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LIGHT INDUCED ABSORPTION CHANGES IN PHOTOSYNTHETIC ORGANISMS

BESSEL KOK

T.N.O. Solar Energy Project, Laboratory for Plant Physiology, Agricultural University, Wageningen (The Netherlands) [1])

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The general procedure for measuring photosynthetic difference spectra is to irradiate the sample under study with weak monochromatic light and observe the change of the light transmission induced by a strong "actinic" cross illumination of the cells. A difficulty involved is that the device registering the light transmission should not directly be influenced by the actinic beam or by fluorescence induced by it. The technique we will describe below was designed on the principle that most of the available photosensitive material in the cells can be charged by a strong lightflash of short duration and that the dark restoration reactions then require a finite time (a few milliseconds) for removing the photoproducts (*cf.* KOK, 1956). An interaction of the actinic and the measuring lights therefore can be prevented by separating them in time. This allowed the registration of complete difference spectra, including the long wavelength region.

APPARATUS

The set up used is shown in Fig. 1. Monochromatic light left a Bausch and Lomb 10 × 10 cm grating monochromator and was concentrated on a small reaction vessel (ø 5 mm). The transmitted and scattered light was collected by a high aperture lens and concentrated on a small opening in a fixed screen. Behind this opening a red sensitive photomultiplier (Mauer Vp 690 J) was mounted. The light path of the actinic beam was arranged perpendicularly to the direction of the monochromatic beam. A d.c. mercury arc was used and the illuminating system was identical to that used in our earlier flashing light work (KOK, 1956). A spinning (600 RPM) disc (ø 50 cm) provided with two slits (20 × 4 mm) interrupted the actinic light beam, which moreover could be darkened by a separate shutter.

During the moments the reaction vessel was illuminated by the flashes ($\sim 3.10^{-4}$ sec.), small blades fastened onto the disk closed off the small aperture in front of the photocell and thus intercepted all fluorescence and stray light induced by the actinic beam. Immediately ($\sim$ 1 m. sec.) after a flash was extinguished, the photocell

[1]) Communication No. 156 of this laboratory; 55th communication on Photosynthesis.

was exposed again to the monochromatic beam and stayed so until ~ 1 m. sec. before a new flash came along.

If no flashing light is given, the photocurrent will stay as constant as the light output of the monochromator, except for the two brief moments of darkening during each cycle. But if the flashes induce short lived absorption changes, this current will show additional cyclic variations.

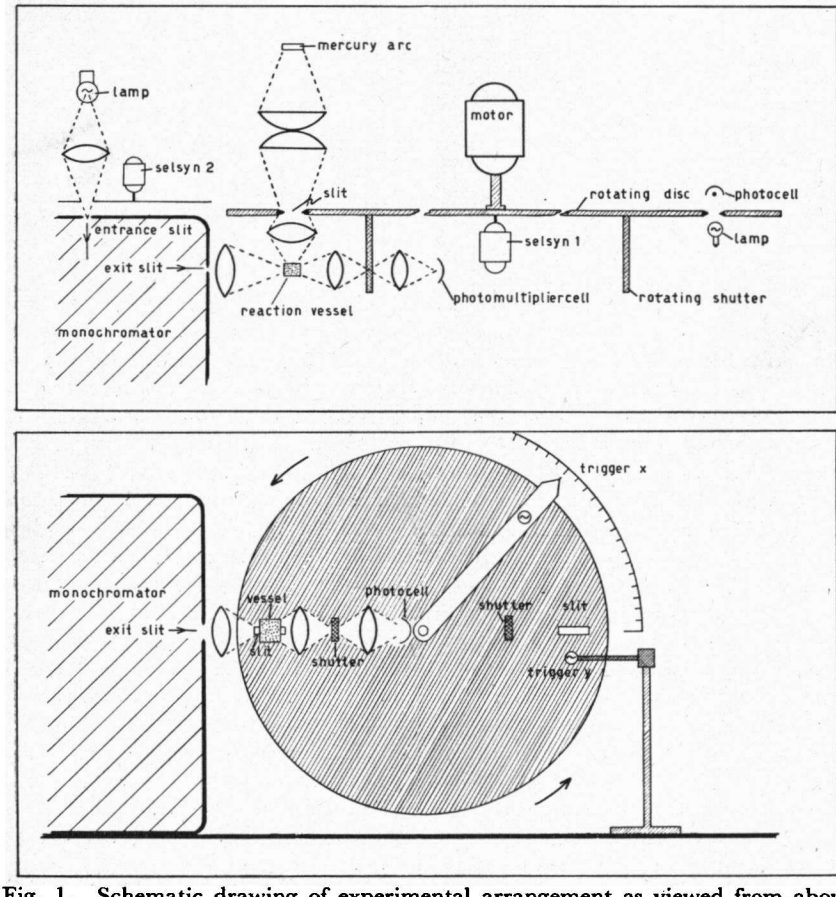

Fig. 1. Schematic drawing of experimental arrangement as viewed from above and from aside. For a further description see text.

The low monochromatic intensity, the inefficient collection of scattered light on the small photocathode and the relatively high noise level of the red sensitive cell made it impossible to significantly observe changes of photocurrent in a single cycle (i.e. in the way WITT, 1955, was able to do with the aid of an oscilloscope screen in his study of the 520 mμ and 475 mμ shifts in green cells). Rather did we have to integrate over a large number of cycles. This was done

in the following way (*cf.* Fig. 2): The anode of the multiplier was tied to the input grid of a d.c. amplifier and was at the same time connected to an adjustable compensating voltage (V_1) via a resistor of 2–4 mΩ (determined by the demand for sufficiently fast time response). The photomultiplier output consists of a d.c. component on which the "noise" is superimposed. The compensating voltage

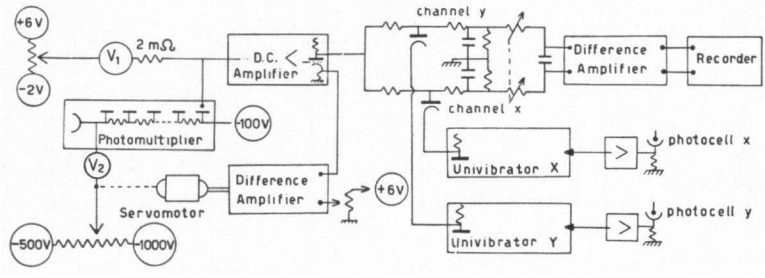

Fig. 2. Arrangement of the electronic circuits described in the text.

was set so as to largely remove this d.c. component and care was taken that the noise component did not exceed the maximum voltage the amplifier could handle with reasonable linearity. The simple three stage amplifier had a gain of 10^4. The average voltage on the cathode of the last tube was compared with a set potential. Any deviation occurring was fed into a Brown recorder, the motor of which actuated a potentiometer correcting the supply voltage (V_2) for the photomultiplier. This servo mechanism automatically compensated for slow drifts of the amplifier, changes in lamp output, settling of algae, etc. (In some experiments it proved to be particularly useful since also long persisting light induced changes of absorption could thus be recorded). The output of the amplifier was split into two identical channels (x and y), which fed the signal via an integrating network into a difference amplifier [1]). The response time of this network could be varied between 5″ and 60″, as dependent on the available effective light intensity (signal to noise ratio). A recorder indicated an eventual difference between the two channels. In addition, both channels were provided with a diode clamp, so that each of them could either be held at a fixed voltage or let the signal pass. Each clamp was actuated via a univibrator, which, if triggered by a brief pulse, opened the channel during an adjustable time interval (usually $\sim$ 4 m. sec.).

In turn each univibrator was actuated by a small size photocell via one stage of amplification. The two photocells were mounted, each opposing a small lamp, close to the spinning disc and actuated the clamp each time a slit passed by. Moreover, one of the photocells (x in Fig. 1) could be swung around the disc circumference and the time location of its triggerpulses in respect to the flashes of the actinic

[1]) A description of most of the used electronic circuits can be found e.g. in ELMORE and SANDS (1949).

beam could be adjusted at will. The other photocell invariably opened its channel (y) during 4 m. sec. immediately before the flash (i.e. at the end of the darkperiod, when the effect of the preceding lightflash is largely restored). Fig. 3 further illustrates this course of events for the case the first photocell opened its channel (x) immediately after the flash.

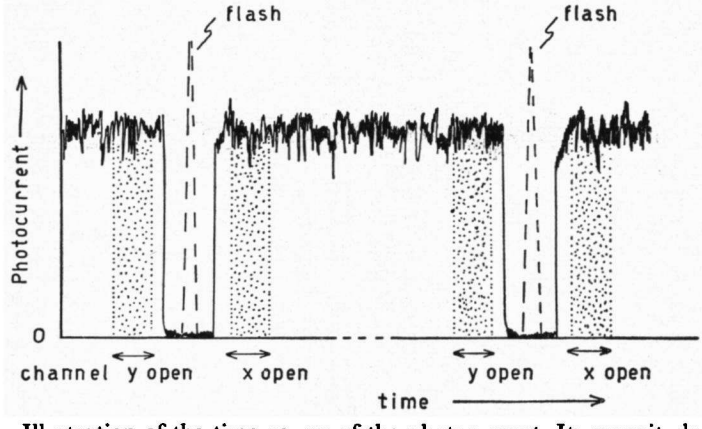

Fig. 3. Illustration of the time course of the photocurrent. Its magnitude during the time intervals y is compared with its value during the intervals x.

If the actinic light is shut off, the two signals, which each influence the difference amplifier during 4 m.sec. per cycle, will be equal and this yields a "zero" reading. If flashes are given, which produce a temporary change of absorption persisting longer than 4 and shorter than 50 m.sec., signal y will differ from signal x. After integration over a sufficiently large number of cycles, a reliable deflection of the recorder will result.

If its photocell is displaced around the disc and channel x opens some time after the flash, a smaller difference will be observed since a partial reversion of the absorption shift has taken place in the meantime. In this way we could measure the time course of the absorption changes.

Finally we may mention the two selsyns shown in Fig. 1: S_2 is governed by S_1 and spins a small disk (at the same speed as the main disk and in the proper phase) in front of the entrance slit. Holes are cut in this disc, which coincide in time with the moments that channels x and y are open. In this way the measuring beam could be made to only illuminate the sample during the required time intervals. The cells then are kept in complete darkness during most of the time and the danger is restricted that the measuring beam would partially prevent the discharge of the photosensitive systems between the actinic flashes (cf. also p. 331).

Performance

In addition to the fundamental ones described above, the method

has a few technical advantages arising from the fact, that only a single sample, light path and amplifier are used and differences are measured between conditions rapidly succeeding each other. Notwithstanding the poor duty cycle of the detecting system, changes in photocurrent of one part in 10^5 could be observed under not too unfavourable conditions of illumination (i.e. generally over a spectral range of 400–750 mμ).

On the other hand, however, significant measurements of such small effects necessitated a very slow time response or averaging the recorder deflections during several minutes (for both a "zero" and a "light" reading). The effects to be studied were not very constant and moreover do prolonged exposures to the bright flashes produce photoinhibition. Therefore, for a survey of an extended spectral region with intervals of 5 or 10 mμ, exposure times of 30–60″ were used and this sacrifice of sensitivity generally necessitated the use of denser cell suspensions, than those currently used in spectral work.

There is no simple way for directly calibrating the system, but a good approximation can be obtained with the aid of small voltage differences applied between the channels and the known values of input voltage and amplification factor.

We made it a point to always keep the compensating voltage (V_1) constant throughout a spectrum measured with a given sample (or, if necessary, make proper recalculations). Only voltage V_2 was (partly automatically) readjusted for each wavelength setting of the measuring beam. In this way the deflections of the difference recorder could always be expressed as a fraction of a constant photocurrent (cf. DUYSENS, 1952, p. 70).

Halfwidth of the monochromatic beam was either 7 or 10 mμ.

RESULTS

In Figs. 4–9 we collected a number of observations made with various types of photosynthetic organisms. Changes of photocurrent are plotted vs. wavelength. Some curves represent the average results of 2–4 measurements, which showed only slight mutual variations as long as closely identical samples were used. In the course of prolonged experiments the effects often tended to decrease -possibly due to photoinhibition caused by the intense flashes. A given wavelength (705 mμ) therefore was measured repeatedly in between and used as a reference. In addition, fresh samples were frequently taken.

Fig. 4 shows some data collected with suspensions of green algae of about equal density (20–40 γ chlorophyll/cm^2). The suspension medium contained 0.05 mol bicarbonate buffer pH 8.7 and 0.5 % agar was added in order to prevent settling. The full and dashed curves were obtained with *Chlorella* (strain A and Myers' strain 1410), the dotted curve was obtained with *Scenedesmus* (cf. also Fig. 8) and reproduces part of a spectrum published elsewhere (KOK, 1957).

In the red spectral region the effects show a great variability; in this respect the examples chosen for Fig. 4 represent more or less the extremes of the available data.

One could conclude that at least with our methods of cultivating and resuspending the algae, the 705 peak predominates the other red shifts in *Scenedesmus*. In *Chlorella A* the shift at 650 and in *Chlorella*

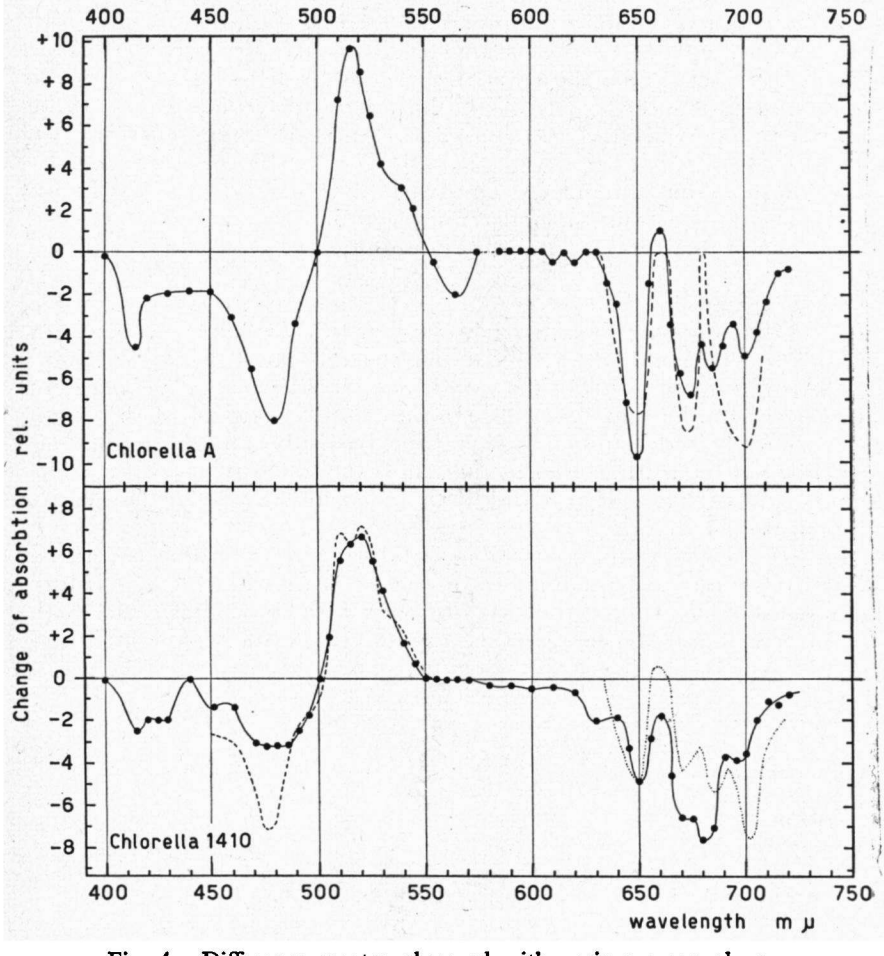

Fig. 4. Difference spectra observed with various green algae.

1410 the change at 680 mμ tends to be the largest. But pretreatment and suspension density play an important role, as is illustrated with the dashed curve (Fig. 4 top), observed with a pale suspension of (nitrogen starved) *Chlorella A* cells. Here we find three clearly separated peaks of about equal height located at 650, 673 and 700 mμ respectively. As will be pointed out in the discussion of Fig. 8, the occurrence of these three bands might well be considered as characteristic for the difference spectra of green algae.

In the short wavelength region ($\lambda < 580$ mμ), Fig. 4*a* shows data

collected with pale, nitrogen starved cells of *Chlorella A*. The ratio between the 520 and the 475 peaks in this case approaches unity and the maximum of the positive change is at 515 mμ. A pronounced shoulder occurs on this band at about 540 mμ. In this experiment a weak negative change is observed between 555 and 565 mμ. In this spectral region the effects appear rather irreproduceable.

One of the examples shown in the left hand side of Fig. 4*b* (*Chlorella* 1410 dashed curve) particularly illustrates the complexity of the positive changes around 520 mμ, where three different maxima can be noticed.

In the blue spectral region we invariably observed negative bands at 475 and 425 mμ, but the deflections eventually occurring in between these two shifts, though doubtlessly significant, were rather unpredictable.

Generally there appears to be very close resemblance between our results and the measurements made in the short wavelength region by DUYSENS (1954), LUNDEGARTH (1954), WITT (1955*a*), STREHLER and LYNCH (1956) and SPRUIT (1956). A decrease of absorption at 650 mμ was also noticed by STREHLER and LYNCH (1956), who used a much slower, but basically similar method as we did for separating the actinic and the measuring beam. The results of COLEMAN, HOLT and RABINOWITCH (1956) are less easily comparable with ours.

Fig. 5 shows results obtained with spinach chloroplasts, which closely resemble the phenomena observed with whole leaves. More than with whole cells, do the data obtained with chloroplasts vary with the pretreatment. Neither are the samples very stable during measure-

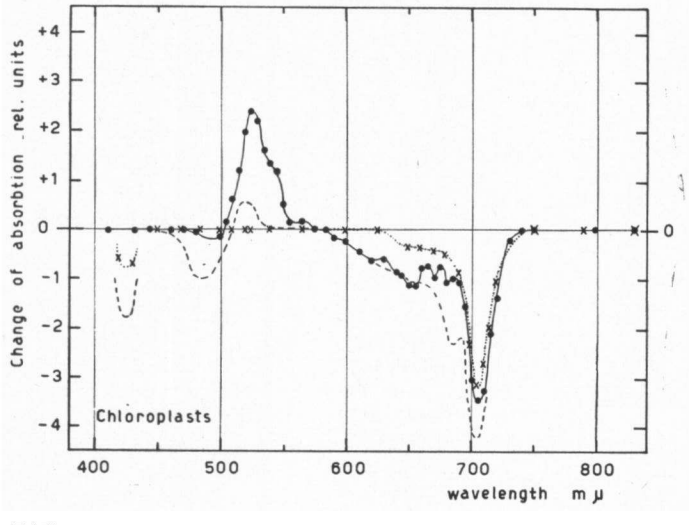

Fig. 5. Difference spectra observed with suspensions of Spinach chloroplasts prepared in different ways.

ments at room temperature. The difference spectra resemble those observed with green algae. The spectrum drawn in full line was obtained with suspensions containing 6 % glucose plus or minus chinon as a hydrogen acceptor. Addition of 2–6 dichlorophenol indophenol (3×10^{-5} mol) left the changes in the red and blue unaffected but strongly decreased the intensity of the 520 mμ band (cf. the dashed curve in Fig. 5). This probably can be correlated with the observation of WITT (1955*b*), that this agent strongly shortens the life time of the 520 change. It then may escape measurement in our apparatus, which requires a lifetime of several milliseconds for detection.

The dotted curve (crosses) shows the peculiar results of a run with the supernatant obtained after low speed centrifugation of a leaf macerate in distilled water. Except for a shift at 425 mμ and a slight decrease of absorption in the red region, only the change around 705 mμ was observed to the full extend. We presently restrict ourselves to the examples given in Fig. 5, which show that a further study of chloroplasts and grana might be quite worthwile.

Fig. 6 gives a difference spectrum observed with the diatom *Nitzschia*, which markedly differs from the ones obtained with green plants.

The decrease of absorption in the blue can be conceived as consisting of two peaks, characterized by maxima at 422 and 436 mμ. The first one probably can be correlated with the corresponding shift observed in green cells.

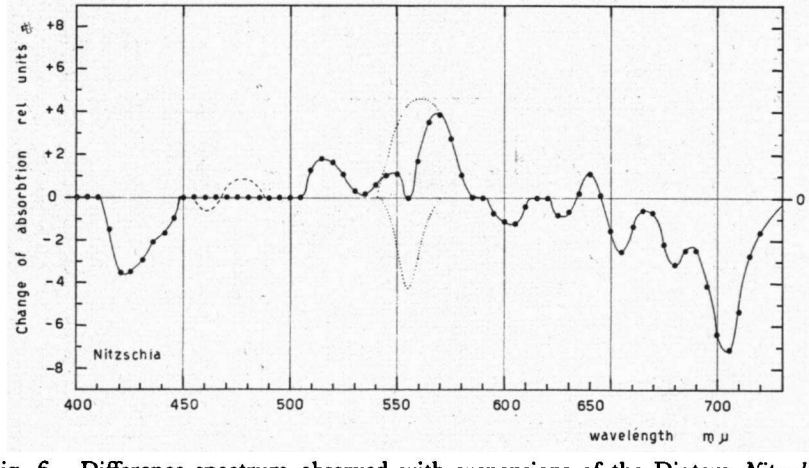

Fig. 6. Difference spectrum observed with suspensions of the Diatom *Nitzschia*.

No negative change was found at 475 mμ; in one experiment the weak changes, plotted as a dashed curve, were observed in this wavelength region. An area of increased absorption, characterized by several maxima, occurs between 505 and 580 mμ. We could tentatively identify the relatively weak band at 515 mμ with the one

observed in green plants. Quite typical is the strong maximum at 568 mμ which is further discussed on page 335.

The two dotted curves in Fig. 6 were drawn in such a way that—if added together—the observed curve results. This construction, though, arbitrary, could illustrate the disappearance of a pigment with a narrow absorption band at about 555 mμ and the appearance of a broad absorption band at 565 mμ.

The negative shifts at 605 and 627 mμ and the positive one at 640 mμ were not observed in green algae, also is the location of the negative change at 655 mμ typical. The bands at 680 and 705 mμ, on the other hand, closely resemble the effects observed with green cells.

Fig. 7*a* (top) shows data obtained with pieces of thallus of the red alga *Porphyra;* the negative shifts with maxima at 426 and 705 mμ are quite pronounced both in absolute and relative measure.

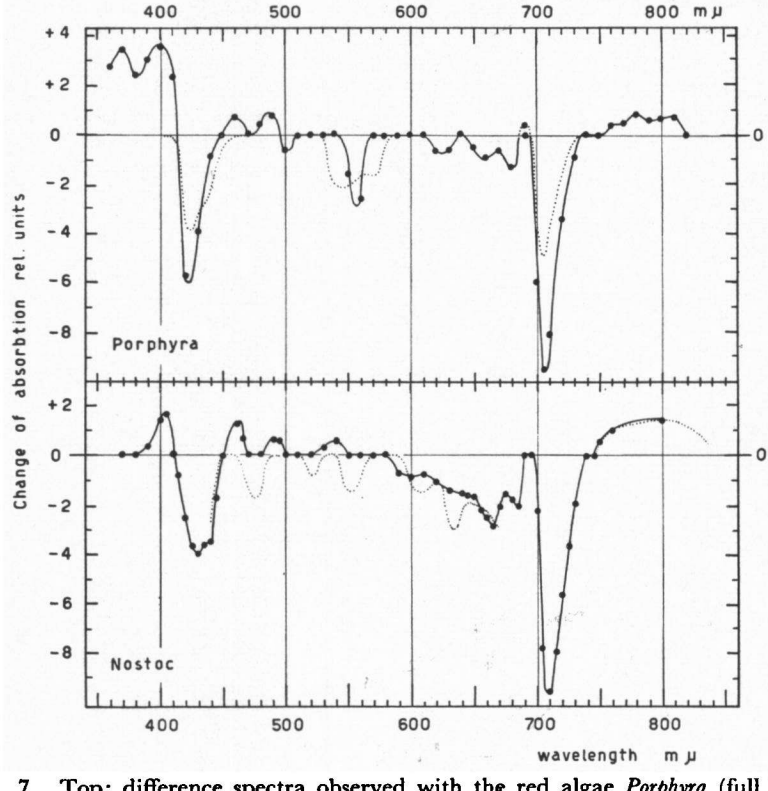

Fig. 7. Top: difference spectra observed with the red algae *Porphyra* (full line) and *Porphyridium* (dotted line). Bottom: difference spectra observed with the blue alga *Nostoc.*

In the blue region this difference spectrum agrees quite well with DUYSENS' (1955) observations made with *Porphyridium.* His interpre-

tation was that transformation of cytochrome f is responsible for the changes at 400, 425 and 555 mμ.

An incidental observation was made with a very dilute suspension of *Porphyridium*, the result is given as a dotted curve in Fig. 7*a*. Noteworth are the broad areas of negative change (410–450 and 530–580 mμ respectively), observed in this experiment. Lack of material so far prevented the collection of further data.

In the red part of the spectrum the 705 change predominates and also its absolute magnitude (approaching a transmission change of one percent) was at least twice the highest values observed even with thick suspensions of green algae and diatoms. The asymmetric shape of the band was discussed earlier (Kok, 1956). Relatively small peaks are observed at 625, 660 and 680 mμ. Zero change is found at 640 and 690 mμ, though at the latter wavelength a possibly significant weak positive change was observed incidentally. Noteworthy is the increase of absorption at wavelengths beyond 740 mμ, extending up to 820 mμ.

This latter increase is also observed in the difference spectrum measured with the blue green alga *Nostoc*, given in Fig. 7 (bottom). The dotted curve in the middle part of this spectrum was derived from another series of measurements. It shows more detailed and pronounced peaks than the full curve, but there is reasonable agreement between the two, if we account for a difference of the zero line. (A change over a broad wavelength area could be involved in one of the spectra).

The intensity of the 708 mμ change is nearly as high as in *Porphyra* and also the other changes in the red region are rather similar. Also the positive effects at 400, 460 and 490 mμ and the negative ones at 425 (400), 475 and 555 mμ can be correlated in both spectra.

Influence of suspension density

Figs. 8 and 9 illustrate the complications encountered if suspensions of varying density are used. Fig. 8 shows the absorption changes observed with a very dense suspension of *Scenedesmus* (open circles, crosses, dashed curve) in comparison with the ones observed with a very dilute suspension of the same algae (dots and triangles, full line). Since the transmission changes in the thin suspensions were much smaller, the latter data have been multiplied with a factor three for the plots in Fig. 8 (the average photocurrent (V_1) was the same throughout these measurements).

The two spectra differ in several respects: Most noteworthy is the increase of absorption between 730 and 820 mμ, observed with the dense suspension only. The similarity of this change with the ones measured with *Porphyra* and *Nostoc* (Fig. 7) is striking.

The experiments further show how the changes in the blue spectral region tend to fully disappear with increasing density. In the dilute suspension the negative shifts at 425 and 480 mμ are pronounced and approach the magnitude of the 705 shift. In vain did we look

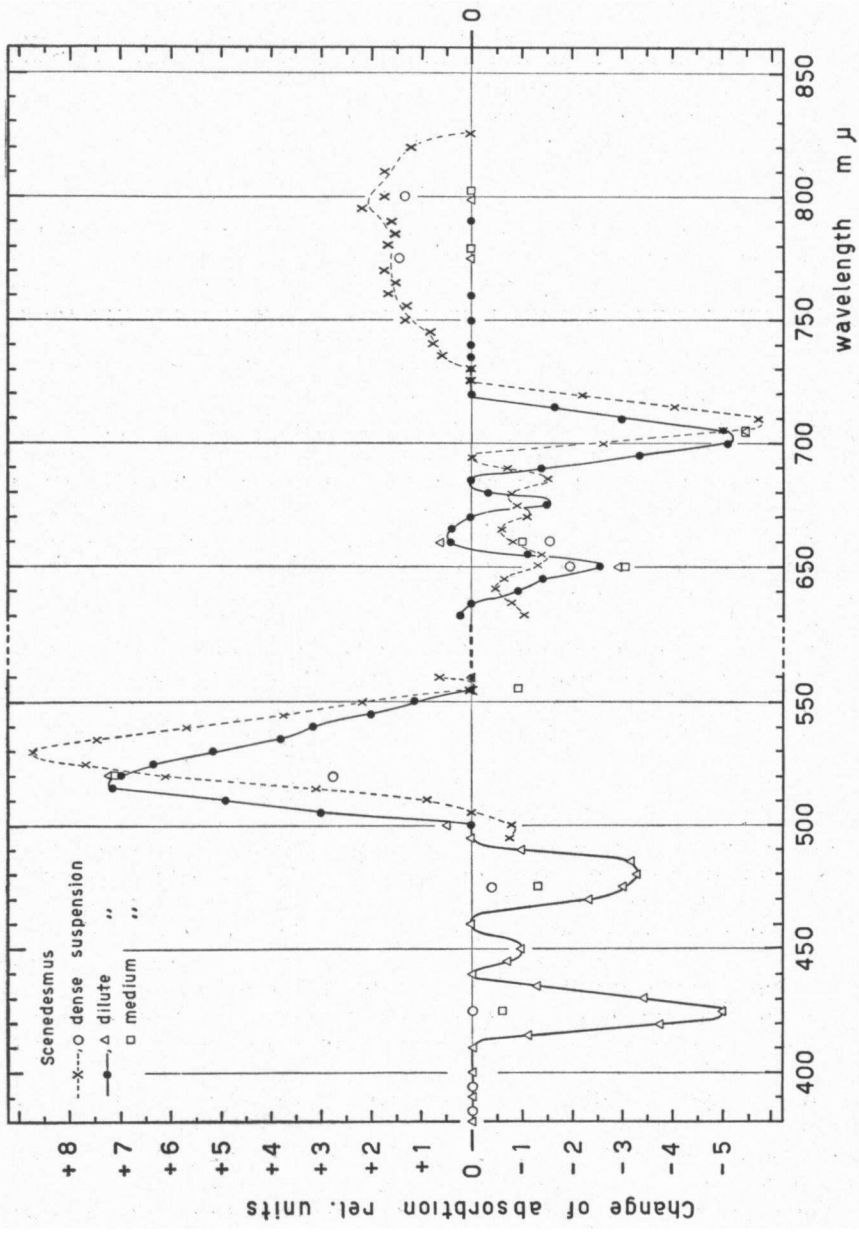

Fig. 8. Difference spectra measured with the green alga *Scenedesmus*, suspended in various concentrations.

for an increased absorption at wavelengths shorter than 400 mμ such as was observed with *Nostoc* and *Porphyra*.

Furthermore, in the dense suspension the blue green band is shifted 12 mμ towards longer wavelengths (from 517 to 529 mμ) as compared with the dilute suspension. In this connection it may be recalled that also in the pale-green suspension used for expt. Fig. 4a this maximum was located at 515 mμ.

The main band in the red is also shifted towards longer wavelengths in the dense suspension (from 703 to 708 mμ) and the other changes in the red differ largely in the two spectra.

Fig. 9 shows data collected with a dense and a dilute suspension of *Nostoc*. The 709 shift had the same shape and location in the two cases (though it was about 3 fold more intense in the thick suspension). But in the blue region did the dilute suspension show far greater changes of transmission—both absolute and relative.

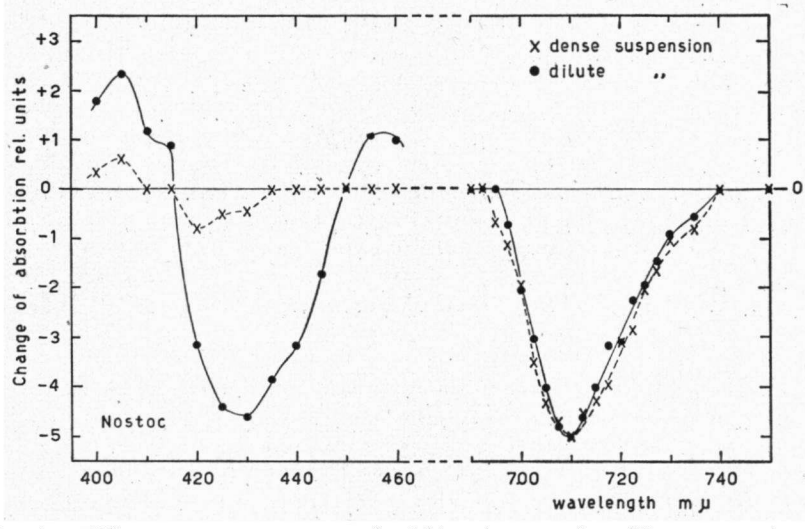

Fig. 9. Difference spectra measured with a dense and a dilute suspension of *Nostoc*.

In dense suspensions, or even in thin suspensions of heavily pigmented cells or chloroplasts, the light absorption may be complete in regions of strong maxima and in extremo only light passing in between algae or reflected by the walls of the reaction vessels will reach the photocathode. A small change in the region of a strong absorption band thus may not be noticed in a too dense suspension. Or, if complete light absorption only occurs in the peak of a shift, the effects may still be noticed on one or both of its shoulders. It thus is feasible that with increasing suspension density a single band of reversible absorption change will be shifted in wavelength location, become asymmetric, or even be split into two bands, located at the sides of the maximum observed in a thin suspension.

On this basis most of the effects of Figs. 8 and 9 in the blue spectral region can be qualitatively understood. The strong absorption by chlorophyll and carotenoids may obscure the 420 and 475 shifts as well as the short wavelength side of the 515 mμ band.

The interpretation of the phenomena in the red is more difficult. In Fig. 8 the dilute suspension showed a negative peak at 676 mμ and zero change at 685 mμ; the dense suspension on the other hand showed a new peak at 685, a minimum of absorption change at 678 and another peak at 671 mμ. This might possibly be an indication that the 676 mμ band is to be ascribed to disappearance of chlorophyll a absorption, which peak in the dense suspension becomes only manifest at both of its shoulders (cf. also the double peaks observed in this wavelength region in the spectra of Fig. 7). This interpretation then could lead one to accept that the 650 mμ band is due to decrease of chlorophyll b absorption (in the dense suspension similar deviations as discussed above are observed). A further consequence of this reasoning then would be that the (often asymmetric) 705 band has to be ascribed to the removal of a separate so far unknown-pigment system. The weak, but significant increase of absorption at 662 mμ indicates that also the formation of new pigment material is involved. Deferring a further discussion of possible interpretations, it should still be remarked that Fig. 8 shows the usefulness suspensions denser than strictly required for sufficient accuracy of measurement. Effects brought about by pigments with a low specific absorption or present in very low concentration could escape observation in too dilute suspensions: the increase of absorption between 725 and 820 mμ is only observed in a dense layer of green algae.

Life time of the absorption changes

The decay of the effects induced by the flashes could be followed by measuring the change of light transmission in relation to the time location of the observation point succeeding the flash.

For effectively preventing the tails of the flashes to influence the photocell, the shutter on the disc overlapped the flash more than a millisecond on both sides. After the shutter opened the pinhole aperture, the photocurrent had to rise from its dark level to within less than a promille of its final value before significant observations during observation time x (Fig. 3) could be made. Therefore in our present apparatus the first few milliseconds after the flash could not be studied. Measurements could only be carried out at room temperature and a further restriction was that only for the major changes the time course could be studied with sufficient accuracy. Most data were collected with green algae, though other organisms showed practically identical behaviour. A few data are shown in Fig. 10.

It appeared that the various changes showed about the same time course, which closely approached an exponential character over the region we were able to study.

The decay halftimes of 5–8 m.sec. were 2–3 times shorter than the shortest time observed by Witt (1955) for the change at 520 mμ.

WITT's data, collected with a number of dark-and flashtimes (a variation which our set up did not allow) show a decrease of halftime with decreasing flash duration and a deviation from first order decay. No flash regime, directly comparable to ours was used.

Finally it may be mentioned that, when a complete spectrum was

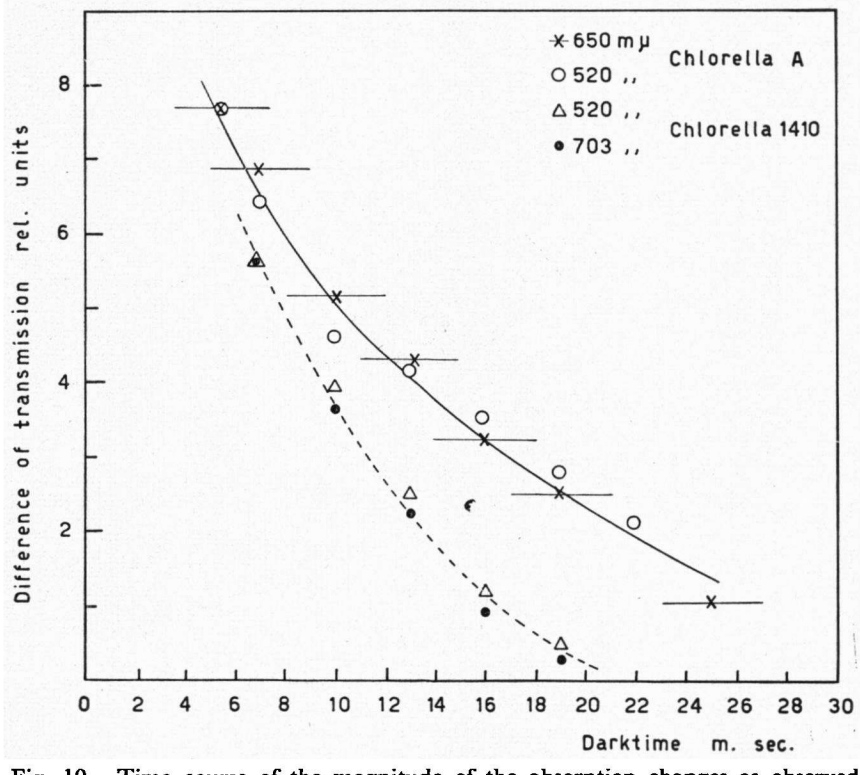

Fig. 10. Time course of the magnitude of the absorption changes as observed at three wavelengths with *Chlorella*.

measured with observation point x located several milliseconds after the flash, all deflections were proportionally decreased and no indications could be found of additional short living intermediates formed during the dark period as transformation products of those formed primarily by the flash.

INTENSITY OF THE ACTINIC BEAM

For the major changes it was possible to study the magnitude of the effects as a function of the intensity of both the actinic and the measuring beam. In Fig. 11 some data, collected in an experiment with *Scenedesmus* are plotted, which show typical saturation curves for the effects. Within the experimental accuracy most changes showed about identical dependency of intensity, except for the 520 shift, which is obviously saturated in much (about fourfold) weaker light.

Evidently the change at 520 has to be ascribed to a system different from the other shifts. This conclusion is further supported by the facts that it is only found in green cells (cf. Figs. 4, 5 and 8) and that it disappears if special acceptors are used in chloroplast suspensions (cf. Fig. 5 dashed curve). It is not possible to decide from the data

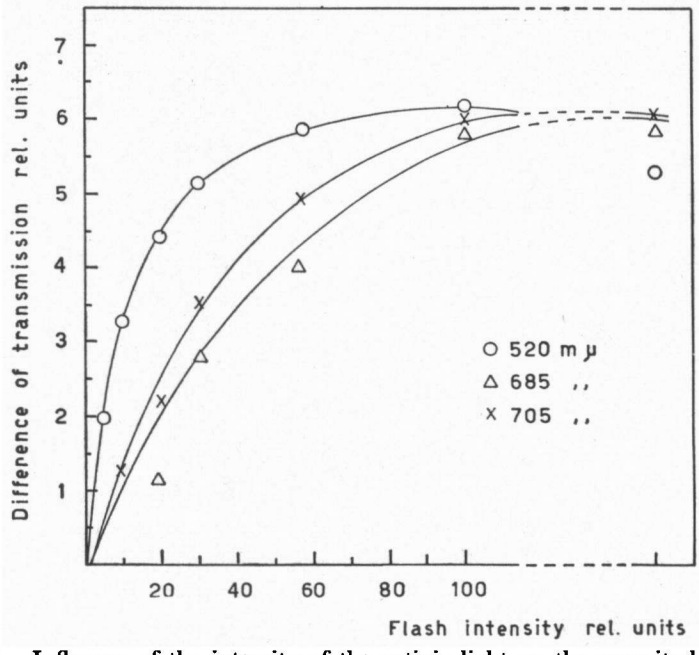

Fig. 11. Influence of the intensity of the actinic light on the magnitude of the absorption changes at three wavelengths observed with *Scenedesmus*.

whether either a different primary light acceptor system is involved or only a separate dark system occurs, driven via the primary reaction responsible for the other changes as well.

In our apparatus the light flashes were induced by the same set up as was used for volumetric oxygen determinations, though the rigorous requirements for separating the two beams necessitated some sacrifice of flash duration and -intensity. Still the intensity requirements for saturating either oxygen evolution per flash or changes of absorption in the red spectral region were of the same order of magnitude. We therefore tend to correlate these changes with the formation and removal of the primary photochemical product(s).

Intensity of the monochromatic beam

Theoretically no influence is expected of the intensity of the monochromatic beam as long as it is sufficiently weak to not induce significant absorption changes or to prevent discharge of the acceptor system loaded by the flash.

On the other hand, a monochromatic intensity as high as possible

should be used to ensure that the observed variations of photocurrent are caused by absorption changes only and not due to phosphorescence of glass or chemiluminescence of the algae (effects, which would simulate a decrease of absorption):

After traversing the sample, the monochromatic beam of intensity I_m reaches the photocell and yields a preset voltage (V_1) across the 2 mΩ resistor (cf. Fig. 2). Thus, $V_1 = g_m I_m$, in which the term g_m contains the spectral sensitivity and the amplification factor of the photomultiplier cell and therefore varies along with voltage V_2.

Suppose a flash increases the intensity transmitted by the algæ with a small fraction f. I_m, so that after the flash: $V_1' = g_m (1 + f) I_m$. In addition the flash induces phosphorescence, the intensity of which (I_p) will be constant as long as the flash intensity is constant. However, V_2 is dependent upon the density of the sample and the wavelength setting of the monochromator, (determining I_m and thus g_m). The amplification of the multiplier cell thus is a function of wavelength and so will be the signal resulting from phosphorescence: $V_p = g_p I_p$. (Due to the spectral variation of the photocathode response, g_p will generally differ from g_m). Thus: $V_1' = g_m (1 + f) I_m + g_p I_p$. The recorder indicates:

$$V_1' - V_1 = \Delta V = f.g_m I_m + g_p I_p = fV_1 + g_p I_p$$

and the computed change:

$$f = \Delta V/V_1 - g_p . I_p/g_m . I_m \qquad (1)$$

As long as $g_p I_p$, is small enough, the measurements correctly indicate the first term of Eq. (1) only. Since I_p is constant and g_p proportional with g_m at any given wavelength, the second term will become increasingly important if the (transmitted) intensity of the measuring light decreases.

A simple way for checking the interference of luminescence thus is to measure a given absorption change with varying intensities of the measuring beam (V_1 constant). In actual checks, under not too extreme conditions, we found f to be constant within experimental error over a 10 to 20 fold range of I_m. With dense suspensions, however, a distortion of the difference spectra is not fully excluded in regions of strong absorption peaks.

Since obviously the two discussed aspects put controversial demands on the intensity of the measuring beam, we arranged the extra rotating disc in front of the entrance slit of the monochromator (cf. Fig. 1). Relatively strong monochromatic light thus could be used during the observations without great danger of annulling the effects of the flashes. The effect of this extra arrangement, however, proved to be only slight.

Significance of the effects

One could wonder whether the small effects brought about by the high intensity-flashes are correlated with truly photochemical reactions.

Scattered light contributes appreciably to the illumination of the photocathode and the temperature increment of the chloroplasts, induced by the flashes, could induce a slight re-arrangement of the pigment molecules (cf. SHIBATA, 1955). In this respect we may also point to some striking similarities in the location of maxima in difference spectra and in the data of GOEDHEER (1955), and LATIMER and RABINOWITCH (1956), concerning birefrigence and selective scattering of green cells.

In this connection Fig. 12 illustrates how the changes in the red observed in expt. Fig. 8 (dilute suspension) could, for instance, be

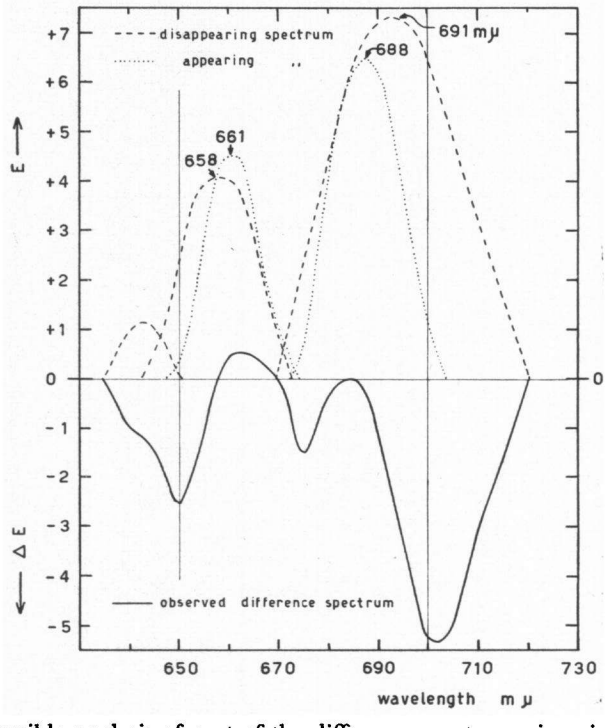

Fig. 12. Possible analysis of part of the difference spectrum given in Fig. 8 (full line).

conceived as mainly consisting of slight shifts of two absorption maxima (normally located at 658 and 691 mμ and shifting 3 mμ in opposite directions), but then a considerable change especially of the width of the long wavelength peak also has to be accepted.

An argument against this interpretation is that the effects disappear or become quite different after the cells are damaged by heat, cold-treatment or poisoning (cf. also WITT *l.c.* and SPRUIT *l.c.*).

Another argument for correlating the described absorption changes, at least partly, to photochemical changes of pigment molecules is, that similar phenomena can be observed *in vitro*, i.e. with true

solutions of chlorophyllous pigments. As an example—merely presented here to illustrate this point—Fig. 13 (open circles) shows a "difference" spectrum, observed in our apparatus with an oxygen free solution of pheophytin a in pyridin to which small amounts of ascorbic acid and thiouria were added. Similar data could, e.g. be

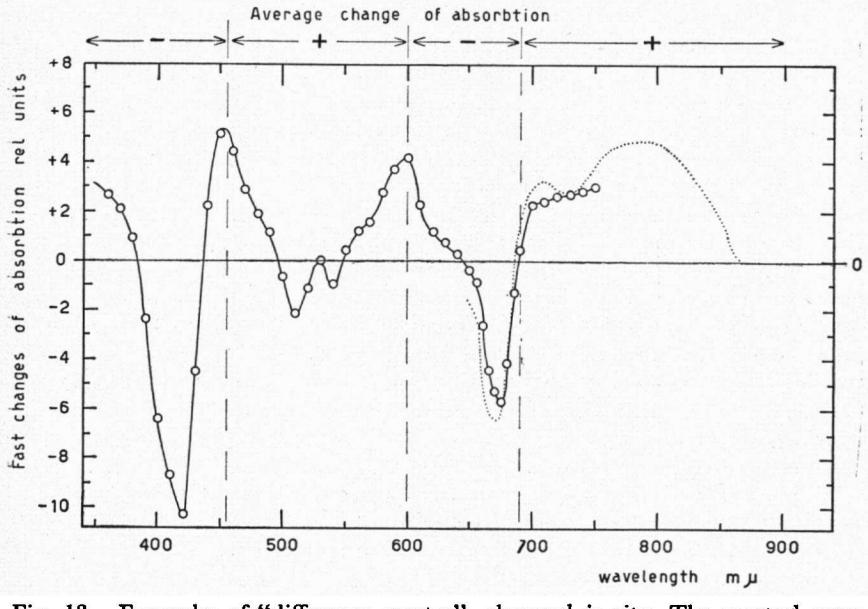

Fig. 13. Examples of "difference spectra", observed *in vitro*. The spectral areas over which the average absorption value slowly increased or decreased are also indicated (top).

obtained with clear solutions obtained by grinding spinach leaves directly in ascorbic acid containing pyridin, as is shown with the dotted curve in Fig. 13. In some respects—such as the decrease of the red and blue absorption peaks and the appearance of new absorption bands in the ultra violet and the infrared, these data qualitatively resemble observations made with living cells.

It must be stated, however, that Fig. 13 is chosen from a number of experiments of preliminary character, which yielded rather complex and variable results. In addition to truly reversible fast absorption changes our apparatus will also respond to unidirectional changes, which are induced stepwise (flash after flash). If such a progressive absorption change occurs, the average photocurrent will vary and this may yield a measurable deflection of the servo mechanism readjusting voltage V_s (also a recording instrument).

With living material such deflections were not observed, which proved that we were dealing with reversible transformations mainly. In *in vitro* experiments strong deflections could occur, which, if in the same direction as the step by step changes pointed to the occurrence of unidirectional transformations. If, on the other hand, the two deflections had opposite signs, more complex phenomena were obviously involved. A still further complication is that in various spectral regions the deflections of each or both of the two recorders may change in sign and magnitude in the course of the reactions (which ultimately result in bleaching of the material).

A further discussion therefore is better delayed until more quantitative data are available, for which purpose an improved technique is designed. We may, however, point to an obvious similarity between the spectral dependency of the "average" changes of expt. Fig. 13 with the changes observed by LINSCHITZ and RENNERT (1952) after illuminating chlorophyll in rigid solvents.

DISCUSSION

The described difference spectra of various plant types do not show great uniformity. Generally observed effects occur only in the far red and at 425 mμ. The red shifts point to a role played by chlorophyllous pigments, but probably none of the known chlorophylls is characterized by a main blue absorption band at 425 mμ *in vivo*. One could speculate that this band is correlated with the same pigment, responsible for the change at 705 mμ. The location of the red absorption maximum of this hypothetical pigment might suggest that it acts as the final light trap in photosynthesis.

On the other hand, the complex phenomena characterizing the energy transfer processes in red and blue algae (cf. HAXO and BLINKS, 1950 and DUYSENS, 1952) and the sharp drop of the quantum yield at wavelengths beyond 680 mμ, observed in all types of organisms studied by EMERSON and LEWIS (1942, 1943) and TANADA (1951) could indicate that the function of this pigment—or part of it—is more involved.

The absorption changes at 440 mμ and those at, or around, 678 mμ can be correlated with the disappearance of chlorophyll a. Similarly, chlorophyll b might be involved in the relatively large changes observed at 475 and 650 mμ in green cells.

But, as was discussed earlier, (cf. Fig. 12 and also KOK, 1957) the complex changes in the red still can be interpreted in different ways. For instance, the two negative shifts in fig. 8 (dilute suspension) at 650 and 675 mμ enclosing a positive change at 663 mμ could be ascribed to a single pigment, normally characterized by an absorption maximum at about 663 mμ. If the light flashes caused this band to become narrower and at the same time more intense, a difference spectrum as observed would result. One could use a similar interpretation for the three shifts observed with *Nitzschia*, located at 625 (—), 640 (+) and 655 (—) mμ respectively. (Such a reasoning is supported by the fact that the absorption spectrum of this organism shows a distinct peak at 640 mμ due to absorption by chlorophyll c cf. WASSINK and KERSTEN, 1946).

In the same wavelength region, also *Porphyra* and *Nostoc* showed bands of decreased absorption. The absorption spectrum of *Nostoc* is characterized by a predominant maximum at 620 mμ (in *Porphyra* this maximum is less pronounced). We therefore are inclined to correlate the absorption shifts in this region with changes in phycocyanin (the dotted curve of Fig. 7*b* shows two maxima at both sides of 620 mμ). It may be recalled (cf. p. 000) that the only run we were able to make with a dilute suspension of *Porphyridium* showed a broad band of decreased absorption between 530 and 580 mμ, possibly due, at least partly, to disappearance of phycoerythrin.

We would like to discuss in slightly more detail the possible role played by carotenoids. The changes at 475–520 mμ in green plants were studied by several workers and accepted as due to the transformation of a single pigment of unknown character. From our data it appears that these bands, typical for green plants, may not be correlated. Only in *Nitzschia* is a small positive change observed at 520 mμ, which might correspond to the one found in green cells. In this organism there is a pronounced additional band of positive change at 560 mμ (cf. Fig. 6 full and dotted curves). This latter peak is located on the long wavelength wing of the fucoxyanthol absorption band. One could ascribe all or part of the observed changes between 510 and 580 mμ (except the 555 shift) in *Nitzschia* to a complex transformation of this carotenoid (cf. KOK 1967b).

In analogy with the phenomena observed with diatoms, it seems likely that the 520 bands in green cells have to be correlated with transformations of carotenoids.

Finally we may mention the role played by cytochromes. If DUYSENS' (1955) interpretation of the blue shifts observed in red algae is correct, it might follow that the generally observed band at 420 mμ is due to the oxidation of cytochrome f. However, the expected increase of absorption at 410 mμ, due to the formation of the ferricytochrome, is observed in blue and red algae only (cf. Fig. 7).

A decrease of absorption at 555 mμ—which could indicate the disappearance of the α band of this cytochrome—was distinctly observed in *Porphyra*, but in red algae also phycoerthrin may be responsible for changes in this spectral area. Changes at 555 mμ have been observed in all organisms which we studied, but the effects were rather irreproduceable. An increase of absorption was found in several cases at 565 mμ, which could be due to the appearance of the α band of ferrocytochrome b.

If our rather ad hoc attempts to correlate the various absorption changes with known plant pigments are justified, it is striking that practically all these pigments can be involved.

SUMMARY

Reversible changes of absorption, induced by flashes of strong light were studied in various photosynthetic organisms. The technique used allowed to include the red and infrared spectral regions into this study. In the short wavelenght region the results showed good agreement with earlier observations largely made with continuous actinic illumination. Though significant differences between various types of plants appear to exist, a decrease in absorption in the red region, characterized by several maxima, was generally observed, the most pronounced effect occurring at 705 mμ. An increase of absorption was observed between 720 and 820 mμ.

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