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SPECTROPHOTOMETRIC AND SPECTROFLUOROMETRIC CHARACTERIZATION OF THE TWO PIGMENT SYSTEMS IN PHOTOSYNTHESIS

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THESIS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biophysics in the Graduate College of the University of Illinois, 1965

Urbana, Illínois

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I. INTRODUCTION

The fundamental phenomena of photosynthesis were well understood before the middle of the nineteenth century. The liberation of oxygen by plants in light, though couched in terms of the phlogiston theory, was described by Priestley by 1771 A few years later, Ingén-Housz discovered that light falling on the green portions of plants was necessary to support the process. A contemporary of Ingen-Housz, Jean Senebier, added the information that carbon dioxide was fixed, and that photosynthesis was the source of carbon for organic matter. The participation of the one remaining constituent, water, was recognized by Nicholas de Saussure in 1804. Then, forty-one years later, Robert Mayer applied his concept of the conservation of energy to the organic world and clearly recognized the energy storage aspect of photosynthesis. The overall description of photosynthesis was now complete

In present day urms, photosynthesis can be described as the reduction of carbon dioxide to the level of a carbohydrate, coupled with the oxidation of water to free molecular oxygen The energy required to drive this endothermic reaction is derived from the absorption of light. Thus, the energy of the photon is stored in the form of chemical bond energy.

The gap between our understanding of what photosynthesis is in its end result, and our knowledge of how this result is actually accomplished, has persisted for many decades. It has only been in the past few years that enough relevant information has been accumulated, so that a model could be tentatively set up, and component reactions and reactants postulated and searched for.

There are many observations that such a model of the mechanism of photosynthesis mus explain. The following experimental results appear basic.

(1) A quantum requirement of 8 ± 1 is now generally accepted for each carbon dioxide molecule fixed or oxygen molecule evolved in steadystate photosynthesis [cf. Emerson and Chalmers (26), Emerson (23), Yuan et al (76) and Brackett et al. (11)], even though lower values have been claimed [cf. Warburg and Negelein (73) and Burk and Warburg (17)].

(2) The flashing light experiments of Emerson and Arnold (24, 25) led to the concept of a "photosynthetic unit" of probably 300 chlorophyll <u>a</u> molecules, and the necessity to postulate energy transfer between pigment molecules within such a unit.

(3) Energy transfer between different pigments was proved by Duysens (21) and Dutton <u>et al.</u> (20), who demonstrated that fluorescence is emitted from the pigment (chlorophyll <u>a</u> in higher plants and algae, or_____ bacteriochlorophyll in bacteria) having the lowest excitation level, whichever of the several pigments present in a cell is excited.

(4) Emerson's discovery of the "red drop" (28) and the enhancement phenomenon (27) led to the concept of two "pigment systems," sensitizing two photochemical reactions in photosynthesis.

(5) By varying the duration of the dark interval between exposures to light absorbed by the two systems used in the demonstration of this enhancement effect. Meyers and French (57) found that the lifetime of the products formed in one system must be several seconds.

Long before there was sufficient evidence to choose a model for the photosynthetic mechanism, Rabinowitch (62) pointed out that the alternative ways of utilizing eight quanta for the transfer of four hydrogen atoms from water to carbon dioxide were to use all of them "in parallel" or to use two sets of four quanta "sequentially." Twelve years later, when the Emerson Enhancement effect was discovered, a sequential two-step mechanism involving two "pigment systems" was advanced by

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Hill and Bendall (37) and Duysens <u>et al</u> (22), as well as Kok and Hoch (50), Witt <u>et al.</u> (74), Bishop and Gaffron (9) and Losada <u>et al.</u> (55). Though each of the authors presents his scheme from a different point of view, all the proposed schemes are versions of the same basic model. This model, which **n** the past few years has received many experimental confirmations, is described below. Though it is by no means to be accepted as final, it provides a good working model to interpret experimental results and to suggest further experiments

The bulk of the photosynthetic pigments present in a plant are thought, in the photosynthetic unit concept, to be chemically inactive, their function is simply to absorb radiation and to pass this energy on to a site where the initial photochemical step takes place These pigments are divided into two functional systems, I and II. Pigment system I consists of a long-wavelength form of chlorophyll <u>a</u>, as suggested by the "red drop" and the Emerson enhancement phenomenon. Pigment system II consists of the so-called accessory pigments, plus a short-wavelength form of chlorophyll <u>a</u>. Analysis reveals that these two forms of chlorophyll <u>a</u> have absorption maxima at 683 nm and 668 nm respectively (see Figure 17 and Table 2) The role of the carotenoids is still unclear

It can be suggested that each of the two systems forms its own "photosynthetic units," each with an associated energy "trap," at which the initial photochemical event occurs. Evidence for the existence of a trap in system I (P700) has been given by Kok and Gott (49), but experiments confirming the existence of a trap in system II are still lacking This is not surprising, as the absorption band of trap I is located on the long-wavelength side of the 683 mm chlorophyll <u>a</u> absorption band, in a position where it is observable, while that of the trap in system II must be sought a little to the long-wavelength side of the 668 nm form of chlorophyll <u>a</u>, where it is more difficult to find because of overlapping with the main chlorophyll absorption band.

As now envisioned, light absorbed by system II is responsible for the photo-oxidation of water -- one of the two postulated photochemical re-The absorption of a photon in this system makes energy available actions. for the transfer of an electron (or hydrogen atom) from water to some intermediate acceptor (now thought to be a cytochrome). This reduced intermediate (or, more likely, some other compound reduced by it in a dark reaction), then serves as reductant for the photochemical reaction sensitized by pigment system I. Absorption of a photon by system I leads to the oxidation of the reduced intermediates produced by system II, and lifting of an electron (or hydrogen atom) to a reduction level high enough to (indirectly) reduce carbon dioxide The overall mechanism is diagrammatically presented in Figure 1. For the production of one oxygen molecule, four electrons (or hydrogen atoms) must be moved through each step, hence the scheme has the "correct" quantum requirement of eight

In the experiments to be presented here, a search has been made for spectral components in the red chlorophyll absorption band of algae and spinach chloroplasts. The long suspected existence of several components in the red chlorophyll <u>a</u> band was first clearly indicated by the "derivative spectral curves" obtained by French (29), using a "derivative spectrophotometer." The first observation of this complex structure by integrating sphere absorption measurements was made by Cederstrand (19) These results were long vitiated by an instrumental problem of a high noise level in the spectrophotometer (see section A of the APPENDIX) Thomas and Govindjee (69) and Govindjee (30) have observed the existence

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Figure 1. The Sequential Two Step Model of Photosynthesis. The cytochromes serve to "chemically connect" the two pigment systems. They are alternately reduced by system II and oxidized by system I. According to Kok (48), the electron (or hydrogen atom) acceptor for system I, designated by X in the figure, may have a redox potential of -0.6 volt. The reduced NADP is supposed to be used, in the Calvin cycle, to reduce an acid to the level of an aldehyde, with the help of ATP.

of spectral details in the red band using a "wet filter paper" technique. The results to be presented here were obtained with the integrating sphere when the source of the noise was identified and eliminated.

In the hope that the pigments associated with the two pigment systems are bound differently in the two, a series of differential solvent extractions was made, and partial separation observed. Boardman and Anderson (10) recently reported a separation of the two systems by total solubilization with digitonin followed by fractional centrifugation. I tried this method, too, with some success. The fluorescence and absorption spectra of the extracts and fractions from digitonin solubilization were examined. Those in which some separation had obviously occurred were used for fluorescence quenching experiments. The results were analyzed in terms of the previously described photosynthetic model.

II. INSTRUMENTATION AND PROCEDURES

A. Spectrophotometer

The spectrophotometer used in this work was unique, in that it allowed for the first time direct observation of the detailed structure of <u>in vivo</u> absorption spectra Its construction was suggested by the late Dr. Robert Emerson Originally, it was intended to measure reliably the absorption of monchromatic light in algal suspensions contained in irregularly shaped manometric vessels. The interest at that time was in exact determination of the quantum yield of photosynthesis in suspensions that were not one hundred percent light-absorbing. Both in achieving the original purpose, and in observing details in absorption spectra, the spectrophotometer worked out well. For ease in presentation, the description of the spectrophotometer is divided into three sections: 1 Monochromator, 2. Integrating "Sphere", and 3. Amplifier.

1. Monochromator

The light source for the monchromator is a G. E. (18A/T10/1P-6V) 6 volt, 18 ampere ribbon-filament lamp. When operated at 21 amperes, the bulb life, though shortened, is still a usable twenty hours. Many other light sources were tried out. None of the gaseous discharge lamps I tried had the required stability. If the entrance slit was opened wide enough to include the wandering of the image of the arc, the resolution became so poor that the measurement of spectral details was impossible. (With a 0.2 inch slit, the limit of resolution is 18 nm or 180 Å) The luminous efficiency of the ribbon filament is the lowest of all the different types of incandescent filaments. It radiates about

9 lumens/watt, while a coiled filament radiates 11 lumens/watt, and a coiled coil filament 13 lumens/watt (45). The recently developed iodinequartz lamps are the most efficient of all; they operate with an efficiency of 19 lumens/watt (75). The reason for choosing a ribbon filament lamp, despite the lower efficiency, was as follows.

A 1000 watt coiled coil projector lamp radiates about ten times as much visible light as the G E. ribbon filament lamp, but when the image of the filament was formed on the entrance slit, the ribbon lamp was found to provide more illumination on the slit. This is due to two factors. First, the radiation from other filaments is more uniform in all directions while that from the ribbon filament is concentrated in a direction normal to its plane. Its radiation pattern is not unlike that of a dipole, while those of the other filaments approach that of a monopole Secondly, the space between the wires in a coiled coil radiates nothing. The greater efficiency of emission cannot make up for the absence of radiation from the spaces between the loops of the wire One of the recently developed iodinequartz lamps, (Sylvania 400 T4Q/CL/F), when operated at twice its rated 400 watt input, was found to surpass the performance of the ribbon filament, in the blue region of the spectrum, it gave 10% more slit illumination than the ribbon filament. Unfortunately, at this overload the iodinequartz lamp deteriorated too rapidly to make it useful. To sum up, the ribbon filament lamp is at present the best source for illumination of a monochromator slit when high output is to be combined with stability. An advantage of the particular G E. ribbon filament lamp used is that it is fitted with a medium prefocused base, and hence is directly interchangeable with the Osram series of spectral lamps, which can be used for collimation and calibration.

The design of a monochromator, as that of any other optical instrument, is influenced by the position and size of the final image to be formed. In our particular case, we wanted to form an image on the absorption cell that would just cover one side of it. This called for a round image with a diameter of 22 mm. If the image were smaller, a part of the absorption cell would contribute nothing to measurements but occupy space in the integrating sphere. This is undesirable, since an integrating sphere works better if the volume occupied by internal foreign objects is as small as possible.

To obtain uniform illumination of the absorption cell, the projection lens (see Figure 2) formed an image of the telescope lens on one side of the absorption cell The diameter and focal length of the projection lens were chosen so that it completely intercepted the cone of light emerging from the exit slit of the monochromator, formed a reduced image of appropriate size on the absorption cell, and provided a cone of light of a sufficiently high f number to pass through the entrance window of the integrating sphere The exit and entrance slits were identical. They were modifications of the bilateral design by Barnes and Brattain (7), in which the jaw mechanism was changed -- it was moved by the action of a compound screw, instead of a rotating cone. Bilateral slits were used so that no shift occurred in the central wavelength as the slit width was changed. The telescope and collimating lenses were a matched pair of achromatic objectives, originally made for an optical range finder. Since both the range finder and the monochromator operate with parallel beams of light on one side of their lenses, the range finder objectives were ideally suited for service in a monochromator. The objective lenses had a focal length of 46 3 cm, the one serving as the telescope lens was provided with a diaphragm giving the limiting f number of the



Figure 2. Plan of Monochromator

monochromator of 10.6. The grating was a (33-53-08-26) Bausch and Lomb flat grating of 600 grooves/mm, ruled over an area of 52 x 52 mm and blazed for maximum reflection at 500 mm. When used in conjunction with the telescope lens, the grating produced a linear dispersion of 1 0 nm/ .011 inch on the exit slit. The platform to which the grating was attached was mounted on a tapered roller bearing and its angular position was controlled by means of a sine bar linkage operated by a micrometer screw (see Figure 3). When properly calibrated, the wavelength could be read directly on the micrometer screw with an accuracy of ± 0.1 nm Since the narrowest bandwidth used in these experiments was 1.0°nm, accuracy of the wavelength measurements was not a critical consideration

The entrance slit was illuminated by a pair of achromatic lenses which formed an image of the ribbon filament on the entrance slit Since the slit extended 7/16 inch up and down from the optical axis, it was necessary to use a little lower \underline{f} number for the condensing lens system than for the monochromator, to obtain uniform maximum illumination of the entrance slit.

All the aforementioned optical elements were screwed down to a base plate of 3/8 inch cold-rolled steel, which was in turn welded to a framework made of 2 inch steel I-beam There was then no problem of insufficient structural rigidity. Some means of fine adjustment was necessary for the optical elements -- these adjustments are quite straightforward and can be inferred from the accompanying photographs (see Figures 3, 4).

2 Integrating "Sphere"

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The integrating "sphere" was not really a sphere at all, but rather a dodecahedron. What was unique about it was not so much its



Figure 3. A photograph of the grating mounting showing the sine bar linkage. The grating cell is fastened to the rotating platform by four push-pull screws, of which two are visible (see top left).

shape as the fact that it had 12 uniformly spaced light detectors rather than the usual single detector located behind an internal baffle. As has been pointed out by Latimer (52), the position of a single detector and its accompanying baffle can be critical in an integrating sphere. I also found this to be true; variations up to 14% were found when a single detector was moved about. A series of experiments were performed in which the light distribution pattern in the integrating "sphere" was changed, using inner surfaces of differing reflectivity and different numbers of detectors. For this purpose, a small mirror was attached to the movable arim that would later hold the absorption cell, 'and the incoming beam of light was directed to different areas on the inner surface of the dodecahedron. The least variation in photocell current with changes in the light distribution pattern was obtained with the "whitest" surface, and with a full complement of detectors (see Table 1).

A question which naturally arises concerns the distribution of photocells on the dodecahedron. Since the normal production run of photocells shows output variations up to 25%, should not the total current (all photocells connected in parallel) vary with the placement of the individual cells? With unmatched cells, the variations in the total current between any two cell distributions did not exceed 2 - 3%. When a matched set of photocells (those with conversion efficiencies between 11 and 12% for white light) were used, the current variation was reduced to less than 1%.

A consideration which cannot be overstressed is the whiteness of the inner surface of the sphere; there is no other single factor which exerts as powerful an influence on how well a sphere will integrate, or how high a photocell current will be obtained Were the inside a perfect diffuse reflector, the only possible source of photon losses in an empty



Figure 4. Photograph of Monochromator

TABLE 1

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Summary of "Sphere" Experiments

Incident Light and Sphere Condition	Photocell Current Deviations from the Average of Sixteen Readings*		
	Average Deviation %	Maximum Deviation %	
Green light Copper surface	20	54	
White light Silvered surface	11	20	
White light Silvered surface MgO coating	16	3.4	
White light Silvered surface MgO coating Baffle, 1 detector	66	14	
White light Silvered surface MgO coating No baffle, l detector	3.6	6.6	
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*Each reading was obtained from a different light distribution pattern.

sphere would be absorption by a photocell, or passage back through the entrance window. In this idealized case, one would expect the decrease in the photocell current, obtained by first directing the light onto one of the photocells and then onto the white sphere surface, to be determined by the ratio between (the area of the photocells) and (the total absorbing area -- photocells plus entrance window). In our particular sphere, the total internal area was 984 cm^2 , the photocells occupied 15.2 cm², and the entrance window 3.7 cm² Hence, if the light were uniformly distributed throughout the sphere, a decrease in photocell current to 15.2/(152+37) or 80% of the original would occur when the light was directed from a photocell to the diffusing sphere surface. Experimentally, a value of 71% was obtained. Provided all photocells used have a linear response to changes in light intensity in the range used, and they all have the same sensitivity, the two factors which prevent the achievement of the theoretical efficiency (80% in our case) are that the photocells are "less black" than the entrance window, and that a "perfectly white" diffusing surface does not exist. If we correct for the fact that the silicon photocells (Hoffman Electronics Corporation, 2A) reflect about 15% of the incident white light, we obtain (assuming the entrance window to be "black") a corrected value for the current decrease expected in a "perfectly" diffusing sphere of 15.2 x 85/ $(15.2 \times .85) + 3.7$ or 78% The remaining lack of agreement between the theoretical and experimental values (78% vs. 71%) is now probably due to the imperfect whiteness of the sphere. The photocells and the entrance window together occupy 1/62 of the total internal area, the other 61/62 being the sphere surface. Hence, it takes many reflections for the average photon to find its way to a photosurface or to the entrance window. If even a very small fraction of the incident energy is absorbed

in each reflection, a measureable energy loss must result.

This suggests a method by which the "absolute efficiency" of an integrating sphere can be calculated It also provides a means for calculating the reflectivity of the sphere's internal surface. As mentioned above, for the integrating dodecahedron of our spectrophotometer, the maximum possible amount of diffuse light absorbed by the photocells was calculated to be 78%, while the experimentally determined value was 71%. Hence, the "absolute efficiency" of this sphere was

$$E = \frac{0.71}{0.78} \times 100 = 91\%$$
 (1)

The physical significance of this "absolute efficiency" is that it expresses what percent of the photons entering an empty integrating sphere are eventually absorbed by the photocells, in relation to the maximum number that could be expected to be absorbed by the photocells. Once the ratios of the reflective area (61/62) and absorptive area (1/62) to the total area are known, the most probable number of reflections a photon will experience before it is absorbed can be calculated. For each impact the photon's chances of being absorbed (p) are 1 in 62, while its chances of being reflected (q) are 61 in 62. The most probable number of reflections before absorption is

$$n_e = \sum_{n=1}^{n=\infty} nq^{n-1}p = 62.$$
 (2)

Since this number, n_e, also represents the number of reflections the average photon experiences before absorption, the reflectivity of the sphere may now be calculated If R is the reflectivity of a white surface, then E, the fraction of energy left after n_p reflections, is

$$E = R^{n}e$$
(3)

E is also the "absolute efficiency", the fraction of energy actually reaching the photocells in relation to the amount expected were the internal surface of the sphere a perfectly diffusing surface. With E determined from equation (1) and n_e from equation (2), equation (3) may be solved for R. A value of .998 is obtained To obtain some estimate of the precision of this calculation, we must allow for a maximum variation of \pm 5% in the sensitivity of the photocells and one of \pm 5% in the linearity of the photocells in the $10^2 \cdot 10^3$ microampere range. Upon propagation of these two errors through the calculations, a final result of 0.998 \pm 0 006 is obtained. The values given in the literature for the reflectivity of magnesium oxide-coated, silvered surfaces for white light run from 0.980 to 0.995 (72). My value of 0 998 is a little higher. This may be due to the non-linearity of the photocells, and -- to a very small degree -- to "nonblackness" of the entrance window

With the above-described sphere, the photocurrent drops by 29% when light is directed first to a photocell and then to the white sphere surface. The output of the Bausch and Lomb spectrophotometer 505 when used with its reflectance attachment as an integrating sphere, can drop by as much as 80% in a similar experiment In Latimer's sphere (52), the current dropped by 60%. Despite the effect of the size of the entrance window

on these values, I would be inclined to attirbute these variations primarily to differences in sphere whiteness.

The universal method of applying a magnesium oxide coating is to smoke it on. Even an electrostatically precipitated smoke layer of 0.8 cm thickness is not completely reflective, but allows some light to be Accordingly, in the construction of the dodecahedron, I transmitted (66). backed up the magnesium oxide coat with a surface of polished silver. Furthermore, since smoked coats of magnesium oxide are destroyed by the slightest touch, a little colorless binder was added to make a mechanically more stable coat. The binder consisted of 40 ml acetone, 13 ml partially depolymerized methyl methacrylate, and 10 ml Dupont 3656 (Lucite paint thinner). The first coat applied to the sphere was the clear vehicle, after which sufficient powdered magnesium oxide was added to the vehicle to make a heavy non-flowing white syrup. Layers of magnesium oxide were built up over a period of about three days to a 2 mm thickness. One should allow several hours between coats, else the effect of shrinkage upon polymerization of the top layer of methyl methacrylate upon the still soft under layers will produce cracks throughout the surface. The methyl methacrylate is a spectrally proper choice for the binder as its spectrum is completely free of absorption bands in the visible region. The closest absorption band is in the ultraviolet, at 340 nm If after building up this densely bound layer of magnesium oxide, the sphere is "smoked" by burning magnesium ribbon, a really first class surface is produced. It is so reflective that if one directs some light into the entrance window and then peers into one of the photocell openings, the sight presented is that of radiation as uniform as that in the window of an operating muffle furnace.

Prior to the definition of this newly defined term, the "absolute efficiency," there has existed no absolute scale upon which to measure the integrating properties of a sphere The problems involved in measuring the absorption spectrum of a scattering material have been discussed by Butler (18). He gives an excellent survey of the literature, and of our knowledge of how an absorption spectrum may be modified in the case of a strongly scattering medium. As an empirical test of the integrating properties of the sphere, Thomas (68) suggested that the measurement of an algal absorption spectrum at three different cell densities is as useful a test as any other. With a good integrating sphere, the base line must remain essentially independent of the cell concentration The absorption curves plotted in Figure 5 show the negligible shift in the base line that occurs in the case of those measurements made with the integrating instrument. This indicates that the integrating sphere is able to distinguish between the increase in scattered light at the higher cell densities, and the increase in absorption.

The dodecahedron is constructed from twelve regular pentagons. The pentagons have sides of 6.9 cm and are cut from 0.032 inch sheet copper. In the center of each pentagon a 0.5 inch hole is punched for the photocell. Since the silicon photocells are thin discs, they may conveniently be mounted by pressing them directly against the 0.5 inch hole with an insulated phosphor bronze finger. The individual copper pentagons (edges chamfered to 58°) were held in a jig and soft soldered together to form the dodecahedron. One side of the dodecahedron was left open; a brass jamb was constructed about this opening into which the door was to fit

To prevent the algal suspensions from settling out of the light path during long experiments, the absorption cell (a cylinder 22 mm in

diameter with a 5 mm path length) was held horizontally, the beam of light entered the dodecahedron at the bottom and fell on the bottom of the absorption cell. Two capillary tubes were attached to the cell for filling The cell 1s held in the center of the sphere by slipping one of its capillary tubes into a split sleeve on the end of an omniposition rod. This rod allows the absorption cell to be precisely located in the light beam. The rod is given the necessary degrees of freedom by clamping it in a hole drilled in a split ball. The split ball is held in a socket. By tightening the socket, the split ball is locked in position and in turn clamps on the rod holding the absorption cell The finished dodecahedron is located inside a closed blackened brass cylinder (see Figure 4), which serves both as a light shield and an electrostatic shield. Further details on the construction of the dodecahedron may be seen in the photograph in Figure 6

3. Amplifier

With the monochromator adjusted to 700 nm, the band width to 1.0 nm, and the lamp current to 21.0 amperes, the energy output of the monochromator amounts to 20 ergs/sec. This light will generate a photocell current of only a 0.1 microampere in the input load resistor. Accordingly, the most difficult problem encountered in the design and construction of this instrument is the one of attaining a suitable signal-to-noise ratio, along with a voltage gain of 80 db (10^4) . A ratio of 1000/1 was required in these experiments if no noise was to appear in the output. Since the noise voltage generated in any amplifier is proportional to the square root of the band width, a tuned amplifier was mandatory To accomplish this, the light entering the monochromator was interrupted at 400 cycles/second by a sector disc (shown in the backgrounds of



Figure 5. Absorption spectra, at different cell densities, of <u>Porphyridium cruentum</u>. Three were obtained with the integrating spectrophotometer, described in the text, and two with a Bausch and Lomb 505 spectrophotometer (without an integrating attachment).

Figures 4 and 6) so that the output from the photocells could be fed into a 400 cycle amplifier. Discrimination against noise at 400 cycles was attained by using a synchronous detector on the signal output of the amplifier. In this manner, only those components of 400 cycle noise that were in phase with the absorption signal could appear in the rectified output. A frequency response curve and circuit diagrams of the amplifier and synchronous detector are given in section A of the APPENDIX

Since the input stage of an amplifier (see Figure 7) determines the zero signal noise level, this stage will be briefly described. The silicon photocells used to drive the input stages are current-supplying devices and are linear, and their currents additive, only if they work into a very low impedance load. Accordingly, they are loaded by a 50 ohm resistor that is in series with the tuned primary of the input transformer. The voltage generated across this 50 ohm load resistor serves to drive the series tuned circuit. The loss in driving voltage which occurs because of the low value of input resistor is in part made up for by resonating the input transformer. Fortunately, as the load resistance is decreased, the drop in driving voltage is about 70% counteracted by the increase in primary oscillating current, brought about by the increase in Q which occurred with the decrease in the series resistance

The input transformer consists of a 100 turn primary and a 10,000 turn secondary wound on a 0 001 inch tape toroidal core (Arnold Engineering Co. No. 4168-S1). The transformer was shielded by potting it in a triple shielded copper and mu-metal case. It was only the availability of the toroidal transformer core that made the 1:100 input transformer technically feasible; without it, it would have been difficult if not impossible to couple the low impedance photocells into the high impedance required for a vacuum tube grid.

C. S.



Figure 6. Photograph of the integrating "sphere" (dodecahedron), showing the absorption cell in measuring position.



Figure 7. The transformer coupled input stage of the amplifier.

All tubes in the amplifier are operated in Class A, with a D.C. heater supply (storage battery) and a voltage-regulated plate supply. The tuning of the amplifier is accomplished by the use of a twin T filter in a negative feedback loop in the second stage and a U.T.C. inductancecapacitance tuned filter in the third stage. The 5879 tubes used in the first and second stages must be selected tubes -- about one tube in six is satisfactory for these positions.

The synchronous detector is quite straightforward. A penlight flashlight bulb generates a light beam that is also chopped by the sector disc and then detected by the 1P42 photocell. This is the source of the signal to drive the synchronous switch. The proper phase relation to the signal channel is achieved by adjustment of the resolver. Section A of the APPENDIX can be consulted for a complete circuit diagram and suggestions on how the amplifier could be improved.

B. Spectrofluorometer

The spectrofluorometer described here is in the process of development by Mr. Jobie Spencer in this laboratory. A detailed paper describing the construction of this instrument will soon be presented by Dr. Govindjee and Mr. Spencer For purposes of completeness, a brief description of the Spencer-Govindjee spectrofluorometer will be given here.

As with many instruments, its conception is simple -- its execution difficult. It uses one monochromator as a source of excitation and a second monochromator to scan the fluorescence. A photomultiplier serves as the detector for this second monochromator The output from the photomultiplier is eventually recorded by a continuous chart recorder. A plan of the instrument is given in Figure 8



Figure 8. Plan of Spectrofluorometer.

Both of the monochromators used are the large Bausch and Lomb f 5 monochromators, model 33-86-45. They are double-pass instruments in small cases, which means that they are plagued by internal reflections about which little can be done. In addition, the cases allow some light to This latter ill can be cured by judicious conenter around the controls struction of stops and shields. Once modified, they will perform their appointed tasks well enough to stop one from undertaking the construction of a better monochromator. The wavelength drums of either or both monochromators can be driven through an 8-speed gear box by a synchronous motor. The source of illumination for the excitation monochromator is a ribbon filament lamp. Light of a chosen wavelength leaves the excitation monochromator, passes through a filter (to minimize light scattered by internal reflections and when necessary to absorb the overlap of higher order spectra), and 1s focused and directed upon the sample by means of a lens and a mirror.

The sample, generally a liquid, is held in a special Dewar flask with a flat window in the bottom, to allow the entry and exit of light. The projection lens forms a reduced image (1 x 10 mm) of the exit slit upon the sample It is this small illuminated rectangle that serves as the light source for the scanning monochromator. Light from this 1 x 10 mm rectangle is reflected by a mirror (not shown in Figure 8) and then focused by a condensing lens through a filter upon the entrance slit of the scanning monochromator. An E. M. I. type 9558B photomultiplier, located at the exit slit of the scanning monochromator, serves as the light detector. Only the availability, in the last few years, of photomultipliers with gains of 10^7 - 10^8 has made this instrument feasible. The anode current of the photomultiplier is fed into the input of a Keithley microvolt-ammeter, model 150A. The Keithley microammeter has an output provision from which the signal can be fed into the Minneapolis-Honeywell Brown recorder The slits of the two monochromators are generally operated at widths of 2.0 mm -- the corresponding band width being 6.6 nm. Synchronizing the chart drive motor of the recorder with the motor that drives the wavelength drum of the scanning monochromator permits us to record a fluorescence spectrum This spectrum is unfortunately affected by the spectral response curves of the different elements of the spectrophotometer, in addition to the actual fluorescence distribution curve. Accordingly, the recorded curve must be corrected for the response curve of the photomultiplier, the transmission curve of the scanning monochromator and of any associated filters, as well as for spectral variations in the photon energy. By so correcting, one can obtain the wavelength dependence of fluorescence intensity expressed as the number of photons emitted for equal numbers of photons absorbed. All the fluorescence curves presented here have been so corrected. When dilution experiments are performed, in which the fluorescence of one sample is to be compared with that of another, one must not only correct for the differences in absorption of the two samples, but also make sure that the volumes of the two samples are identical and that their absorbances are low. I always used a sample volume of 2.0 ml, the corresponding optical path length being 1.0 mm. When possible, the absorbancy was adjusted so that only 5% of the incident light was absorbed at the absorption maximum.

C. Growth and Preparation of Plant Material

The plant material used in the experiments described in this thesis consist of spinach chloroplasts and three algae. They were grown and prepared as follows.

The spinach was Spinacea oleracea, grown from seed supplied by the Burpee Seed Company (American Spinach #5058). The plants were grown in a greenhouse in natural light. As soon as the leaves reached full size, but before they started to wrinkle, they were cut off and used for the preparation of chloroplasts (with older, wrinkled leaves, the solubilizing effect of the detergent was found to be markedly decreased). Approximately 200 gms of leaves were harvested for each experiment. All the following operations were carried out at 5° C. The leaves were ground up with a little sand in a 0.4 M sucrose, 0.05 M tris, 0.01 M sodium chloride buffer solution of pH 7 2. The solution was squeezed through eight layers of cheese cloth to remove the pulp and then centrifuged for 30 seconds to remove the sand. After the sand was removed, the supernatant liquid was recentrifuged for 10 minutes at 1000 g to form a chloroplast pellet. The pellet was resuspended in a detergent (Digitonin) and allowed to incubate for 30 - 60 minutes. The resuspension medium consisted of 0.05 M phosphate buffer, pH 7.2, 0.01 M potassium chloride and 0.2 percent Digitonin. While a longer incubation period leads to an increase in the amount of chloroplast material that is solubilized, it also brings about a decrease in chloroplast activity. Upon the end of incubation, the chloroplasts are centrifuged at 1000 g for 10 minutes, 10,000 g for 30 minutes and 50,000 g for 30 minutes. All the pellets obtained are collected and resuspended. The final supernatant liquid is saved. This solubilization procedure is very similar to the one described by Boardman and Anderson (10). The chloroplast fractions are then kept in the cold and in darkness until used; measurements with them should be started promptly because of the decrease in activity with time.
The algae used were <u>Chlorella pyrenoidosa</u>, <u>Anacystis nidulans</u> and <u>Porphyridium cruentum</u>. These algae were grown in inorganic media in culture flasks through which gas mixtures of 5% CO_2 in air were ______ bubbled. They were continuously illuminated with white light and harvested in 4 - 7 days, after a 40 x increase in cell population had occurred. Section B of the APPENDIX gives the chemical composition of the growth media.

III. EXPERIMENTAL RESULTS

A. Absorption Bands of Chlorophylll In Vivo

1. Basic Considerations, and Experiments Indicating the Validity of the Results

The purpose of the following mea surements was to determine what details of the absorption spectra of live cells and chloroplasts can be observed in the long-wavelength (red) chlorophyll absorption band by careful, low-noise measurements.

The ratios of pigment concentrations in plant cells can be made to vary over a wide range by changing the growth conditions. Changes in both the <u>intensity</u> and the <u>wavelength</u> of light used for growing the algae can cause drastic alterations in the relative amounts of pigments present. An illustration is given in Figure 9, in which the absorption spectrum of <u>Anacystis nidulans</u> grown in white light is compared with that of the same alga grown in red light (isolated by means of a Corning No. 2408 red filter, which transmits only wavelengths longer than 630 nm): The phycobilin/ chlorophyll ratio in the latter is about 20% smaller than in the former. What we are interested in here, however, is only the number of identifiable spectral components of chlorophyll present, rather than the relative intensities of the bands belonging to the several pigments.

The absorption curves to be presented here are for algae grown in continuous white light, and for spinach chloroplasts, derived from plants grown in a greenhouse in natural light.

The bandwidth of the measuring beam was 1.0 nm (10 Å) for all wavelengths longer than 550 nm. For wavelengths shorter than 550 nm, it was either 2.5 or 3.0 nm, as marke c on the curves. This increase in bandwidth in the blue region is made necessary by the decrease in both

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Figure 9. Absorption spectra of two cultures of Anacystis nidulans, one grown in white light, the other in red light. $\Delta \lambda$ is the bandwidth of the measuring beam.

the energy output of the tungsten lamp, and in the sensitivity of the detectors (silicon photocells). For these two reasons, the signal-to-noise ratio decreases toward the blue end of the spectrum. The signal level had to be increased by doubling or tripling the bandwidth to maintain the desired high signal-to-noise ratio (in the neighborhood of 1000.1). Since the energy output from a monochromator varies as the product of the slit widths, doubling the slit width results in a fourfold increase in the energy output. Of course, as the bandwidth is increased, fewer details become observable in the spectrum; this is unfortunate, but our first experimental requirement is a sufficiently high signal-to-noise ratio

The absorption curves presented below are graphs of the absorbance, A, as a function of wavelength, λ , or wavenumber, $1/\lambda$, where

$$A = \log_{10} \frac{I_o}{I}$$
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$$I = \text{transmitted light intensity}$$

Since the spectrophotometer used was a single-beam instrument, it was necessary to determine separately I and I_0 . The first set of readings $(I_0) = f(\lambda)$ is obtained with water in the absorption vessel, a reading being made every 5 nm. The water is then removed and the vessel filled with the suspension of cells or chloroplasts to determine I. This second set of readings is taken, at closer intervals, down to 1.0 nm in the red, where the bandwidth of the measuring beam also is 1.0 nm. The data are then transferred to punch cards and computation of the absorbance performed on the University's 7094 I. B. M. computer.

For this calculation, I-readings are available every 1.0 nm, I_o -readings only once every 5.0 nm. However, since the I_o curve 1s smooth (see Figure 10), the missing I values can be obtained by linear interpolation. The computer does this automatically. That this causes little loss in accuracy is shown by Figure 11 It suggests that there is little change in the absorption curve, whether we measure I at each 1.0 nm, or only at every 5.0 nm and interpolate in between. Since hundreds of readings are to be made through a hand lens, a reduction of 80% in the number of I readings means a significant saving of time, and lessening of the observer's fatigue. (Even so, each absorption curve took about two hours to measure!) The usual procedure was to measure I from 550 to 730 nm at 5.0 nm intervals and then to place the algae in the absorption vessel and to measure at every 1 0 nm from 730 back to 550 nm. At this point, the bandwidth was increased from 1.0 to 3.0 nm, and the I-readings continued at 2.0 nm intervals to 400 nm The suspension was then replaced with water, and I_{o} measurements made at 5.0 nm intervals back to 550 nm.

The crucial question is the origin of the shoulders and peaks noted on the absorption curves (see Figures 12 and 17). Since these details represent changes in absorbance of the order of only 0.2%, it is important to make sure that they are not due to fluorescence or another cause, rather than to changes in absorption. Curve 1 in Figure 12 was obtained with live <u>Chlorella pyrenoidosa</u> cells, Curve 2 with a pigment extract. It is gratifying to see that no fine structure appears on Curve 2, although the amount of fluorescence originating from the extract must have been five to ten times greater than that originating from the live cells. In fact, since the measurements were made with an integrating



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WAVELENGTH Figure 11. Absorption curves of <u>Chlorella pyrenoidosa</u> for I values determined by two different methods. The upper curve was calculated with I_0 values measured each 1.0 nm, while the lower curve was calculated with I_0 values interpolated between the points measured at each 5.0 nm.



sphere, the effect of fluorescence must have been to decrease the value obtained for the absorbance, thus depressing slightly the whole absorption curve, but adding no new details to it.

There was no suggestion of fine structure in the extract (Figure 12, Curve 2), in the extracted cells (Figure 12, Curve 3), or in a suspension of extracted cells in the extract (Figure 13) We observed this structure in the red absorption band only with the pigment in its natural association in the cell.

Incidentally, not only does the pigment have to be in the cells to display the fine structure shown in Figure 12, but the cells must have been recently engaged in photosynthesis Cells that have been removed from the culture room and allowed to stand in the dark, or in the refrigerator, for a few days, gradually lose the doublet structure typical of "fresh" cells. (Experiments seem to be called for on the induction of photosynthesis in such dark-incubated cells, and the return of the fine structure!)

An obvious question is that of the reproducibility of the details. Figure 14 shows four consecutive absorption measurements on a <u>Chlorella</u> suspension. All four curves show the double-humped shape of the 675 nm absorption band. Curve 1 was the first and Curve 4 the last measured. In proceeding from Curve 1 to Curve 4, we note a steady reduction in the sharpness of the structure This demonstrates the aforementioned observation that cells that had recently been engaged in photosynthesis show the fine structure most clearly. The time between the first measurement and the last was probably 45 minutes. This indicates a rather rapid deterioration of fine structure, usually, its loss was noted only after several hours of standing in the dark or in low light.

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Figure 13. Absorption spectrum of extracted <u>Chlorella pyrenoidosa</u> cells suspended in their methanol extract.



Figure 14. <u>Chlorella pyrenoidosa</u> absorption curves displaying the complex character of the red absorption band. Gradual change in the shape of the band is shown by the four curves. (Curve 1 first, Curve 4, 30 - 60 min later.)

A final question concerns variations from culture to culture, when similar growing conditions are used. Variations do occur between such cultures, the two absorption curves in Figure 15 show several of them, in the red peak as well as in the blue. No attempt to obtain spectrally uniform cultures was made.

2. Representative Curves and Their Resolution into Components

To resolve an absorption band into spectral components, one must first choose the shape of the component bands to be postulated in the analysis. A justifiable choice is to assume that the shape of the component bands are similar to that of the red chlorophyll a absorption band in solution, and look for a function most closely matching this curve. With absorption plotted as a function of wave number, the Gaussian error curve, $y = ae^{-bx^2}$, was often used for this purpose in the past. I found it to provide a close fit for the red chlorophyll a absorption band in ether (see Figure 16). It "mismatched" slightly on the long-wavelength "tail" of the band and more strongly on its short-wavelength end, where vibrational sub-bands begin to overlap with the first band. On the whole, though, the match is surprisingly good. In the case of Chlorella (cf. Figure 17), the matching was achieved by choosing three Gaussian components, each with an arbitrary amplitude, bandwidth, and location, and then having the computer sum the three components, each one of the nine variables being allowed, in turn, to assume a series of gradually changing values. The summation curves were then compared to the experimental absorption curve. After each comparison, an adjustment was made in one of the nine variables, and a new series of summation curves calculated. This trial and error procedure was repeated thousands of times until a satisfactory fit was obtained. The same analysis was performed with the



Figure 15. Two representative <u>Chlorella pyrenoidosa</u> absorption curves. Note the consistent appearance of the four bands in the region of carotenoid absorption (450 - 500 nm) and the variation in the red chlorophyll <u>a</u> absorption band (650 - 700 nm).

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Figure 16. A comparison between a Gaussian error curve and the red chlorophyll <u>a</u> absorption band (in ether).



Figure 17. The resolution of the red chlorophyll absorption band of <u>Chlorella</u> into Gaussian components. The solid line gives the sum of the Gaussian components, while the circles show the shape of the measured absorption curve. The dash-dot component curve represents chlorophyll b; the dashed curve, the short-wavelength chlorophyll <u>a</u> component; and the dotted curve, the long-wavelength chlorophyll <u>a</u> component.

absorption curves of spinach chloroplasts, and the cells of <u>Anacystis</u> <u>nidulans</u> (blue-green alga), and <u>Porphyridium cruentum</u> (red alga) (cf. Figure 18). A summary of the results is given in Table 2, which is discussed in section IV A.

B. Separation of Pigment Fractions

If the components into which the absorption bands of chlorophyll a in vivo can be resolved correspond to different molecular species, one can try to separate these species. They could correspond, for example, to different degrees of aggregation of the pigment molecules It has been observed by Jacobs et al. (41), Brody (12), and Love and Bannister (56) that the absorption maxima of aggregated chlorophyll shift with changes in the size of the particles. Alternatively, the differences could be due to different carriers (protein molecules) or associate solvent molecules to which the chlorophyll a molecule may be bound. The chlorophyll bands are known to shift with the polarity of the solvent. For example, the absorption maximum of chlorophyll a shifts from 660 nm in hexane to 672 nm in carbon disulfide. In vivo, chlorophyll is usually assumed to be bound to a protein, but we must remember that lipids constitute about 40% of the chloroplasts, and that upon separation of lipids all pigments tend to follow the latter. Thus, chlorophyll a molecules may be associated in vivo with lipids, instead of, or in addition to, being bound to proteins.

If the pigment-carrier bonds are different for the different spectral components, it may be possible to rupture them in one and leave those in the other intact. With this possibility in mind, stepwise extraction of pigments from the chloroplasts by acetone and methanol was studied The extracted material, as well as the material left in the chloroplast, were examined by absorption and fluorescence spectroscopy--the latter both at



Figure 18. Absorption curves resolved into Gaussian components. The circles show the original absorption curves, the solid lines are the sum of the Gaussian components, and the broken lines are the several Gaussian components.

TABLE 2

SUMMARY OF RESOLVED CHLOROPHYLL COMPONENTS

IN VIVO

Name of Plant –		Chlorop	hyll <u>a</u>		Ratio of Heights	Chlorophyll b	
	Short-wavelength Component		Long-wavelength Component		of Chlorophyll a Components		
	Peak Location nm	Half-band- width nm	Peak Location nm	Half-band- width nm	Short λ component Long λ component	Peak Location nm	Half-band- width nm
Chlorella pyrenoidosa	<u>a</u> 668.4	17.0	683. 1	17.7	. 988	651.0	178
Chlorella pyrenoidosa	<u>a</u> 668 4	17.0	683. 1	17.7	921	650 2	20. 1
Spinach Chloroplast	ts 666.7	17.0	680. 3	17.6	. 769	650. 2	19.8
Anacystis nidulans	666. 7	29.6	681.2	26.0	. 902		
Porphyridi cruentum	<u>um</u> 669. 3	23.3	684. 0	24. 4	. 905		

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room temperature and at the temperature of liquid nitrogen.

As another method of component separation, used also by Boardman and Anderson (10), the chloroplasts were suspended in a detergent (digitonin), and the solubilized material fractionated by centrifugation. These fractions, too, were examined by absorption and fluorescence spectroscopy. The particle size was determined by electron microscopy, and, to provide information about the degree of orderliness of the pigment molecules, a measurement was made of the degree of polarization of chlorophyll a fluorescence excited by polarized light.

1. Differential Solvent Extraction

Freshly cut spinach leaves were ground up with a little quartz sand in a sucrose buffer, as previously mentioned (see II C) After washing and centrifugation, the chloroplast pellet was suspended in a 0.05 M tris buffer of pH 7.2 Aliquots of 0.5 ml were immediately removed and transferred to two series of test tubes. One series contained 5 ml portions of aqueous acetone, with acetone concentration from 0 to 100%, increasing in increments of 10%. The other group contained a similar series of methanol-water mixtures After shaking, the solutions were kept, as far as possible, in the cold (about 0° C), and in the dark. The chloroplasts were allowed to stand in contact with the extracting solutions at this temperature for ten minutes, after which they were centrifuged (at 0° C) at 12,000 g, for 10 minutes. The supernatant liquids were separated from the pellets and kep in screw cap test tubes. The pellets were resuspended in 5 ml portions of tris buffer, and kept in the cold and in the dark until needed. Since chlorophyll is known to decompose rapidly when removed from its chloroplast environment, spectral measurements were made as soon as possible. The absorption spectra

were measured by a Bausch and Lomb 505 spectrophotometer, fitted with a reflectance attachment modified to serve as an integrating sphere.

a. Absorption Spectra

Representative examples of the absorption spectra of resuspended chloroplasts are given in Figure 19. The sequence of band heights in this figure (suggesting that more chlorophyll is extracted by 20% than by 50% acetone) will be examined later. The curves show a shift of the red absorption maxima, and disappearance of the chlorophyll <u>b</u> shoulder at 650 nm, as the solvent concentration is increased. The formation, at solvent concentrations of 50 - 60%, of a chlorophyll complex absorbing at 740 nm is also clearly shown This band was previously reported by Govindjee (32), and by Aghion (1) and Aghion <u>et al.</u> (2), no further investigation of this band was undertaken in this study.

Figure 20 is the plot of the position of the main red absorption maximum of the <u>resuspended chloroplasts</u> after extraction with acetone or methanol of different concentrations. The locations of the red absorption maxima are only plotted for solvent concentrations up to 80%, because beyond this point, so little pigment remains in the chloroplasts that absorption measurements become unreliable. The plotted maxima were measured with a precision of ± 0.5 nm (With one exception, the same limits of precision hold for all of the following absorption data.) A salient feature of the two curves in Figure 20 is the constant position of the absorption maximum up to approximately 26% for acetone extractions and 38% for methanol extractions. Beyond these concentrations, the maximum shifts rapidly to shorter wavelengths. It is interesting to note that the solvent concentrations at which these drops begin correspond to similar dielectric properties of the solvent. The average



Figure 19. Absorption spectra of resuspended spinach chloroplasts after extraction with acetone solutions of different strengths.

dipole moment of a 26% acetone solution is 1.55 debyes, while that of a 38% methanol solution is 1.57 debyes. The corresponding dielectric constants are 67 and 64 respectively (39).

The absorption spectra of the extracts form a similar series of displaced absorption peaks, plotted in Figure 21. At low solvent concentrations (less than 10% for acetone, and less than 25% for methanol), the absorption peaks remained at the same location as in the original chloroplasts. As the concentration of the solvent was increased, the peaks began to shift. At solvent concentrations above 55% for acetone and 65% for methanol, the absorption peaks reached limiting positions. A simple explanation would be that at low solvent concentrations, chlorophyll remains attached to its carrier, while at the higher concentrations, chlorophyll is removed from its carrier. When comparing the concentrations at which the peaks began to shift in the resuspended chloroplasts (Figure 20) to those at which the shift began in the extracts (Figure 21), we note that the latter occurred at 10 - 15% lower concentrations. This suggests that the extracted chlorophyll-carrier complex is dissociated more readily than the complexes remaining in the chloroplast. At the higher solvent concentrations (greater than 50% acetone, or greater than 60% methanol) the dissociation of chlorophyll from its carrier in the extract becomes complete, and the spectral shift ceases; we now have solutions of molecularly dispersed chlorophyll. The unextracted chlorophyll also undergoes a change, ultimately approaching (but not quite reaching) its state in the molecular solution.

To gain more information about the spectral components present in the extracts, the half-bandwidths of the extracts were measured (see Figure 22). The half-bandwidth of the chlorophyll-carrier complex extracted with dilute solvents was 22 - 24 nm. It increased with the



Figure 21. Location of the red chlorophyll a absorption band in spinach chloroplast extracts, made with acetone and methanol of different concentrations.



Figure 22. Curves showing the half-bandwidth of the red chlorophyll a absorption band as a function of solvent concentration in extracts from spinach chloroplasts.

concentration of the solvent and reached a maximum at 30% acetone or 40% methanol; this is halfway down the slope of the curves in Figure 21. A mixture of extracted complexes and molecular chlorophyll probably exists at this concentration. The half-bandwidths of the low-concentration extracts are subject to considerable error because of the low absorption of the extracts. However, they are a little less than the half-width of the original chloroplasts, which was (excluding the chlorophyll <u>b</u>) about 29 nm.

The relative <u>amounts</u> of chlorophyll in the different extracts were estimated from absorbancy measurements. These results are plotted in Figure 23. The most conspicuous feature of these curves is the sudden increase in the amount of extracted chlorophyll above 60% acetone, and 75% methanol. We note that the increase occurs at solvent concentrations corresponding to the complete dissociation of chlorophyll from its carrier (Figure 21).

In Figure 23 we note a rather unexpected minimum in the amount of material extracted in the 50 - 60% region. This is the concentration region at which the broad 740 nm band (chlorophyll microcrystals?) appears in resuspended chloroplasts (cf. Figure 19).

In the case of resuspended chloroplasts (Figure 24), absorbance rises with increasing solvent concentration in the 20 - 40% range. This seems at first paradoxical -- the more chlorophyll we remove from the chloroplast, as shown by the increasing absorbance of the extracts (see Figure 23), the more there appears to be left in the chloroplast! However, the phenomenon has a plausible explanation. With the break-up of the pigment complexes in the chloroplasts, the mutual shading of pigment molecules (the "sieve effect") is decreased. This is why the integrals of the absorption bands are lower in vivo than in solution. Since we have evidence that dilute solvents extract a chlorophyll-carrier complex, it is not unreasonable to suggest that at a little higher solvent concentration, the solvent, in addition to removing more of the chlorophyll complex, also disrupts the organization of the chlorophyll-carrier complex remaining in the chloroplasts, so that the chlorophyll-carrier complex becomes more evenly distributed, and thus acquires a higher apparent extinction coefficient.

If the chlorophyll were homogeneously distributed in the cell suspension, and the red absorption band strengths were the same in vivo as in vitro, then the sum of the band integrals of the extract and the corresponding chloroplast residue would be the same for all solvent concentrations (see Figure 25). Experimentally, however, the sum of these integrals is found to vary by a factor of two to three. In general, the expected increase in total absorption with increasing extraction is observed. The dip in the curves that occurs around a 60% solvent concentration is most likely due to the formation of the 740 nm chlorophyll complex (microcrystals?) in the resuspended chloroplasts. When the band integrals are extended to include the 740 nm region, the decreases at 60% disappear.

b. Fluorescence Spectra

The fluorescence spectra of the solvent extracts and of the resuspended chloroplasts were measured at room temperature, and at liquid nitrogen temperature. As in the case of the absorption spectra, the location of the fluorescence peaks cannot be measured with a precision greater than ± 0.5 nm. Figure 26 shows representative fluorescence curves obtained at room temperature by excitation at 420 nm. A main band appears at 675 nm in the acetone extract, and at 685 nm in the chlorplasts; both are followed, at the longer wavelengths, by the first





vibrational band at 735 - 740 nm. When the locations of the main fluorescence peaks are plotted, the curves shown in Figures 27 and 28 are obtained. In the case of the resuspended chloroplasts (Figure 27), we note that the shift in the position of the fluorescence peaks occurs at the same concentrations as the shift in the corresponding absorption peaks (Figure 20). This parallelism in the displacement of the absorption and the fluorescence bands was to be expected. At low solvent concentrations (less than 25% acetone, less than 40% methanol), the absorption and the fluorescence peaks remain at wavelengths characteristic of intact chloroplasts, beyond these concentrations, the absorption and the fluorescence bands begin to shift toward shorter wavelengths. When the locations of the fluorescence maxima in the extracts are examined (Figure 28), a more complicated picture appears While in Figure 21 the absorption peak of the extract remains for a while in the position characteristic of intact chloroplasts, the fluorescence peak begins to shift at once In methanol extracts, the fluorescence peak displacement ends at 60% -- at the same concentration as the displacement of the absorption peak ends. In acetone, instead of the displacement ceasing at 60% (as in the absorption spectra, Figure 21), the fluorescence peak shift continues up to 100%.

We have previously noted that chlorophyll in the extracted chlorophyll-protein complex seems to be more susceptible to solvent attack than the chlorophyll-protein complex remaining in the chloroplast. Fluorescence spectra are generally more sensitive than absorption spectra to slight displacements of the Morse potential curves that can arise through chemical interaction At the low solvent concentrations, at which "unchanged" chlorophyll-carrier complexes are extracted, methanol and acetone nevertheless disturb the chlorophyll energy levels enough to be detected as shifts in the fluorescence bands. At the higher solvent concentrations,

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Figure 27. Location of the chlorophyll a fluorescence maxima at room temperature in resuspended spinach chloroplasts as a function of solvent concentration. One curve is for extraction with acetone, the other for that with methanol.



SOLVENT CONCENTRATION- % Figure 28. Location of the chlorophyll fluorescence maxima in extracts obtained from spinach chloroplasts by solvents (acetone and methanol) of different concentrations.

greater than 50%, chlorophyll is entirely dislodged from its carrier. Two factors may contribute to the continued shift of the fluorescence band in molecular solutions First, there is a continuing change in the dielectric constant of the solvent as the concentration of the solvent increases, second, an increasing number of the molecules of the solvent attach thems elves to the chlorophyll molecule at the expense of dislodged water molecules. The differences between the curves for acetone and methanol suggest differences in their effectiveness in displacing water molecules, due perhaps to different chemical affinity and different molecular size.

The relative fluorescence yields were calculated for both extracts and resuspended chloroplasts (Figures 29 and 30) In the case of the extracts, we note that at low solvent concentrations (less than 20%) the fluorescence yield of extracted material (excited in the blue to the second singlet level) is about one tenth of that at the highest solvent concentrations. This is added evidence that the initially extracted material is the natural chlorophyll-carrier complex, which is known to fluoresce with a yield of only about 3% (when excited in the blue band). In solutions, the chlorophyll a fluorescence yield is of the order of 30% and is independent of the wavelength of the exciting light The fluorescence yield of the resuspended chloroplasts merely doubles after treatment with concentrated solvent. The relative smallness of the effect can be attributed to the continued attachment of the carrier (protein?) to the non-extracted chlorophyll. The ups and downs of the curves in Figure 29 are remarkable. They may be indicative of the existence in the chloroplast of different chlorophyll complexes, with different fluorescent capacities. The minimum yields, observed in 50% acetone and 60% methanol, most likely are due to the formation, in this region, of pigment complexes (perhaps

Figure 29. Fluorescence yield (quanta emitted/quanta absorbed) of resuspended spinach chloroplasts, after extraction with solvents (acetone and methanol) of different concentrations.



Figure 30. Fluorescence yield (quanta emitted/quanta absorbed) of extracts obtained from spinach chloroplasts. The extracts were made with acetone and methanol of different concentrations.

microcrystals) giving rise to the 740 nm absorption band (Figure 19). The fluorescence spectra of the resuspended chloroplasts at -196° C, to be presented later, give additional indications of the complexity of the changes that occur during chloroplast extraction, in addition, they strongly suggest differential extraction of the different complexes.

That the material extracted from chloroplasts at low solvent concentrations contains chlorophyll-carrier complexes and not aggregated chlorophyll molecules, is confirmed by observations on colloidal chlorophyll

The details of the preparation of colloidal chlorophyll solutions were as follows.

Spinach leaves were ground in an ice-cold mortar in dim light. When they were reduced to a pulp, 20 ml of acetone were added, and the resulting slurry immediately centrifuged at 12,000 g for about three minutes. The supernatant, containing dissolved chlorophylls, was isolated and its absorbance adjusted to a predetermined value by the addition of more acetone. A series of 10 ml acetone-water solutions of increasing acetone concentration were made, and 0 5 ml of the pigment extract was added to each. The absorbance of the chlorophyll in these colloidal solutions was the same as in the solvent extracts studied previously.

Colloidal solutions obtained in this way contained the carotenoids as well as the chlorophylls <u>a</u> and <u>b</u>. They thus approximate any colloids which might be obtained by the extraction of the spinach chloroplasts with dilute solvents. (All chloroplast pigments were present in these extracts.) Upon addition of the pigments, a turbidity was immediately noted at acetone concentrations under 40%. (Turbidity was also noted in the low solvent concentration extracts.) This suggests that particles with linear dimensions of at least a hundred nanometers were present in both cases.

Although the properties of a colloid change with the degree of aggregation, and it may yet be possible to prepare pigment colloids with physical properties similar to those of the cell extracts in dilute solvents, the colloids I was able to prepare differed greatly from the extracts. Figure 31 shows shows the red absorption peak in the colloid to be located at 670 - 672 nm, while in the extracts (Figure 21), this peak lies at 677 nm. At acetone concentrations less than 40%, the fluorescence yield of the colloidal pigment (Figure 31) is low -- only 4% of that in pure acetone. In the case of cell extracts in dilute acetone, the fluorescence yield starts at about 12% of that in pure acetone (Figure 30) and rises to 50% by the time the acetone concentration reaches 40% The location of the fluorescence peak in colloids (Figure 32) also displays an entirely different behaviour from that in the extracts (Figure 28). All this confirms the supposition that what is extracted from the chloroplasts by dilute solvents is a chlorophyllbearing complex, rather than a colloidal chlorophyll aggregate.

The fluorescence spectra of the extracts, of the resuspended chloroplasts, and of the chlorophyll colloids, were measured also at -196° C. At this low temperature, excitation energy losses, both by internal conversion and by bimolecular quenching, are lowered. Therefore, pigments which, at room temperature, fluoresce only weakly or not at all, often become strongly fluorescent. In these experiments, 2.0 ml samples were frozen on the window in the bottom of the Dewar flask, and then covered with liquid nitrogen to a depth of 5 cms. A significant increase, both in the total <u>intensity</u> of fluorescence and in the complexity of the band <u>structure</u> occurred at this low temperature. (Results relating to the intensity of fluorescence must, however, be treated with caution because of the non-uniform thickness of the frozen samples.)



We will discuss the results of the experiments at -196° C in order of increasing complexity. The simplest fluorescence spectra were obtained with the colloidal solutions, prepared as before by the extraction of chloroplasts with pure acetone, and subsequent dilution with water. These spectra were similar to the room temperature fluorescence spectra of the same colloids. The only obvious difference was a small shift of the fluorescence maximum toward the longer wavelengths Both resembled the simple spectra illustrated in Figure 26.

At solvent concentrations greater than 50%, the chloroplast extracts of acetone and methanol also display simple fluorescence spectra similar to Figure 26. This type of spectrum must be characteristic of [^] both molecularly dispersed chlorophyll, and of certain colloidal aggregates.

At lower solvent concentrations (less than 40%), where we assume the existence of a chlorophyll-carrier complex, the fluorescence spectrum at -196° C is much more complex. The acetone extracts show a second, broad fluorescence peak in the neighborhood of 710 nm. Figure 33 shows the disappearance of this peak at acetone concentrations above 35%. Since we previously noted that methanol is less effective than acetone in removing chlorophyll from its carrier, we expected to find stronger evidence of the presence of complexes in the methanol extracts than in the acetone extracts. This is indeed the case. As many as four strong components can be discerned in the fluorescence spectra of dilute methanol extracts at -196° C (see Figures 34 and 35).

The fluorescence spectra of dilute methanol extracts are plotted in Figure 35, along with a spectrum representative of a concentrated methanol extract (dashed line). An unmistakeable trend is seen, complexes with fluorescence bands at the longest wavelengths appear most strongly at the lowest methanol concentrations (10%), while those
represented by the shorter wavelength bands appear more strongly in the 30 - 40% region. Finally, at concentrations greater than 50%, the 674 nm fluorescence band, characteristic of molecular solutions of chlorophyll <u>a</u> in methanol, emerges as by far the strongest component (see Figure 34). The curves obtained in the 30 - 40% methanol are somewhat similar to those observed in the dilute acetone extracts, where a single broad band at 710 nm (see Figure 33) replaces the several separate peaks displayed in the dilute acetone. All the long-wave bands disappear when the complexes dissociate and release free chlorophyll molecules; as often noted before, this dissociation occurs in methanol extracts at concentrations about 10% higher than in acetone.

The fluorescence spectra at -196° C of the chloroplasts resuspended after fractional extraction display a clear three-peak pattern. Representative members of this family of curves are given in Figure 36. These three components are separated by about 30 nm, compared to the much more narrow separation in the extracts (10 - 15 nm). As a result, there is considerably less shifting of the maxima as the relative amplitudes of the components vary. The sequence of spectral changes occurring in the resuspended chloroplasts is very clear. Initially, in the nonextracted chloroplasts, three components are present, two strong ones and one weak one. The long-wave band at 735 nm predominates over the 700 nm band, the short-wave 680 nm band is still weaker. The band at 735 nm decays very rapidly with increasing solvent extraction; it is essentially gone when a 50% solvent is reached. The 700 nm component is not appreciably weakened up to 50% methanol, but it, too, disappears in more concentrated solvent. The 680 nm band is relatively weak in chloroplasts extracted with low concentration methanol, but as more concentrated solvents are used, it becomes predominant in the residue from extraction,



Figure 33. Fraction of total fluorescence belonging to the 710 and the

Figure 34. Strength of the four fluorescence band components as a function of the methanol concentration. (Fractional contribution of each component to the total fluorescence calculated from the height of the several peaks.)



WAVELENGTH - nm Figure 35. Fluorescence spectra of dilute methanol extracts of spinach chloroplasts at -196° C for different solvent concentrations.

until finally it is almost the only component left in the extracted chloroplasts.

In Figure 37, the contribution of the three components to the total fluorescence is plotted as a function of methanol concentration. Comparable results were obtained with the acetone-extracted chloroplasts; they are summarized in Figure 38. The broad band at 710 nm was tentatively resolved into two components.

To summarize these results the components with long-wavelength fluorescence bands seem to be extracted from the chloroplasts first (1. e., in the weakest solvent) and appear most strongly in the extracts prepared with such solvents. At the higher solvent concentrations, the 685 nm component also leaves the chloroplast and becomes a strong component in the fluorescence spectrum of the extract. Upon still further increases in in solvent concentration (greater than 60% methanol), all chlorophyllcarrier bonds are broken and we obtain, in extracts, a fluorescence spectrum peak at 675 nm characteristic of molecularly dispersed chlorophyll. The traces of chlorophyll left in the chloroplasts after such drastic extraction have a fluorescence band at 680 nm -- not quite identical with that of molecular chlorophyll in organic solvents, but possibly corresponding to a molecular solution in a solvent of very high dielectric constant, such as a lipid.

2. Digitonin Solubilization

A digitonin-solubilized chloroplast solution was separated into four fractions by means of the ultra-centrifuge. The fractions were kept at 0° C. until used; five hours usually separated the grinding of the spinach \cdot leaves and the beginning of the measurements. Details of the solubilization



Figure 36. Fluorescence spectra at -196° C, showing the changes which occur with changing methanol concentration.



Figure 38. Fraction of total fluorescence belonging to each component as a function of acetone concentration. The amount of fluorescence was estimated from the height of the fluorescence band.

Figure 37. Fraction of total fluorescence belonging to each component as a function of methanol concentration. The amount of fluorescence was estimated from the height of the fluorescence band.

and fractionation procedure are given in Section C (Growth and Preparation of Plant Material) of section II.

a. Absorption Spectra

The 580 - 750 nm region of the absorption spectrum of the initial solution of digitonin-solubilized chloroplasts, "Fraction O", and the four fractions, 1 - 4, from the ultra-centrifuge, are presented in Figure 39. Examination of these curves shows increased chlorophyll b content in Fraction 1 (compared to "Fraction O") and its decrease in Fractions 3 and 4. The distribution of accessory pigments among the different fractions is presented in a graph (see Figure 40). We note the accumulation of chlorophyll b, with its absorption band at 650 nm in Fraction 1 and its corresponding decrease in Fractions 3 and 4. The only reliable position to measure carotenoid content is at the 480 nm shoulder, and here absorption is 55% by chlorophyll b and 45% by carotenoids. The results here are, however, consistent with those obtained at 650 nm for chlorophyll b. (Figure 39 does not show the carotenoid region of the absorption spectrum.) This shows that as the natural pigment complexes are broken down by the detergent, chlorophyll \underline{a} is accumulated in the smaller particles and more accessory pigments are left in the larger particles. This agrees with the picture of two types of pigment complexes being present in photosynthesizing cells, one containing mostly chlorophyll a, and the other, accessory pigments with some chlorophylla. Complexes of the first kind are associated with the smaller particles, those of the second kind with the larger ones.

The locations of the red absorption maxima were measured and plotted in Figure 41. The red absorption maximum of whole chloroplasts lies at about 676 nm, between those of Fractions 1 and 2 on the short-



Figure 39. Absorption spectra of the digitonin-solubilized spinach chloroplast fractions. These fractions were adjusted (by dilution) to comparable absorbancies before measurements were made. Curves are displaced vertically to permit clear comparison of their shapes.

wavelength side and those of Fractions 3 and 4 on the long-wavelength side. This could be due entirely to differences in chlorophyll \underline{b} content, and thus would provide little additional evidence for the existence of two spectral types of chlorophyll a.

We can, however, imagine the smaller particles to be enriched in the "long-wavelength" type of chlorophyll <u>a</u>, while the larger particles are enriched in the "short-wavelength" type of chlorophyll <u>a</u>, and chlorophyll <u>b</u> The wavelength maxima were measured very carefully, and although their accuracy is still not better than + 0.5 nm, their precision is + 0.2 nm.

b. Fluorescence Spectra

The fluorescence spectra of the fractions obtained from the digitonin-solubilized chloroplast preparations were measured both at room and at liquid nitrogen temperatures. The room temperature spectra displayed the simple fluorescence pattern -- a main peak followed at the longer wavelengths by a vibrational sub-band. Examples of this type of spectrum are presented in Figure 26. At liquid nitrogen temperatures, a more complex structure appears. Representative fluorescence spectra at -196° C are presented in Figure 42, in which two strong components and a third weaker component are indicated. We note that the components are distributed unequally among the fractions. In the smaller-particle fractions, there is more of the 735 nm component compared to the 695 nm component (which may be identical with the 700 nm component in Figure 36). Again, the "long-wavelength" chlorophyll <u>a</u> appears to accumulate in the smaller particles; we have noted before that it is also preferentially extracted by low-concentration solvents.



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Figure 40. Variation in the distribution of pigment components between the 1 -- 4 digitonin-solubilized fractions, as compared to the original suspension. The absorption curves of all fractions were normalized in the peak of the chlorophyll a red band. The differences were measured at 650 nm (region of high chlorophyll b absorption) and at 480 nm (region of carotenoid and chlorophyll b absorption).



Figure 41. Location of the red chlorophyll a absorption band in different fractions of digitonin-solubilized chloroplasts.



Figure 42. Fluorescence spectra at -196° C of digitonin-solubilized spinach chloroplast fractions.

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The photochemical properties of these fractions were investigated by means of fluorescence-quenching experiments. The results are presented in Table 3. The first thing to note is that the observed changes in fluorescence intensity were very small; however, the following observations seem of some interest. (The limit of precision of these measurements is in the region of $\pm 0.2\%$.) The fluorescence of the original preparation, "Fraction O", was unaffected by the addition of quenching agents -- perhaps due to the great bulk of the particles. The higher fractions, representing the smaller fragments of chloroplasts, should permit more intimate contact of the pigment with the quencher.

We note that the fluorescence of the smaller size fractions, 1 and 2, which contained a greater proportion of the "695 nm component", is measurably quenched by the addition of oxidized cytochrome, the effect on Fractions 3 and 4 is smaller. The action of DCMU (3-3, 4-dichlorophenyl-1, 1-dimethylurea), a commonly used poison for photosynthesis, was the same on all fractions. it abolished the effects of the addition of cytochrome c Sodium ascorbate, on the other hand, affected the fluorescence of Fractions 3 and 4 more than that of Fraction 2. (The effect was in all cases enhancing, rather than quenching.) The effect of reduced cytochrome c with and without NADP is too slight for meaningful interpretation.

c. Particle Size and Polarization of Fluorescence

The size of the particles in the four fractions from the digitonin-solubilized and centrifuged spinach chloroplasts was estimated from photographs taken with an electron microscope, using a chromium-shadowing technique. Examples of such photographs are shown in Figure 43. The particles in Fraction 4 had a uniform size of 200 ± 50 Å



Figure 43. Electron Photomicrographs of the Digitonin-Solubilized Fractions.

TABLE 3

PERCENT CHANGES IN FLUORESCENCE

SOLUTIONS ADDED	FRACTION				
			(20 ml al:	alıquots used)	
	0	1	2	3	4
0.4 ml H ₂ O	*	*	*	*	*
0.2 ml oxidızed cytochrome c 0.2 ml H ₂ O		-0.9	-2.6	-1.6	-0.5
0.2 ml oxidized cytochrome c 0.2 ml DCMU					
0.2 ml reduced cytochrome c 0.2 ml H ₂ O			+0.2	+0 5	+0.4
0.2 ml reduced cytochrome c 0.1 ml TPN 0.1 ml H ₂ O			+0.2	+0.5	
0.2 ml reduced cytochrome c 0.1 ml TPN 0.1 ml DCMU					
0.4 ml Na ascorbate	not measured	not measured	+1.5	+3. 4	+2.6
Oxidized cytoch DCMU TPN Sodium Ascorba Reduced cytoch:	rome c ite rome c	1. 6	$5 \times 10^{-4} M$ $10^{-4} M$ $8 \times 10^{-3} M$ $10^{-3} M$ $0 \times 10^{-4} M$ $0 \times 10^{-4} M$	A f oxidized cyto sodium asco	ochrome c rbate

The reduced cytochrome c was made up by the addition of sodium ascorbate to oxidized cytochrome c. The chemical equilibrium, as checked by measurement of the absorption spectra, was shifted far toward the reduced cytochrome side.

* A dilution correction derived from experiments in the first line of the table has been applied to each figure in the table.

-- No significant fluorescence change

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(comparable to that of the macromolecules observed in the chloroplast lamellae, and perhaps identical with the "photosynthetic units"). Fraction 3 consisted of discs, and clumps of discs, about 1000 Å in diameter, with very few of the small particles that formed the bulk of Fraction 4. Fraction 2 consisted of large discs (4000 - 5000 Å in diameter) and apparently amorphous bodies in which the smaller 1000 \tilde{A} discs of Fraction 3 appeared to be imbedded. In addition, there was a background of threadlike filaments of 100 Å thickness, with lengths up to 1000 Å. Some of the small round bodies (macromolecules?) that appeared in such great abundance in Fraction 4 were also scattered about in this fraction. Fraction 1 appeared to be made of particles only a little larger (about 7000 Å in diameter) than the larger particles of Fraction 2. An occasional 1000 A disc and a 200 Å "macromolecule" also appeared in Fraction 1. For the most part, the large discs appeared to be featureless, they lacked the smaller 1000 $\stackrel{o}{A}$ discs that were a feature of Fraction 2.

To obtain information concerning the orderly arrangement of chlorophyll <u>a</u> molecules in each fraction, measurements were made of the degree of polarization of chlorophyll <u>a</u> fluorescence excited by polarized light. Depolarization can occur by rotation of the excited molecule during the excitation time, and energy transfer to a slightly differently oriented molecule. The results obtained on Dr. G. Weber's fluoropolarimeter are shown in Table 4. The one significant result is the increase in polarization that occurs in the smaller fractions, 3 and 4. In other words, chlorophyll in the 200 $\stackrel{\circ}{A}$ particles (photosynthetic units?) prevalent in Fraction 4 seems to be better organized than that accumulated in the other fractions.

TABLE 4

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POLARIZATION OF FLUORESCENCE . OF DIGITONIN SOLUBILIZED FRACTIONS

Fraction	Polarization of Fluorescence
0	2.66%
1	2.92%
2	3.01%
3	5.13%
4	5.40%

This table shows the degree of polarization of chlorophyll fluorescence when excited by polarized light of 420 nm.

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IV. DISCUSSION'

The experiments will be discussed here in the same general order in which they were presented. Accordingly, the first topic to be considered is the resolution of the red chlorophyll <u>a</u> absorption bands <u>in vivo</u> into components (experiments presented in section III A).

A. Analysis of the Red Absorption Band of Chlorophyll a In Vivo

The shape of the red absorption band of chlorophyll <u>a in vivo</u> first led Albers and Knorr (3) to suggest the presence of several components within the band envelope. Evidence for such complex character of the band then came from French's experiments (29) with a "derivative spectrophotometer." This instrument plotted the first derivative of the absorption curve instead of the curve itself, and showed that apparently smooth absorption curves often concealed changes in slope indicative of the superimposition of several components. With the aid of a curve analyzer, Brown and French (16) reconstructed the derivative curve of <u>Chlorella pyrenoidosa</u> as a sum of the derivatives of several Gaussian curves, $y = ae^{-bx^2}$; they concluded that the red chlorophyll band in <u>Chlorella</u> contains <u>four</u> components, one assignable to chlorophyll <u>b</u> and three to chlorophyll <u>a</u>. Finally, using a very precise integrating spectrophotometer, Cederstrand (19) obtained direct evidence of the complex structure of the absorption band of chlorophyll <u>a</u> in live cells, without resorting to differentiation.

Absorption curves of algae which had been engaged in photosynthesis within the last 30 minutes before measurement consistently suggested the presence of only one chlorophyll <u>b</u> and <u>two</u> chlorophyll <u>a</u> components. More than two chlorophyll <u>a</u> components generally appeared only in algae

that had been kept in the dark or in low light for several hours. Figures 5, 15, and 17 are representative of the first, Figure 14 of the second kind. The latter suggests three strong chlorophyll <u>a</u> components, plus one weak long-wavelength component Thomas (67) found evidence of as many as <u>eight</u> spectral components (irregularities in the shape of the absorption curve obtained with a Beckman DK 2) in the absorption spectra of chloroplasts from <u>Aspidistra elatior</u>. I, however, have never observed an absorption spectrum suggesting the presence of anywhere this number of components.

As mentioned above, Brown and French had obtained an excellent match between the sum of the derivatives of four Gaussian components and the derivative absorption curve of Chlorella. To achieve this match, they had to add a third, weak band of chlorophyll <u>a</u> at 694 nm on one end, and to displace a broadened chlorophyll b band toward shorter wavelengths on the other end. I have doubts about both proceedings. I would expect the shape of the absorption curve of each spectral component to be more similar to that of the red band of chlorophyll a in solution than to a Gaussian curve. Since the solution curves are not strictly Guassian, I would expect a sum of Gaussian components to "mismatch", particularly in the tails of the absorption curves (see Figure 16). Therefore, the need to postulate a 694 nm component to reconstruct the long-wavelength tail, as proposed by Brown and French appears doubtful to me (even if other reasons exist for postulating a chlorophyll a band in vivo somewhere in this location!). On the short-wavelength end of the red band, the presence of the first vibrational band of chlorophyll a is sufficient to make a mismatch inevitable Hence, I prefer to choose the position and the half-width of the chlorophyll b band in such a way as to obtain the closest fit in the immediate neighborhood of the chlorophyll b peak and to neglect deviations

at the shorter waves, rather than tool to follow French and Brown in shifting and broadening the chlorophyll \underline{b} b d band to **a** chieve **b**etter matching at the shorter waves.

Typical results of both are sreprese nted; Figure 17 shows what I would consider a reasonable fit for Chlorest rella, w hile Figure 18 shows the closer match obtained by using a broadene os ned and displaced chlorophyll b band (see also the chlorophyll b column of TaHs Table 2). An additional interesting observation suggested by Table 2 is that the locations of the chlorophyll a components remain the same (within the property + 1.5 nm) for all the spectra resolved, the short-wavelerne enth ch_10 rophyll a peak lies at 668 nm and the long-wavelength peak at 683 EB3 m; t De chloro phyll b component, if present, is located at 651 nm. It is i specific is that the half-bandwidths of the chlorophyll a components in the sachney stis and Porphyridium spectra are about 50% broader than in Chlorio orella; their positions are the same as in the Chlorella cells, and their rel Is elative i mtensities do not differ from those in Chlorella by more than 10%0% in fact, the greatest deviation in this ratio was found in spinach chlorologolas \mathbf{e} s, where the height of the 668 nm band was only 75% of that of the see Mnr band. (Perhaps, in this case, part of the 668 nm complex was leaoseached or it in the preparation of the chloroplasts.)

It is remarkable that the row relative amounts of the two components -chlorophyll <u>a</u> 668 and chlorophyll <u>a</u> $\underline{sa}(\emptyset)$ -- are the same in red and bluegreen as in the green algae. Emergeners eff ect studies suggest that in green algae, chlorophyll <u>a</u> 668 belongs primerimeril **t** to the so-called pigment system II, and chlorophyll <u>a</u> 683 to pigment instage I.

The early "red drop" in this the phy cobilin-containing algae, and the absence of a 670 nm peak in the activizion spectrum of the Emerson enhancement effect, suggest that the 668 nm main component in these algae does not

belong to system II -- a disturbing conclusion. We would like to associate the doublet structure of the main chlorophyll <u>a</u> band with the division of chlorophyll <u>a</u> between the two pigment systems -- but this, unfortunately, seems not to apply to the two phycobilin-carrying types of algae.

To sum up: if we assume that the true shape of the chlorophyll <u>a</u> (or <u>b</u>) absorption curve components <u>in vivo</u> is best represented by the shape of the absorption curves of chlorophyll <u>a</u> (or <u>b</u>) in solution, then our analysis suggests the presence of only two components in the "light-adapted" state of chlorophyll a and of one or two more in some "dark-adapted" states.

The doublet nature of the chlorophyll <u>a</u> absorption band <u>in vivo</u> is supported by many indirect observations. For example, Govindjee and Rabinowitch (33), when repeating Emerson's measurements (27) of the action spectrum of the enhancement effect more precisely, found evidence of the presence of two kinds of chlorophyll <u>a</u> in <u>Chlorella</u>, one with a band at 670 nm, producing the enhancement effect, and one absorbing at and above 680 nm and not contributing to it. The half-width of the 670 nm band determined by these enhancement measurements was found to be about 19 nm, agreeing well with the half-width of about 18.0 nm determined by Gaussian analysis.

B. Analysis of the Fluorescence Spectrum of Chlorophyll a In Vivo

With two components in the absorption band, one might expect two components in the fluorescence band. The existence of a second component in the fluorescence band of chlorophyll <u>in vivo</u> was discovered by S. Brody (12), he observed, in <u>Chlorella</u>, at -196° C, a strong fluorescence peak at 720 nm, in addition to that at 685 nm, observed at room temperature. Later, Govindjee (31), Kok (47), Brody and Brody (13), Bergeron (8) and Litvin et al. (54) noted that, at low temperatures, the 685 nm peak itself is composed of two components. This raised the total number of fluorescence bands at -196° C to three; their locations varied according to the plant material used (in blue-green and red algae, a fourth band at 740 nm appears even at room temperature).

The complex character of the fluorescence band of chlorophyll a in spinach chloroplasts at room temperature was suggested by Govindjee and Yang (35), on the basis of an analysis of the fluorescence spectrum by Weber's "matrix" method (measurement of the relative intensity of fluorescence at different wavelengths, excited by absorption at different wavelengths). By observing directly the shape of the fluorescence spectrum, they found that it is affected by various reagents, suggesting the presence of (at least) two fluorescence components with different sensitivities to quenching. Later, Krey and Govindjee (51) found that the shape of the envelope of the fluorescence band at room temperature could be changed by using different excitation intensities Further evidence for doublet structure of the fluorescence band was provided by Lavorel (53), who noted an increase in the polarization of fluorescence in Chlorella when excited by light absorbed primarily by chlorophyll a in pigment system I, 1 e., in the region above 680 nm. A similar behaviour was observed by Govindjee and Weber (34) in Porphyridium. They, too, concluded that polarized fluorescence may be associated with the "long-wavelength form" of chlorophyll a in pigment system I. Perhaps this polarized fluorescence may be due to chlorophyll a molecules, whose regular arrangement was demonstrated by Olson et al. (59). They proposed that these molecules are closely associated with an enzymatic reaction center ("trap"). According to Kok (46), the absorption band of the chlorophyll a molecules associated with the "trap" for system I (P 700) lies in the neighborhood of 700 nm. Olson et al. (60) described experiments suggesting

that this polarization is due to a fluorescence band component at 716 nm. We can, therefore, postulate that this polarized fluorescence is due to Kok's "P 700".

The fluorescence decay curve of chlorophyll <u>a</u> in <u>Chlorella in</u> <u>vivo</u> was found, by Murty and Rabinowitch (58), to consist of two component curves, both with decay constants in the nanosecond range distinct from the much slower decaying "delayed" fluorescence of Arnold and Davidson (4)]. It remains to be seen whether these two decay times correspond to different fluorescence bands.

If our resolution of the red band in "light-adapted" cells into two components (Figures 17 and 18) is considered as certain, we must conclude that the <u>half-bandwidth</u> of each component is relatively narrow -approximately 19 nm in green-algae, and 23 - 26 nm in blue and red algae The first value is less than the average half-bandwidth of chlorophyll <u>a</u> in solution, 19 nm in ether, 20 nm in benzene, isobutanol, and carbon tetrachloride, 22 nm in octanol, and 23 nm in methanol (36). This result was a little unexpected. The high density (10^{-2} M) of the pigment system <u>in vivo</u> [cf Rabinowitch (63)] could be expected to <u>widen</u> the band, although certain types of aggregation are known <u>in vitro</u> which sharpen the absorption band cf. Jelley (42). This sharpening is due to rapid energy migration, destroying the coupling of electronic excitation with intramolecular vibrations, whether energy migration in "photosynthetic units" can account for the relative sharpness of the chlorophyll <u>a</u> 668 and chlorophyll a 682 components in vivo remains to be seen.

A crystalline structure of more than a very small portion of the chlorophyll <u>a in vivo</u> is improbable, because the position of the red absorption band at 675 nm is still far from its position in crystalline

chlorophyll of 740 nm reported by Rabinowitch <u>et al.</u> (64) and Jacobs and Holt (40).

The relative narrowness of the absorption band components <u>in</u> <u>vivo</u> also speaks against <u>photoconduction</u> as the primary effect of light absorption. Substitution, <u>in vivo</u>, of a conductance level for the molecular excitation level of chlorophyll <u>in vitro</u>, first suggested by Katz (43) and later by Arnold and Davidson (5), Arnold and Maclay (6), and Kearns <u>et</u> <u>al.</u> (44), should have caused a broadening of the absorption band, as well as a greater displacement than is actually observed.

C. Fractionation of Chlorophyll a by Extraction and Solubilization

Our objective is to present a consistent picture of the results of the absorption and fluorescence experiments on the several fractions obtained by extraction and solubilization. The spectral components observed can be then related to the pigment groups postulated in the twostep mechanism of photosynthesis (see Figure 1).

Since we believe that there are two main spectral components in the chlorophyll <u>a</u> band <u>in vivo</u>, we must accordingly postulate two major kinds of chlorophyll <u>a</u>-bearing complexes. A widely accepted picture is that these groups correspond, at least in green algae, to chlorophyll <u>a</u> associated with pigment system I, and pigment system II, respectively. The difference in the location of the absorption peaks of these two components may be due to differences in the carrier to which the chlorophyll <u>a</u> molecules are bound.

The similarity of absorption maxima (676 - 675 nm; cf. Figures 20 and 21), and fluorescence maxima (683 - 683 nm; cf. Figures 27 and 28), and the changes in fluorescence yields (cf. Figures 29 and 30) observed in vivo and in extracts made with dilute solvents, suggest that

these chlorophyll-carrier complexes are extracted from the chloroplasts essentially intact. [Thomas (70), too, has concluded that unchanged chlorophyll-carrier complexes are present in extracts obtained with dilute acetone from chloroplasts of <u>Aspidistra elatior</u>.]

Although absorption measurements on whole cells indicate the presence of two major chlorophyll <u>a</u> groups <u>in vivo</u>, we have no substantial evidence for their existence in the absorption spectra of the extracts, even in those made with dilute organic solvents.

The half-bandwidths observed in dilute solvent extracts are, however, consistent with the hypothesis that both kinds of chlorophyllcarrier complexes are present in these extracts (see Figure 22). The difficulty encountered in the measurement of both absorption and fluorescence bands in such extracts is that the amount of pigment extracted by the dilute solvents is small --too small to observe the slight irregularities in the band envelope which reveal the presence of two chlorophyll <u>a</u> components. When the solvent concentration is increased, and more pigment is extracted, the solvent begins to break the chlorophyll <u>a</u>-carrier bonds. The released molecular chlorophyll <u>a</u> possesses absorption and fluorescence bands which strongly overlap those of the original complexes. This makes attempts to observe the complexity of band structure as difficult in the stronger solvents as it had been in the more dilute solvents

The measurement of fluorescence spectra at -196° C revealed the presence of many band components. Since the samples could be melted and refrozen many times and would still display the same fluorescence spectra, the additional components do not arise from "ice-ruptured" pigment groups. As stated previously (IV B), the total number of fluorescence components displayed in vivo at -196° C is three. This triplet structure of the fluorescence band at -196° C is particularly clear in the fluorescence of resuspended chloroplasts (see Figure 36). The partitioning of the fluorescent components among the different fractions, in both solvent extracts and digitonin-solubilized fractions, strongly suggests that a partial separation of the pigment systems occurs in these processes.

The short-wavelength component (680 - 685 nm) present at -196° C in the florescence spectra of both the resuspended chloroplasts (see Figure 36) and the digitonin-solubilized fractions (see Figure 42), probably originates from the "bulk" of the chlorophyll <u>a</u>. Whether this fluorescence band originates in one or in both kinds of chlorophyll <u>a</u> is not suggested by our measurements. The half-bandwidth of the single fluorescence band (at room temperature) is about 25 nm in dilute extracts and thus comparable to the 29 nm half-width of the whole absorption band. The half-bandwidth of the fluorescence band of chlorophyll <u>a</u> in acetone is 22 nm. This is not incompatible with both kinds of complexes contributing to fluorescence, but the presence of molecular chlorophyll in the same sample makes the conclusion very uncertain (see below). The fluorescence band of molecular chlorophyll in acetone, located at a slightly shorter wavelength, could itself account for the broadening of the band.

The fluorescence spectra of the extracts at -196° C (see Figure 35) display four component bands which overlap so strongly as to make determination of the bandwidth of the component at 685 nm impossible. Its location at 685 nm suggests that it arises from the short-wavelength chlorophyll <u>a</u> absorbing at 668 nm in system II, rather than the long-wavelength chlorophyll <u>a</u> absorbing at 683 nm in system I. This suggests that a portion of the chlorophyll <u>a</u> in system I is non-fluorescent, a conclusion supported by the life-time measurements of Brody and Rabinowitch (15) and Tomita and Rabinowitch (71).

The 685 nm component in the extracts (see Figure 35), is preceded, on the short-wavelength side, by a fluorescence component at 670-675 nm, which may be attributed to "free", i.e., molecularly dissolved, chlorophyll <u>a</u>. This assignment is supported by the fact that this band grows as the solvent concentration in the extracts increases.

In accordance with an interpretation by Govindjee (31), the two long-wavelength fluorescence components, which appear at -196° C in all digitonin-solubilized fractions, as well as in all extracts in dilute solvents, and in resuspended chloroplasts after extraction, may arise from chlorophyll <u>a</u> molecules associated with the two "traps", postulated to exist in the two pigment systems. The band component at 740 nm may be associated with the "trap" in system I (absorption band at 700 nm), and that at 700 nm with the hypothetical "trap" in system II (absorption band somewhere near 680 nm). At room temperature, the fluorescence from these two "traps" is very faint because of quenching by the initial photochemical step of photosynthesis. At -196° C, cessation of all chemical activity makes energy available for fluorescence.

The preceding assignment of the several chlorophyll <u>a</u> fluorescence components to different functional groups is by no means certain. It is, however, consistent with the results of the cytochrome quenching experiments (Table 3), and with the NADP-reduction experiments of Boardman and Anderson (10). The larger particles from the digitonin solubilization, which displayed an accumulation of the chlorophyll-complexes giving rise to the 700 nm fluorescence band, which we have assigned to system II, were found incapable of reduction of NADP. Their fluorescence was quenched by the addition of <u>oxidized</u>, but not of <u>reduced</u> cytochrome c. The smaller particles from the digitonin solubilization, which showed an accumulation of the chlorophyll complexes giving rise to the 740 nm fluorescence band, and which we assigned to system I, were capable of NADP reduction (10). Their fluorescence was not quenched by oxidized cytochrome c.

An additional bit of information which the extraction experiments provide concerns the relative solubilities of the different complexes. Examination of Figure 36 reveals that the chlorophyll <u>a</u> which we have associated with pigment system I is more readily extracted by dilute solvents than the chlorophyll <u>a</u> associated with system II. This could account for the decreased half-width (22 - 24 nm, see Figure 22) of the absorption band of the most dilute extract, compared to the half-bandwidth (28 nm) of the intact chloroplasts.

Examination of the electron photomicrographs of Figure 43 shows that although the heavier fractions are mixtures of fragments of different sizes, Fraction 4 is composed of uniform 200 Å spheres. These may be the particles, observed by Steinman (65), which Park and Pon (61) named "quantasomes", and suggested to be the photosynthetic units. They displayed both a fluorescence spectrum and a photochemical activity which suggests enrichment of pigment system I. Polarization experiments (Table 4) show a more strongly polarized fluorescence from these 200 Å bodies, compared to that of the larger particles. Lavorel (53) and Govindjee and Weber (34) noted a greater degree of polarization of fluorescence when pigment system I was excited, and suggested that the pigment in system I may be more orderly than in system II. These results are consistent with the observations, described above, on Fraction 4 from the digitonin solubilization.

D. Summary

A series of algal absorption curves were measured with an

integrating spectrophotometer especially constructed for the purpose of examining in detail the shape of the red chlorophyll <u>a</u> absorption bands <u>in vivo</u>. On the basis of the curves obtained, the bands were resolved into two Gaussian components, tentatively identified, in the case of green algae, with the two pigment systems in photosynthesis. While the halfwidth (excluding chlorophyll <u>b</u>) of the band envelope in <u>Chlorella</u> is 32 nm, the half widths of the two components were found to be about 18 nm, those in <u>Anacystis</u> and <u>Porphyridium</u> were about 50% wider. The number and positions of the component bands were about the same in all algae studied, 668 nm and 683 nm, their relative heights differed between 0.7 and 0.9, with the 668 nm band always being the weaker one. In red and blue-green algae, the short-wave component, chlorophyll <u>a</u> 668, despite its unchanged position and intensity, does not seem to belong to "pigment system II", as in green algae.

Isolation of the two spectral components of chlorophyll <u>a</u> was attempted by extraction with aqueous acetone and methanol, and by digitonin solubilization, followed by differential centrifugation. In the case of the solvent extracts from spinach chloroplasts, the results suggest extraction of a complete chlorophyll-carrier complex by dilute solvents, and its breakdown in more concentrated solvents. Both the absorption and fluorescence spectra of the solvent-extracted, and of the digitoninsolubilized fractions suggest partial separation of the two spectral components. An indication that these two separated components are associated, in spinach chloroplasts, with the two proposed photochemical steps in photosynthesis, was noted in the effect of oxidized cytochrome c on the fluorescence of the several fractions.

Fluorescence spectra were measured at -196^oC for all fractions. A characteristic three-component spectrum was always observed in the digitonin-solubilized chloroplasts and in the chloroplasts extracted with dilute solvents. These three components (with fluorescence maxima at 685 nm, 695 - 700 nm, and 735 nm) partitioned themselves between the fractions in a manner that was consistent with partial separation of the two pigment systems. The 685 nm fluorescence band was associated with "bulk" chlorophyll <u>a</u>, probably partly or entirely in system II. The 695 - 700 nm band could be tentatively assigned to the "trap" in pigment system II, and the 735 nm band to the "trap" in pigment system I.

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APPENDIX

A. Suggestions for Further Development of the Spectrophotometer, and Circuit Diagrams

1. If an iodine quartz ribbon filament lamp could be made, a worthwhile increase in the energy available in the blue might be attained

2. As it is presently operated, the motor which drives the sector disc is driven from a 60 cycle frequency standard. This was necessary because of frequency variations in the University power line Otherwise, the sector disc, being locked to the line frequency by the synchronous motor, would faithfully follow the University master generator This superimposed upon the absorption signal a second signal generated in the synchronous switch by the phase changes produced because of the different frequency determining elements in the signal and chopper amplifiers. The spectrophotometer was operating as a frequency meter monitoring the University line frequency! The elegant solution is to redesign the amplifier channels so that they are phase tracking. This could be done by stagger tuning a pair of twin T filters in each channel, spaced to give a flat topped frequency response curve, and, as a consequence, increased skirt selectivity. The reduction of skirt response would reduce the noise level a little. The problem of noise being generated by frequency variations would then be incontestably eliminated.

3. Examination of the chopper amplifier reveals a pair of pushpull 6AQ5's driving the transistor chopper (see Figure 44). Originally, this output stage was designed to drive a mechanical chopper; it could easily be eliminated and the transistor chopper driven instead by cathode follower. A slight reduction in noise would be realized by driving the transistor chopper with a square wave. There would be a gain from two sources by doing this. first, the time indeterminacy about when the transistor switches would be reduced, second, since any noise in the chopper amplifier leaks, through a transistorized chopper and into the signal channel, the more noise-free top of the square wave would be of benefit.

4. The 5879 used in the input stage (see Figure 45) should be replaced by a 7543, a recently developed pentode with a little lower noise and higher gain.

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Figure 44. Circuit Diagram of Chopper Amplifier.



Figure 45. Circuit Diagram of Signal Amplifier.

B. Chemical Composition of Culture Media

The chemical composition of the inorganic growth media used for the algal cultures is presented in Tables 5 and 6.

TABLE 5

CHEMICAL COMPOSITION OF CULTURE MEDIA

Salt	Concentration of stock solution	Aliquot of stock solution/liter of culture medium		
		Chlorella and Anacystis	Porphy- ridium	
MgSO ₄ · 7H ₂ O	50 gm/l	5 ml	50 ml	
кн ₂ ро ₄ `	25 gm/l	75 ml		
Na_2HPO_4	25 gm/l	l ml		
к ₂ нро ₄	25 gm/l		20 ml	
KNO ₃	2 5 gm/l	50 ml	50 ml	
CaCO3	8 gm/l	2 ml		
Ca(NO ₃) ₂ . 4H	2 <mark>0 25 gm/l</mark>		10 ml	
KC1	25 gm/l	l ml		
KCl	320 gm/l		50 ml	
NaCl	250 gm/l		50 ml	
KI	10 gm/l		5 ml	
KBr	10 gm/l		5 ml	
A ₂	*	l ml		
A ₅	*		5 ml	
в ₉	*		5 ml	
ABC	*	0.5 ml		

*For composition of micronutrients A_2 , A_5 , B_9 and ABC, see Table 6.

TABLE 6

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MICRONUTRIENTS

A 2	MnCl ₂ ·4H ₂ O	1.81 g/l
	н ₃ во ₃	2.86 g/1
~ 3	ZnSO ₄ ·7H ₂ O	0.22 g/1
	CuSO ₄ ·5H ₂ O	0.079 g/l
	(NH ₄) ₆ M ₂₇ O ₂₄ ·4H ₂ O	0.20 g/l
-A ₅	A ₂ + A ₃ , with slight modifie Hoagland, (38)	cations taken from
1B ₉	(after Brody and Emerson, modifications)	(14) with slight –
		mg/l
	$Al_2(SO_4)_3Na_2SO_4$ ·24H ₂ O	. 786
	KBr	. 119
	KI	. 083
	$Cd(NO_3)_2 \cdot 4H_2O$. 154
	Co(NO ₃) ₂ ·6 H ₂ O	. 145
	NiSO4 · 6H2O	. 131
	Cr(NO ₃) ₃ ·9H ₂ O	. 040
	Na ₃ VO ₄ ·16H ₂ O	. 041
	$Na_2WO_4 \cdot 2H_2O$. 033
<_ ₁₀	As203	6. 61
١	srs0 ₄	10.49
	HgCl ₂	6. 77
,	PbCl ₂	6. 71
	LiCİ	30.55
	RbCl	14.20
	K ₂ TiF ₆ ·H ₂ O	5.00

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TABLE 6 (Continued)

	mg/l
NaSeO ₄	11.96
Be(NO ₃) ₂ . 3H ₂ O	103.7
Uranyl nitrate	10.0

ABC $5 \text{ ml } A_3 + 5 \text{ ml } B_9 + 5 \text{ ml } C_{10}$ made up to 100 ml.
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