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A STUDY OF THE PIGMENTS PRODUCED IN DARKNESS BY CERTAIN GREEN ALGAE

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(WITH FIVE FIGURES)

An observation which has become well established is that certain green plants (*e.g.*, some of the green algae) are able to produce their chlorophyll in the dark. The experimental data characterizing the pigments so produced, however, are indeed meager. It therefore has seemed advisable to reinvestigate the pigments formed by such plants in the dark with the improved spectroscopic methods now available. In the first place there is no conclusive evidence that tells whether or not chlorophyll *b* is formed in darkness. Secondly, it is to be hoped that a study of these rather exceptional cases of chlorophyll formation in the dark may well be a foothold in the study of the general process of pigment development. And thirdly, it is conceivable that spectroscopic differences between "light" and "dark" pigments might be found which could later be correlated with the beginnings of photosynthesis.

This paper is therefore a report of the data so far obtained in a spectroscopical and physiological study of the pigments formed by green algae in the dark.

Although it had previously been noted (*e.g.*, HEINRICHER (7), SCHIMPER (15), and KLEBS (8)), the first unqualified demonstration of the greening by algae in the dark seems to have been made by BEIJERINCK (3) in 1890 on a pure culture of *Chlorosphaera limicola*.[1] A later paper by the same worker reported (4) that various pure cultures of green algae (including *Chlorella vulgaris, Stichococcus bacillaris,* and *Scenedesmus acutus*) had been grown for several years in absolute darkness. The cultures developed quantities of green cells which again became autotrophic when brought into light. The work of ARTARI (1) also demonstrated the greening of algae and lichen gonidia in darkness. ÉTARD and BOUILHAC (6) compared spectroscopically

[1] A colored plate shows green cells that had been grown in the dark on a sucrose-peptone gelatin.

the alcoholic extracts of the blue-green alga, Nostoc, grown on sugar solution in darkness with extracts of green leaves. Their methods gave no significant differences between the two. RADAIS (**14**) made a somewhat more detailed spectroscopic study of the alcohol and carbon disulphide extracts of parallel light and dark cultures of *Chlorella vulgaris*. He obtained identical absorption spectra for the extracts of the two cultures. In addition to the "end absorption" below 511 mμ he observed three absorption bands with mean axes at 667, 618, and 577 mμ. His carbon disulphide extract gave bands with mean axes at 679, 659, 625, and 583 mμ. The occurrence of a distinct additional absorption band in the red at 659 mμ for the carbon disulphide extract suggests the presence of the *b* component. It is rather difficult, however, to see why the alcoholic extracts failed to show a similar band. And by analogy to the absorption curves in other solvents (*e.g.*, ZSCHEILE (**25**)) it seems rather surprising that a chlorophyll *b* peak would show up so clearly in this region unless the *b* component were present in an unusually high proportion.

DANGEARD (**5**) cultivated *Scenedesmus acutus* for eight years in darkness and found that it maintained its green color. The algae brought into light at the end of that time showed within five hours a photosynthetic activity (liberation of bubbles of oxygen). Unfortunately DANGEARD's technique of observing photosynthesis was neither rapid enough nor sufficiently quantitative. Considerable pigment changes might have taken place in less than five hours or might still have been going on at the end of that time (*cf.* SEYBOLD, **16**).

While the available data are consistent with the view that the pigments formed in darkness by certain green algae are photosynthetically effective, in the light of more recent knowledge it has seemed advantageous to reinvestigate this problem with the improved methods of pigment study and photosynthesis measurement now available.

Since the completion of the experimental work of this paper, there has come to the author's attention the recent publication of VAN HILLE (**22**). Incidental to his study of the relation between rate of photosynthesis and chlorophyll content this worker studied *Chlorella pyrenoidosa* grown in darkness. He states that methanol extracts of cells grown in light and in darkness showed the same absorption spectra, although he includes no data and apparently worked only at wavelengths greater than those absorbed by the carotenoids. VAN HILLE also found for cells grown in darkness (p. 748) that "The readings per half-hour of photosynthetic measuring are constant from the beginning and show the normal time of induction (SMITH, 1937)." Again no data are included. Although VAN HILLE's findings have been duplicated, the present paper is considered justified by the more detailed and comprehensive data obtained.

Spectroscopy of the pigments

Three species of green algae were used in this study: *Chlorella vulgaris*, *Protococcus* sp., and *Chlorococcum* sp.[2] These were grown in pure culture on the agarized Detmer's solution diluted to $\frac{1}{3}$ as recommended by MEIER (**11**) in flat one-liter medicine bottles with loose cotton plugs. Bacteriological technique was observed. Three parallel series were used: (1) in darkness with added organic nutrient; (2) in light with added organic nutrient; (3) in light without added organic nutrient. Added organic nutrient consisted of 0.5 per cent. dextrose and 0.2 per cent. peptone. Cultures in light were continuously illuminated by tungsten filament bulbs with Corning Aklo filters to reduce the infra-red. Intensity at the level of the cultures was 65 foot candles as recorded by the Weston photronic cell.

Common methods of extraction employing cold solvents proved ineffective. The use of hot solvents was considered dangerous in view of the labile nature of the pigments. A method has been devised which is a modification of the method used by MUDD and co-workers (**13**) for the extraction of labile bacterial antigens. A suspension of algal cells, washed off the agar surface, is placed in a metal ball-mill[3] and rapidly frozen around the inside rim by rotating the mill in a $-80°$ C. bath (dry ice in alcohol). Freezing is completed in less than one minute. The ball-mill is then quickly attached to a high vacuum line in series with a low temperature condenser held at $-80°$ C. The ice is thus rapidly sublimed off and drying is complete in 12 to 16 hours. The mill is then returned to the low temperature bath and two steel balls inserted. After an hour and a half grinding the bath is removed, 90 per cent. acetone introduced into the ball-mill, and grinding continued for another hour. The acetone extract is removed and the pigments transferred to ethyl ether. (Anesthesia ether, Malinckrodt, containing 2.5 per cent. alcohol was used throughout because of its very low peroxide content.)

Unfortunately the above procedure is not quantitative in that not all of the algal cells are crushed and extracted. Conditions are nearly optimal, however, for the preservation of labile and easily oxidizable cellular materials such as the chlorophylls and carotenoids.

Spectral absorption curves on the ether solutions have been determined, using the photoelectric spectrophotometer previously described by MILLER (**12**) and observing the precautions which he suggested. At some time during each run the calibration curve for prism setting was checked ($\pm$ 2 Å)

[2] Identified by Professor FELIX MAINX of the German University at Prague. Obtained through the courtesy of Dr. C. E. SKINNER by whom they had been isolated from soil.

[3] Kindly provided by the Department of Veterinary Science, the University of Minnesota.

against the 6402 Å neon line. The slit-widths used varied from 0.04 mm. at 4000 Å and 0.02 mm. at 4200 Å to about 0.002 mm. at 6000–6800 Å. In the absorption curves to be presented intensity of absorption is designated merely as $\log \frac{I_o}{I_x}$ according to Beer's Law, since concentrations are not known. The wavelength axes are in Ångström units.

A method previously used by STRAIN (**20**) has been followed in order to separate into two groups the chlorophyll and carotenoid pigments. This is demonstrated by the curves of figure 1 for the extracted pigments from

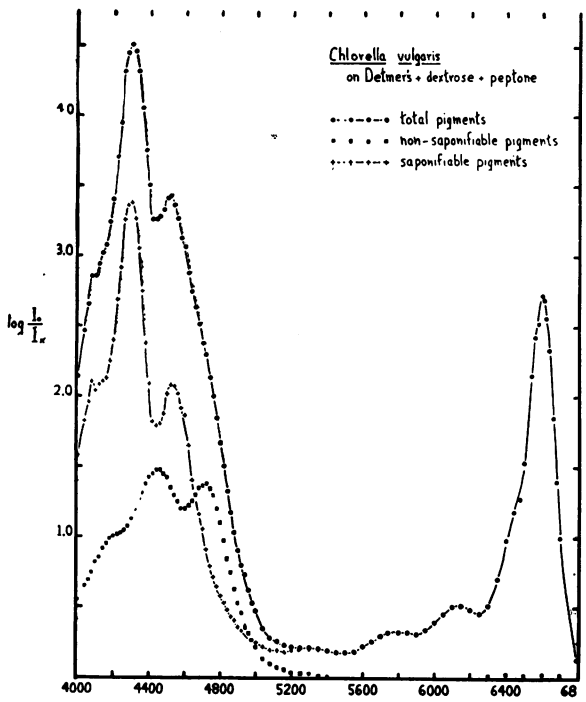

FIG. 1. Absorption spectra of pigments in ethyl ether + 2.5 per cent. alcohol extracted from *Chlorella vulgaris* grown in darkness. Curves refer to equal aliquots of the original extract. For further explanation see text.

Chlorella vulgaris grown in darkness. The upper curve describes the light absorption of the total pigments. If an equal or known aliquot of this ether solution is saponified and the non-saponifiable pigments taken up in ether, the lower curve is obtained. This may be considered the absorption curve for the carotenoid pigments. If the lower curve is now subtracted from the upper curve, point-by-point in the original data, the intermediate curve is obtained. This may be considered the absorption curve of the saponifiable pigments, *i.e.*, of the chlorophylls.

The method of course depends upon the assumption that the saponification removes all the chlorophylls but destroys none of the carotenoids. In my hands the common cold saponification has failed to remove all the chlorophylls, as shown by residual light absorption at 6400–6600 Å. A hot saponification has been required.[4] Comparison of hot and cold saponification on equal aliquots of the pigments of *Chlorococcum* sp. grown in the dark is given in figure 2. Intensity of absorption is significantly higher after the

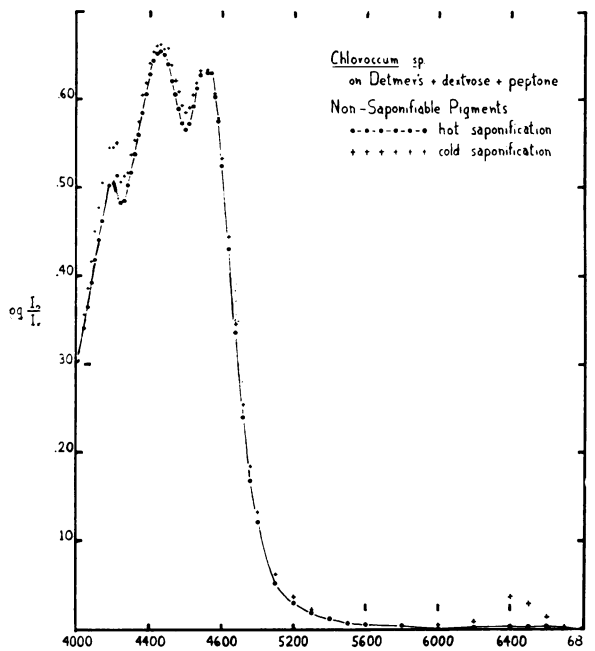

FIG. 2. Absorption spectra of the pigments remaining after hot and after cold saponifications. Pigments originally extracted from *Chlorococcum* sp. grown in darkness.

cold saponification only in those spectral regions where the chlorophylls show high absorption.

Figure 3 shows the absorption curves for the total pigments extracted from *Protococcus* sp. grown under three different conditions. For two of these curves all values of $\log \frac{I_o}{I_x}$ have been multiplied by a factor so chosen

[4] To 4 ml. of 30 per cent. KOH in methanol refluxing over a water bath, 5–10 ml. of the ether solution are added and refluxing continued for 3 minutes. The total solution is then poured into separatory funnel containing ice water and about 10 ml. of ether. The resulting ether solution of carotenoids is washed 5 times with water, separated, and made up to the desired volume. For cold saponification the same time, quantities, and reagents were used and the entire procedure carried out in a separatory funnel.

as to give identical values for the intensity of absorption at 6600 Å on all curves. This merely brings the curves together for ease of comparison. The corresponding absorption curves for the chlorophyll pigments, obtained as described above, are presented in figure 4. Agreement is rather striking, especially through the longer wavelengths of the spectrum. Noticeable variations occur in the height of the bands in the blue: the 4520 Å peak of chlorophyll *b* and the 4100 Å and 4300 Å bands principally due to chlorophyll *a*. These variations may be accounted for in part by variations in the ratio of *a* to *b*.

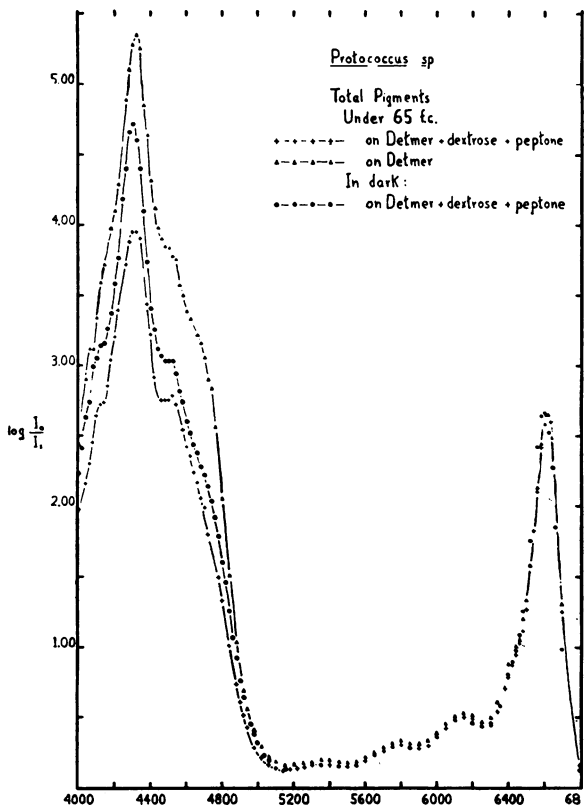

FIG. 3. Absorption spectra of the total pigments extracted from *Protococcus* sp. grown under three different conditions.

Comparison of the chlorophyll curves has been made with the absorption curves of the isolated components obtained by ZSCHEILE (**25**) on the same type of instrument. In general the curves of figure 4 (for ether + 2.5 per cent. alcohol) show a displacement of the peaks toward the red of about 10 to 25 Å. Because of this shift a quantitative analysis of the curves for percentages of the components *a* and *b* by the method of ZSCHEILE (**25**), and

based on his curves for the chlorophylls in ether solution, is impossible. Only anomalous results can be obtained.

The presence of the well-defined 4520 Å band in all the curves of figure 4 as well as in the intermediate curve of figure 1 makes it clear that chlorophyll *b* (or something very similar) is formed in the dark by these cultures of Protococcus and *Chlorella vulgaris*. And inspection of the curves of figure 4 shows that in Protococcus the chlorophyll *a*: *b* ratio is at least of the same

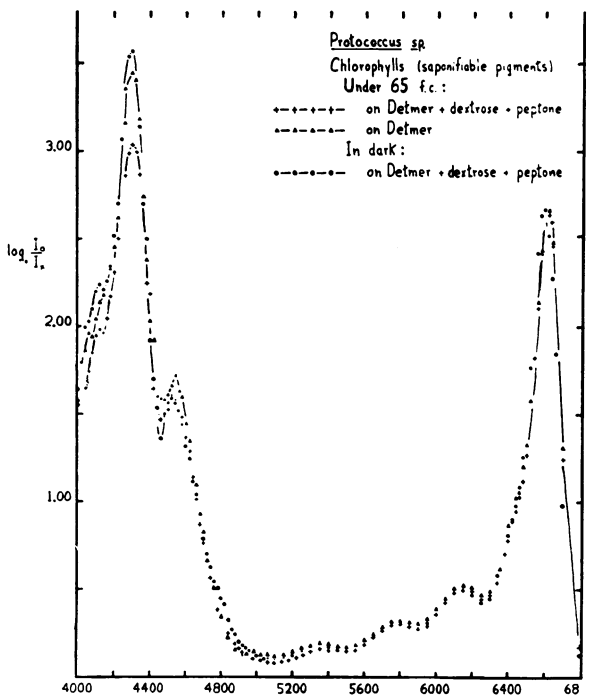

FIG. 4. Absorption spectra of the chlorophylls produced by *Protococcus* sp. grown under three different conditions.

order of magnitude for cells grown in light or in darkness. The formation of chlorophyll *b* in the dark has been confirmed by the appearance of distinct and characteristic chlorophyll *b* zones on sucrose chromatograms prepared according to the method of SEYBOLD and EGLE (17). Comparison of the curves of figures 3 and 4 indicates that the carotenoid: chlorophyll ratio varies considerably under the different conditions of growth. More detailed examination of the carotenoids should be made to determine whether these variations are qualitative or merely quantitative in nature. Further chromatographic work planned for the study of the yellow pigments has not as yet been carried out.

Development of photosynthetic activity

It was advisable at this point to consider the development of photosynthetic activity in algal cells which had previously grown only in darkness. Essentially this involved a comparison of the induction periods of cells grown in darkness and in light. Measurements were made by the familiar Warburg technique, using one illuminated flat-bottom vessel and a non-illuminated thermobarometric control. The volume of the experimental flask was 14.22 ml. to the level of Brodie fluid in the manometer, as calibrated with mercury. When used as described below 1.0 mm. increase in pressure corresponds to an oxygen evolution of 0.56 cu. mm.

The constant-temperature bath was held at 26° C. ± 0.01° as checked by a Beckmann thermometer. Light was provided by an optical system mounted horizontally beneath the bath. The light from a projection bulb, suitably condensed by two lenses, was reflected vertically up through the glass bottom of the bath by a concave mirror. The area of the light beam in cross-section at the level of the experimental flask was large enough so that the flask was always illuminated during the three-cm. amplitude of its shaking cycle. This arrangement was designed to give very high light intensities, since previous work [WARBURG (**24**), SMITH (**19**), MCALISTER (**9**)] indicates that the induction period is longer, and therefore more easily measurable, under higher intensities. At very high light intensities, however, (about 5,000–20,000 f.c.) an inhibition of photosynthesis was observed. A study of this inhibition is to be reported elsewhere. For measurement of the induction period an intensity of 2,200 f.c. was used.

Preliminary results indicated that more consistent results could be obtained for cells grown in liquid culture than for those grown on solid media as in the pigment study. It was also found that *Chlorella vulgaris* was much better adapted for this part of the study than the other algae available. *Protococcus* sp. could not be centrifuged out cleanly and showed such low rates of photosynthesis that it was more difficult to work with. Cultures were grown in 500-ml. Erlenmeyers with a current of air bubbled through. To obtain darkness, flasks were wrapped in photographic light-proof paper and kept in covered iron pails. Cultures grown in light were placed uniformly around a 5.5-cm. water jacket surrounding a 300-watt bulb. The intensity at the illuminated side of the flasks was about 450 f.c. Sterile precautions were observed only when glucose was added to the nutrient solution, although microscopic tests for purity were made in all cases.

Measurements were made with the cells suspended in the potassium carbonate-bicarbonate buffer corresponding to the sodium carbonate-bicarbonate buffer no. 9 of WARBURG (**23**) (0.015 M K_2CO_3, 0.085 M $KHCO_3$). Immediately before an experimental run the cells were centrifuged out of the nutrient solution, taken up in the buffer, and centrifuged out again in

a graduated tube. To the packed cells fresh buffer was added to give a suspension in which 1.0 ml. contained 0.05 ml. of cells. The suspension was then kept in the dark and aliquot portions withdrawn in the dark by an automatic pipette. When cells were grown in darkness, all further operations except the brief reading of cell volume were performed in the dark.

One ml. of cell suspension was added to 7.2 ml. of buffer in the experimental flask. The flask and manometer were placed in position without illumination and about five minutes allowed for adjustment to equilibrium. Four or five consecutive five-minute readings were taken to establish the course of respiration. Light was then turned on for $\frac{1}{2}$ minute. Respiration in the succeeding dark period was again established by at least four readings. This procedure was repeated with light exposures of 1, 1.5, 2, 3, and 4 minutes, using a fresh batch of cells for each exposure. The displacement between the respiration curves before and after the light exposure is a measure of the amount of photosynthesis which took place. This graphical method is illustrated by the upper curves of figure 5. It is an "integrational" method necessitated by the lag in the Warburg instrument.[5]

The method is of course limited to the accuracy of extrapolation of the respiration curves. Occasionally a deviation in the rates of respiration before and after illumination was observed, but never of more than about 5 per cent. It is recognized that such a variation introduces a possible source of error in the extrapolation. Several preliminary experiments seemed to indicate that more consistent results were obtained when a fresh aliquot of cells was used for each time interval and when 0.5 per cent. dextrose was added to the stock suspension kept in the dark. These conditions were therefore observed throughout.

The induction curves obtained for *Chlorella vulgaris* as described above are shown in figure 5. The close coincidence of the curves for cells grown with 0.5 per cent. dextrose, with and without light, clearly answers the problem of this investigation. *The pigments produced by this green alga in darkness are adequate for the process of photosynthesis.* Cells grown in the light without dextrose (upper curve) attain a somewhat higher photosynthetic rate and seem to have a somewhat longer induction period than cells grown with dextrose. The *induction loss,* however [extrapolated intercept on the photosynthesis axis, *cf.* McALISTER (**9, 10**)], is practically identical for all three lots of cells.

In considering the above data as a further description of the induction phenomenon, a possible limitation of the integrational method must be taken into account. McALISTER (**10**) has demonstrated that after high rates of photosynthesis there is an appreciable "pick-up" of CO_2 after the plant is

[5] This is essentially the same method previously used by WARBURG (**24**), VAN DER PAAUW (**21**), and SMITH (**19**).

darkened. Making the reasonable assumption that there is also a continued evolution of O_2 by a plant on darkening, comparable to the "pick-up" of CO_2, he has suggested that there may be an inherent error in the integrational method used here. What is probably measured for each period of illumination is the actual amount of photosynthesis during illumination plus the "pick-up" (in this case the extra evolution of oxygen) on darkening. Each point on the induction curve is therefore too high by an amount equal to the "dark pick-up" involved in its determination. So an induction curve measured by the integrational method is probably somewhat in error in regard to its shape and *induction loss*. From the characteristics of the "dark pick-up," however, it seems unlikely that the indicated length of the induction period will be appreciably in error.

The papers of McAlister (**10**) and Aufdemgarten (**2**) have shown that the induction period is a function of the preceding dark rest when photosynthesis is measured by the rate of CO_2 uptake. Aufdemgarten (**2**) states that the induction period in Stichococcus is lengthened by increasing dark rests of up to fifteen minutes, beyond which no further effect was noted. McAlister's (**10**) more comprehensive data for wheat show a rapid increase in *induction loss* with dark rests increasing up to about one minute, followed by a more slowly progressing effect up to about eight hours. These papers had not yet appeared when the experimental work was done. No attempt was made to control the preceding dark rest, which varied from about two to eight hours during each run for cells grown in light. The experimental points, however, were not obtained in any definite order and any appreciable change in the true induction period during this time would hardly have permitted the points for the two, three, and four minutes illumination to fall on a straight line. Of course, the final straight line may not be truly constant in slope. Indeed, in continuous light all three batches of cells showed photosynthetic rates increasing slowly with time and always slightly higher than the final rates indicated by the curves in figure 5. But following the usual interpretation, the induction period is clearly over somewhere between one and two minutes of illumination. Certainly dark rests of more than two hours could have had little if any additional effect on the characteristics of the induction period in *Chlorella vulgaris*.

It is also of interest to compare the induction curves (fig. 5) with those obtained by other workers using the same method, which is based on the rate of evolution of oxygen. Smith (**19**), using a preceding 30-minute dark rest, found for Cabomba induction periods of about two to five minutes depending on the light intensity and CO_2 concentration. Van der Paauw's (**21**) curves for Hormidium show an induction of $1\frac{1}{2}$ minutes at 26° C. (length of dark rest not stated). Warburg (**24**) observed for *Chlorella vulgaris* an induction period of $1\frac{1}{2}$ minutes after a five-minute dark rest. His conditions

of light intensity, CO_2 concentration, and temperature were almost identical with those maintained in the present study. The induction period obtained by WARBURG after a five-minute dark rest is identical with that shown in figure 5 for the same organism after an infinitely long dark rest. This is also in line with WARBURG's data for one- to five-minute periods of intermittent light.

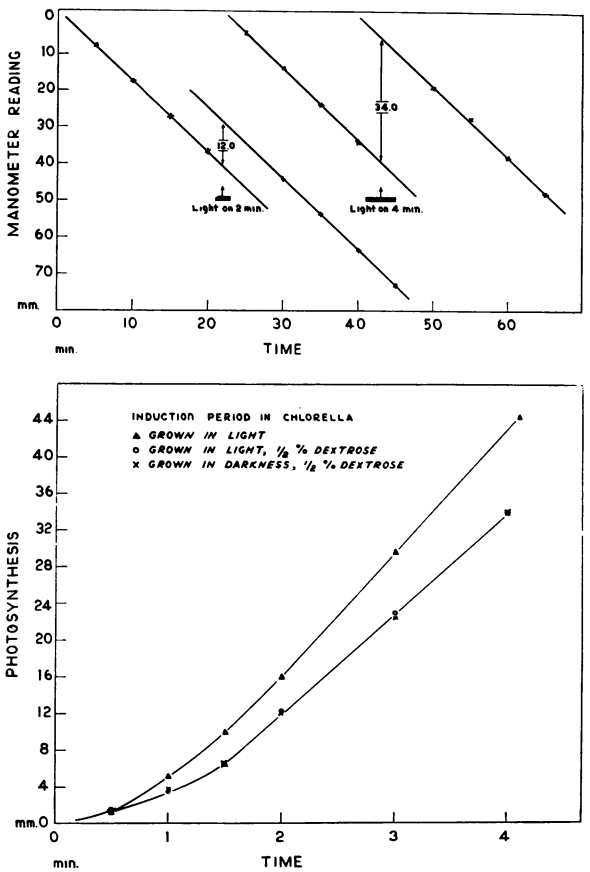

FIG. 5. The induction period in *Chlorella vulgaris*. Ordinates are readings from the Warburg manometers in mm.; multiply by 0.56 to obtain cu. mm. of O_2. The upper set of curves describes the method by which each point in the lower set of induction curves was obtained. For further explanation see text.

Any detailed study of the effect of the preceding dark rest on the induction period was not a planned part of this investigation, and the results are certainly not critical in this respect. However, after comparison of the data of WARBURG and of this paper on one hand with those of MCALISTER or of AUFDEMGARTEN on the other, it seems that the induction in O_2 production

and the induction in CO_2 uptake are not equally dependent upon the preceding dark rest, and are therefore probably dissimilar in other respects.

Summary

1. The pigments produced by *Protococcus* sp. and *Chlorella vulgaris* in darkness have been studied with a photoelectric spectrophotometer and compared with the pigments produced in light. For this purpose a method of extraction not previously used in pigment work has been described.

2. No significant qualitative differences have been found between the chlorophyll pigments produced in light and in darkness.

3. The induction periods for cells grown in light and in darkness have been studied by the WARBURG technique for a strain of *Chlorella vulgaris*. Only slight differences have been found between the induction curves of cells grown in darkness and in light.

4. The pigments produced by this strain of *Chlorella vulgaris* in darkness are adequate for the process of photosynthesis.

5. From a comparison of the data obtained with other data in the literature on the induction period it has been suggested that there is a dissimilarity between the induction in O_2 evolution and the induction in CO_2 uptake.

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