relative energy required in different parts of the spectrum to produce the same physiological effect. This energy value can be calculated rather than directly determined providing certain facts are known. First, the relation between chlorophyll formation and relative energy must be known for at least two different wavelengths, because if the curves for any two wavelengths are well defined and are parallel, it is safe to assume that all wavelengths would give parallel curves. And second, the amount of chlorophyll formed for any particular relative energy must be determined for each wavelength region one wishes to study.

By placing both of these data on the same plot, that is the curves of λ_1 and λ_2 and single determinations of chlorophyll formation for λ_3, λ_4, etc. one can construct an effectiveness spectrum. By shifting the curve of λ_1 along the abscissa (log relative energy) until it superimposes on a single experimental point of λ_3, one can read from the λ_1 curve that value of log relative energy for λ_3 corresponding to a desired amount of chlorophyll (the ordinate). This saves the work of determining complete curves of chlorophyll formation against log relative energy for each wavelength.

In the experiments reported here only 96 hour plants were used. Consequently, the 96 hour curves of Figs. 4 and 5 were chosen for calculating the effectiveness spectrum. In Table V are found the determinations of chlorophyll concentration for a convenient log relative energy for sixteen filter combinations. Using these data and the curves in Figs. 4 and 5 in the manner described above, the relative energy necessary to form a chlorophyll density

of 0.0280 was found. In Table VI these reconstructed values and their reciprocals are given along with the dominant wavelengths of each filter combination. These are plotted in Fig. 6. There are three definite peaks of "effectiveness," one in the blue, one in the yellow, and one in the red. A suggestion of a peak is present at 545 mμ and it is drawn as such, because reports on the absorption bands of protochlorophyll in the living leaf viewed spectroscopically (Scharfnagel, 1931) indicate that such a peak is present. There is little other justification for drawing it this way, however, since the

TABLE V

Chlorophyll Formation for Various Corning Filter Combinations

Dominant wavelength	Corning filter combinations	Log relative energy	Chlorophyll density
mμ			
436	306 + 511	1.43	0.0356
442	038 + 511	1.37	0.0428
452	038 + 554 2 std.*	1.23	0.0265
471	368 1/2 std. + 554 2 std.	1.29	0.0113
480	368 + 554 2 std.	0.96	0.0043
520	503 1/2 std. + 352 + 430	0.90	0.0028
537	348 + 503	0.87	0.0042
552	512 + 350 + 430	1.37	0.0164
575	348 + 430	1.14	0.0159
599	246 1/2 std. + 978 2 std.	1.12	0.0103
615	246 + 978	1.31	0.0280
624	244 + 978	1.11	0.0212
645	243 + 978 1/2 std.	1.37	0.0385
676	Δ(244 + 555) − (241 + 241 + 241)	1.22	0.0222
686	Δ(241 + 555 1/2 std.) −(244 + 555)	1.61	0.0153
710	Δ(244 + 585) − (244 +555)	2.20	0.0040

*Standard thickness.

precision of the points in this spectral region is extremely poor. This is due to the small amount of energy available here.

The curve in Fig. 6 may be considered a first approximation effectiveness curve because two refinements can be made. The first limitation arises because of the use of filters and the approximation introduced by using a dominant wavelength for each filter combination. It will be recalled that each combination transmits over a range of 40 mμ, although most of the light is contributed by a 20 mμ portion in the center. When the reciprocal of the relative energy is plotted against wavelength in the effectiveness curve, the points on the wavelength scale are placed at the dominant wavelength of the energy distribution curves in Fig. 2. This gives equal weight to all the energy under each curve as far as effectiveness in chlorophyll formation is concerned. When an effectiveness curve obtained with filters contains steep peaks as the

TABLE VI

Reconstructed Values o Log Relative Energy for a Constant Amount of Chlorophyll Formed for Various Corning Filter Combinations

Dominant wavelength	Log relative energy	Relative energy	Reciprocal of relative energy
mμ			
436	1.30	19.95	0.0301
442	1.19	15.49	0.0646
452	1.26	18.20	0.0550
471	1.76	57.60	0.0174
480	1.78	60.30	0.0166
520	1.82	66.10	0.0151
537	1.69	49.00	0.0204
552	1.66	45.70	0.0219
575	1.44	27.60	0.0362
599	1.62	41.70	0.0240
615	1.31	20.40	0.0490
624	1.27	18.60	0.0538
645	1.19	15.49	0.0646
676	1.36	22.90	0.0437
686	1.93	85.10	0.0118
710	3.05	1122.00	0.00089

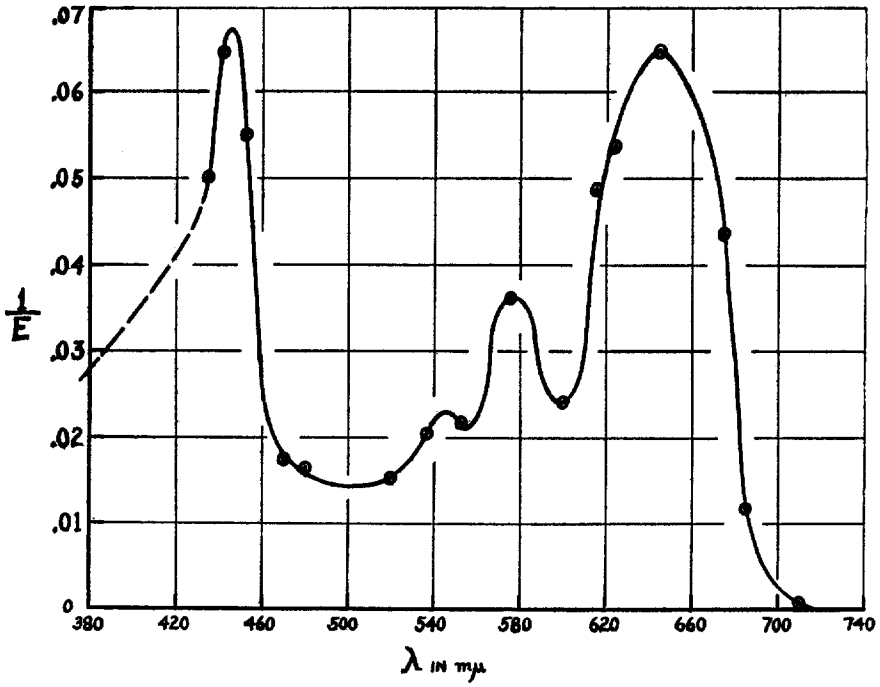

FIG. 6. First approximation effectiveness spectrum of protochlorophyll. The reciprocal of the relative amount of energy required to form a constant amount of chlorophyll is plotted against wavelength.

protochlorophyll curve does, such an approximation introduces a considerable error. The energy on one side of the center of gravity of the filter may be responsible for most of the chlorophyll formed, the other half contributing only an insignificant amount. This necessitates the calculation of a new dominant wavelength. By multiplying the reciprocal of the relative energy from the effectiveness curve at any wavelength by the energy transmitted at that particular wavelength by the filters, a new factor can be obtained for that

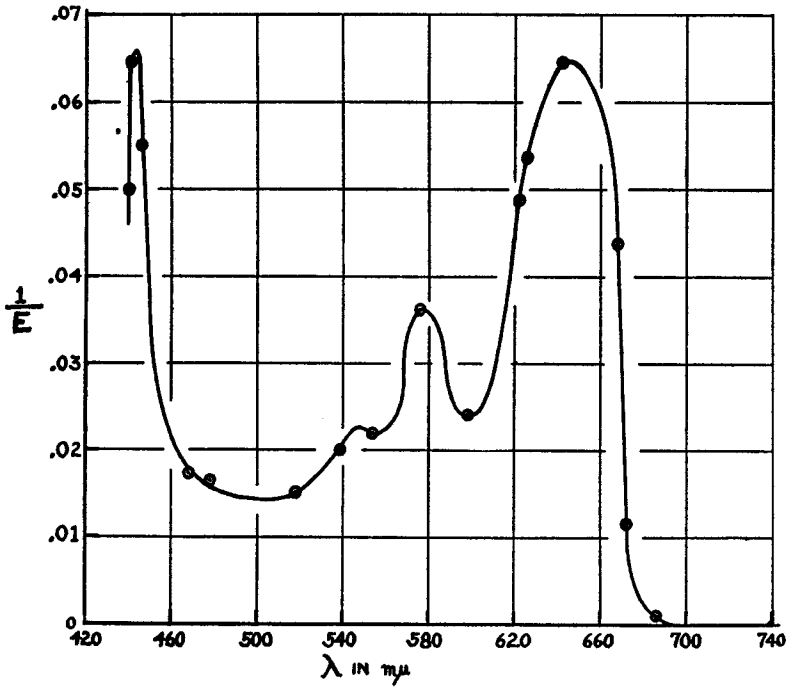

FIG. 7. Second approximation effectiveness spectrum of protochlorophyll, corrected for the error in estimating the dominant wavelength of the filter combinations.

wavelength. In this way new curves of effective energy distribution against wavelength are constructed for each filter combination and new centers of gravity determined corresponding to the dominant wavelengths. In order to apply this correction for the filters transmitting in the blue it was necessary to extrapolate the effectiveness curve beyond 440 mμ where there are no experimentally determined points. The curve was extended into the blue in conformity with what is known of the way most porphyrins absorb there. The extrapolation is indicated as a dotted line in Fig. 6. The values of dominant wavelengths are the corrected abscissa points in the second approximation effectiveness curve plotted in Fig. 7. What has been accomplished is essen-

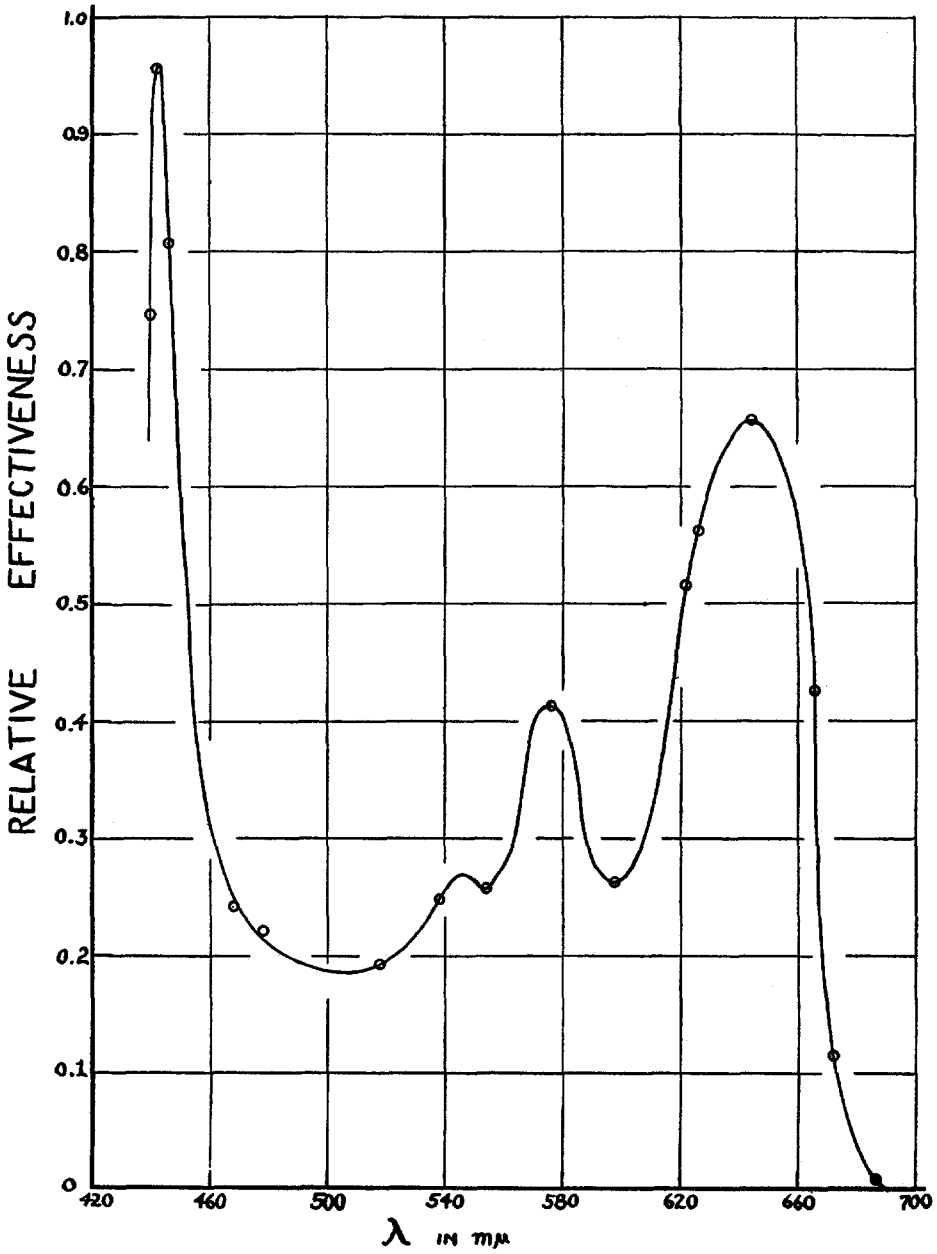

FIG. 8. Effectiveness spectrum of protochlorophyll, corrected for the quantal content of the incident light.

tially a shifting of the points on the slopes further toward the peaks resulting in a steepening of the two main absorption peaks.

A further refinement similar to the quantum correction first made on the luminosity curve for human dim vision by Dartnall and Goodeve (1937) can be applied to this curve. It is well known that light is absorbed in discrete units and that the energy of a quantum of one wavelength is different from that of another. Expressing an effectiveness curve on an energy basis does not take into account this information, since it is the quantal content of the light that one is interested in. A correction of the incident energy to an incident number of quanta can be made by multiplying the reciprocal of the relative energy at a particular wavelength by the energy of the quantum at this wavelength, which is given by Planck's formula. This value is plotted against wavelength and a new effectiveness curve results which is plotted in Fig. 8. What has been altered is the relative height of the blue and red peaks, the blue peak becoming higher than the red.

The major peaks in Fig. 8 are found at 645 mμ, 445 mμ, and two minor ones at 575 and 545 mμ. This effectiveness curve is related to the absorption spectrum of protochlorophyll in the condition found in the living leaf. When the pigment is extracted in organic solvents a shift in the absorption peaks is to be expected, as is the case with chlorophyll. This is borne out by examining the curves of methyl alcohol extracts of *Avena* seedlings shown in Fig. 3. The absorption found in the red portion of the spectrum of an etiolated plant extract in methyl alcohol is presumably due to protochlorophyll. There is a peak of absorption at 630 mμ which undoubtedly corresponds to the 645 mμ peak of the effectiveness curve. The other peaks cannot be detected in Fig. 3 since the absorption of total pigment extracts is recorded here and the carotenoids mask these protochlorophyll peaks.

DISCUSSION

Red light has always been considered most effective in chlorophyll formation, a finding which has been reported repeatedly by previous investigators (Lubimenko, Sayre, Rudolph, Strott, Seybold and Egle). On the whole these studies consisted in comparing the effectiveness of three broad wavelength regions, red, green, and blue. In this paper a more complete study has been presented of the relative effectiveness of various wavelengths in forming chlorophyll, the visible spectrum being divided into sixteen portions. It has been possible to show that both blue light and red light are highly active but blue light of dominant wavelength 440 mμ is most effective in forming chlorophyll, a result not previously reported.

In the introduction to this paper several criticisms of the earlier work have been discussed, any one of which might account for the divergent result obtained here. First, the amount of chlorophyll formed for a constant duration

of light stimulus was compared for different wavelengths in the early work instead of the relative energy necessary for a constant amount of chlorophyll formed. Secondly, the duration of the light was varied as a means of controlling the total relative energy, instead of varying the total energy directly and holding the duration of the stimulus constant. And thirdly, the possible screening effect of the carotenoids in the blue region of the spectrum where they absorb heavily was not evaluated.

There is a fourth reason for the discrepancy in results, however, which offers the most likely explanation. If the curve in Fig. 8 is studied closely one can see that though the blue peak is higher than the red one, it is much narrower. Consequently, if the relative effectiveness of three broad spectral regions *of equal energy* is studied as was done by the previous workers, the result they obtained becomes understandable. The "effective" absorption of these three regions, red, green, and blue can be evaluated by multiplying the absorption spectrum of protochlorophyll by the energy distribution of the filter combinations these workers used. This product will be small for the blue end of the spectrum because the protochlorophyll absorbs only in a narrow spectral region, and largest in the red where the pigment absorption is broad and continuous over a relatively large spectral region. The result of such an investigation would be that red light is most effective in forming chlorophyll and blue light least since this rough method gives the total area of absorption, not the position of the peaks or their relative heights. A more detailed investigation of the effectiveness of small spectral regions as conducted here provides this additional information and shows that red and blue are both effective in chlorophyll formation.

The similarity but non-identity of the protochlorophyll curve to chlorophyll itself is noteworthy. Chlorophyll in aqueous digitonin extract has a peak at 675 mμ and at 445 mμ, with several minor peaks in the red (Smith, 1941). The relative heights of the blue and red peaks in the protochlorophyll spectrum are more similar to chlorophyll a than to chlorophyll b but the position of the bands closely approximates that of chlorophyll b which in ether extract has peaks at 645 and 445 mμ (Zscheile, 1935). The ratio of the blue to red peaks in the effectiveness spectrum of protochlorophyll is 1.47, in the absorption spectrum of chlorophyll a it is 1.35, and in the absorption spectrum of chlorophyll b it is 2.87. Clearly, protochlorophyll is a pigment remarkably similar to the chlorophyll pigments in whose synthesis it is involved.

The real question of whether the protochlorophyll studied here is identical with the substance investigated in the inner seed coats of the pumpkin by Noack and Kiessling and Fischer and his coworkers has yet to be considered. If the identity of the two could be established we would know that protochlorophyll differs from chlorophyll a only in lacking two hydrogens in the 7, 8 position and that the task of the light action in the synthesis of chlorophyll is to hydrogenate the protochlorophyll molecule. Since the pigment

in the pumpkin seeds has never been isolated in pure state, the absorption spectrum of the substance is unknown. Four absorption bands have been observed spectroscopically by Noack and Kiessling which are believed to be characteristic of the molecule obtained from the pumpkin seed. They are, in the order of the intensity of the bands, at 620 to 629 mμ, 560 to 576 mμ, 523 to 527 mμ, and a very dim band at 596 to 602 mμ. Allowing for the shift in absorption which one would expect for a pigment in an organic solvent as compared with its presence in the living leaf, there is an excellent correspondence between the absorption bands reported for the pumpkin seed protochlorophyll and the peaks in the *Avena* effectiveness spectrum. Only the dim 596 to 602 mμ band reported by Noack and Kiessling has no counterpart in the effectiveness spectrum curve, but this is due to an experimental limitation. In order to demonstrate this small peak it would have been necessary to determine the effectiveness at 5 mμ intervals between 580 and 620 mμ, which is not possible with filters.

The blue peak found in the effectiveness curve on the other hand has no exact counterpart in the report of the absorption properties of the pumpkin seed protochlorophyll molecule. Noack and Kiessling make the interesting statement that in dilute solution, when the extract appears greenish, there is a band in the blue "but it is unimportant" for their purposes. (In more concentrated solution the extract appears brownish.) It must be borne in mind that these authors were not dealing with pure protochlorophyll extracts and the presence of any other plant pigments would reflect itself in the absorption in the blue region of the spectrum. It was undoubtedly for this reason that the absorption there was not considered diagnostic of protochlorophyll. Until the chemical isolation of protochlorophyll can be accomplished both from the pumpkin seed and from etiolated seedlings it will not be possible to state decisively what the relation of the pumpkin seed compound is to the actual precursor of chlorophyll. But the existence of the protochlorophyll effectiveness spectrum will serve as the test of the functional significance of any such purified pigments.

An interesting insight into the morphological relation of the plastid pigments is suggested by the demonstration that the carotenoids do not filter the light from the protochlorophyll molecules. The only way for the carotenoids to be present but not to act as screens is for them to be located *behind* the protochlorophyll molecules. This would suggest that the carotenoid molecules are located inside the plastids, with the protochlorophyll molecules on the outside surface. Whether this can be applied generally to other plants would have to be experimentally determined.

SUMMARY

1. Although the carotenoid pigments are present in large concentration in the plastids of etiolated *Avena* seedlings as compared with protochlorophyll,

the pigment precursor of chlorophyll, it is possible to show that the carotenoids do not act as filters of the light incident on the plant in the blue region of the spectrum where they absorb heavily. This suggests that the carotenoids are located behind the protochlorophyll molecules in the plastids.

2. Since the carotenoids do not screen and light is necessary for chlorophyll formation, an effectiveness spectrum of protochlorophyll can be obtained which is the reciprocal of the light energy necessary to produce a constant amount of chlorophyll with different wavelengths. The relative effectiveness of sixteen spectral regions in forming chlorophyll was determined.

3. From the effectiveness spectrum, one can conclude that protochlorophyll is a blue-green pigment with major peaks of absorption at 445 mμ, and 645 mμ, and with smaller peaks at 575 and 545 mμ. The blue peak is sharp, narrow, and high, the red peak being broader and shorter. This differs from previous findings where the use of rougher methods indicated that red light was more effective than blue and did not give the position of the peaks of absorption or their relative heights.

4. The protochlorophyll curve is similar to but not identical with chlorophyll. The ratio of the peaks of absorption in the blue as compared to the red is very similar to chlorophyll a, but the position of the peaks resembles chlorophyll b.

5. There is an excellent correspondence between the absorption properties of this "active" protochlorophyll and what is known of the absorption of a chemically known pigment studied in impure extracts of seed coats of the Cucurbitaceae. Conclusive proof of the identity of the two substances awaits chemical purification, but the evidence here favors the view that the pumpkin seed substance, which is chemically chlorophyll a minus two hydrogens, is identical with the precursor of chlorophyll formation found in etiolated plants.

I wish to express my sincere gratitude and indebtedness to the members of the Biophysics Laboratory: to Professor Selig Hecht, for much prized friendship and stimulating teaching throughout the time I have been in his laboratory; to Dr. Simon Shlaer, for his valuable direction and suggestions given so generously, especially in connection with the research reported here; to Mr. Charles Hendley, for his friendly help in the years of our association.

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