

# A QUANTITATIVE STUDY OF CHLOROSIS IN CHLORELLA UNDER CONDITIONS OF SULPHUR DEFICIENCY

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(WITH TEN FIGURES)

## Introduction

The rôle of chlorophyll in the process of photosynthesis is not clearly understood.<sup>1</sup> Present evidence indicates that under certain conditions there is a direct relation between chlorophyll content and photosynthesis in *Chlorella* cells (1, 4). On the basis of experiments with intermittent light, EMERSON and ARNOLD (2) suggested the existence of a chlorophyll unit in the light reaction in photosynthesis. Later work has shown, however, that such a relation does not always exist and that the concept of a fixed chlorophyll unit in photosynthesis is no longer tenable (3). It would be of considerable interest to know how the efficiency of chlorophyll changes with varying degree of chlorosis. A thorough investigation of this type is not available. Prerequisite to such a study is a quantitative analysis of the development of chlorosis and of recovery from chlorosis. The present work is an investigation of this nature under conditions of sulphur deficiency.

## Methods

The strain of *Chlorella* used in this investigation was originally isolated from soil by WANN (12) and is known as *Chlorella* no. 11. It is the same strain that was used in the studies of HOPKINS and WANN (5, 6, 7), FLEISCHER (4), PEARSALL and LOOSE (10), LUDWIG (9), and KENNEDY (8). FLEISCHER'S (4) modification of the nutrient solution employed by EMERSON was used, the composition being: Na citrate—1.00 gm.; KNO<sub>3</sub>—1.25 gm.; MgSO<sub>4</sub> · 7H<sub>2</sub>O—2.46 gm.; KH<sub>2</sub>PO<sub>4</sub>—1.22 gm.; Fe (as FeCl<sub>3</sub>)—0.01 gm.; glucose—15 gm.; H<sub>2</sub>O—1 l. With the exception of the Na citrate (Baker and Adams, C. P.), (fig. 1) all the chemicals used were J. T. Baker's C.P. analyzed. The sulphur deficient medium was prepared by replacing the MgSO<sub>4</sub> with an equivalent amount of MgCl<sub>2</sub>. Three-liter Florence flasks containing 1.5 l. or 12-l. flasks containing 8 l. of nutrient solution were used as culture vessels. The cultures were sterilized by autoclaving at 15 lb. for 25 minutes or for 35 minutes when the larger vessels were used. The initial pH of the cultures after sterilization was about 5.3. Suspensions of cells grown in a full nutrient solution were employed for inoculation. In some experiments a suspension of cells in sterile distilled water was used, the cells having been rinsed previously in distilled water (three times, by repeated centrifugation and decantation, observing aseptic precautions).

Large culture vessels were used so that the studies could be confined to cultures in which the population density did not exceed 2,000 cells/mm.<sup>3</sup>

<sup>1</sup> Examination of the literature leads to the conclusion that there is little direct data concerning the rôle of chlorophyll.

The possibility of secondary effects due to significant changes in the composition of the nutrient solution was thus minimized.

The cultures were grown in an insulated light chamber at 25 or 30° C.  $\pm 0.5^\circ$ . A 500-watt tungsten filament bulb placed in a reflector over the top of the chamber served as a light source. Infra-red radiation was filtered out by a  $\frac{1}{2}$ -inch layer of circulating water. The light intensity was of the order of 500 foot candles as measured on the floor of the chamber with a Weston Illuminometer. The cultures were shaken for 5 minutes every half hour, a mobile platform being in the chamber.

The population density of the cultures was determined with a haemocytometer. A photoelectric colorimeter was used to determine the chlorophyll content of methanol extracts of the cells. Interference by carotenoids was avoided by using a Corning signal red filter having a cut-off at 6300 Å. The colorimeter was calibrated with a preparation of purified chlorophyll extracted from corn leaves following the procedure of SCHEITZ (11). Chlorophyll determinations were always made immediately after removal of the sample from a culture.

#### EXPRESSION OF DATA

The population density of the culture ( $N$ ) is in terms of cells per mm.<sup>3</sup> of culture. The rate of cell division is the slope of the curve giving the logarithmic increase in cell number per hour,  $\left(\frac{\Delta \log N}{\Delta T}\right)$ . Chlorophyll is expressed in micromoles per 50 ml. of culture, ( $C$ ), or on a basis of  $\mu$  moles per 10<sup>9</sup> cells ( $C_n$ ). The rate of chlorophyll formation is the slope of the curve giving the logarithmic increase in chlorophyll,  $\frac{\Delta \log C}{\Delta T}$ .

### Results

#### DEVELOPMENT OF CHLOROSIS IN SULPHUR-DEFICIENT CULTURES

Practically no growth will occur if a suspension of washed cells, grown in a full nutrient solution, is used to inoculate a culture to which no sulphate has been added. Thus neither previous accumulation of sulphur in cells nor sulphur impurities in the chemicals used, are significant. In the experiments reported here, sufficient sulphate was added to support growth up to a population density of the order of 2,000 cells per mm.<sup>3</sup>

Data showing typical behavior during growth in a sulphur deficient culture are presented in figure 1. It is seen that cell division and chlorophyll formation gradually cease. It is significant that the rate of chlorophyll formation decreases more rapidly than the rate of cell division. Thus there is an initial decrease in the amount of chlorophyll per cell while chlorophyll formation is still occurring. Even after cessation of chlorophyll formation there is a slight increase in cell number. This results in a further decrease in the chlorophyll content per cell. Development of chlorosis continues due to decomposition of chlorophyll in the cells. Decomposition is initiated about the time cell division ceases.

To get a quantitative measure of the degree of chlorosis the chlorophyll deficit of the chlorotic cells can be expressed as a percentage of the normal chlorophyll complement.

$$\text{Percentage of chlorosis} = \frac{C_{N_0} - C_{N_1}}{C_{N_0}} \times 100$$

where  $C_{N_0} = \mu$  moles chlorophyll/ $10^9$  normal cells = 3.50;  $C_{N_1} = \mu$  moles chlorophyll/ $10^9$  chlorotic cells. On this basis it is found that when chlorophyll synthesis stops, the cells are 45 per cent. chlorotic. The slight increase in cell number found after this point results in 64 per cent. chlorosis when cell division stops. These values for the degree of chlorosis at cessation of chlorophyll formation and at cessation of cell division appear to be quite

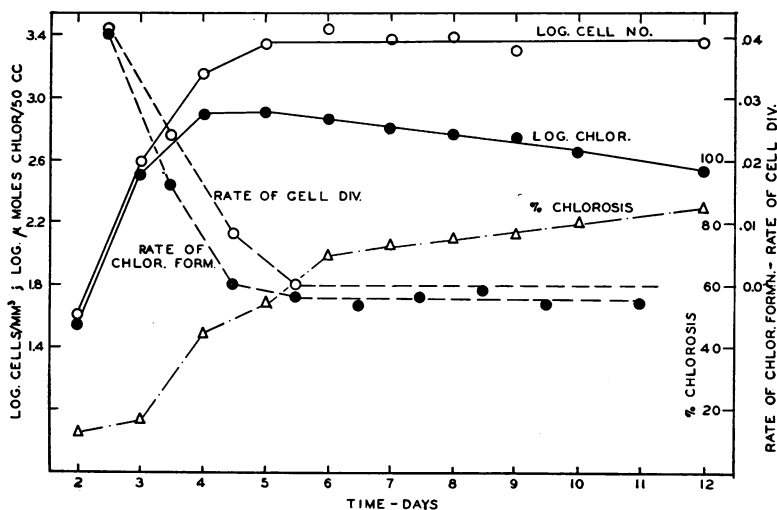


FIG. 1. Growth in culture lacking sulphur showing the development of chlorosis at 25° C. (The ordinate log. chlorophyll has been shifted up 4.75 units.)

constant. Decomposition of chlorophyll results in a gradual increase in the degree of chlorosis. Complete loss of chlorophyll (100 per cent. chlorosis) is attended by death of the cells since they no longer respond to addition of sulphate. Such cells contain significant amounts of carotenoids. The carotenoids decompose gradually in the dead cells.

Microchemical tests of the chlorotic cells with Sudan IV in lactophenol and with I-KI show an accumulation of considerable amounts of fat and little or no starch. This behavior is also found if sugar is omitted from the medium. Normal cells in full nutrient solution do not contain sufficient fat or in a form to be demonstrated with Sudan IV.

#### RECOVERY FROM CHLOROSIS

To follow the process of recovery an aliquot of a sterile, standard solution of  $K_2SO_4$  was added aseptically to cultures containing sulphur deficient, chlorotic cells. Samples were removed aseptically at intervals for chloro-

phyll determinations. In some experiments lasting only about 24 hours, aseptic precautions were not observed. In such cases bacterial or fungal growth was insufficient to have any noticeable effect. The cultures were kept in the growth chamber during recovery.

Chlorophyll formation is the first visual evidence of recovery—(macro- or microscopic). This can be detected about five hours after the addition

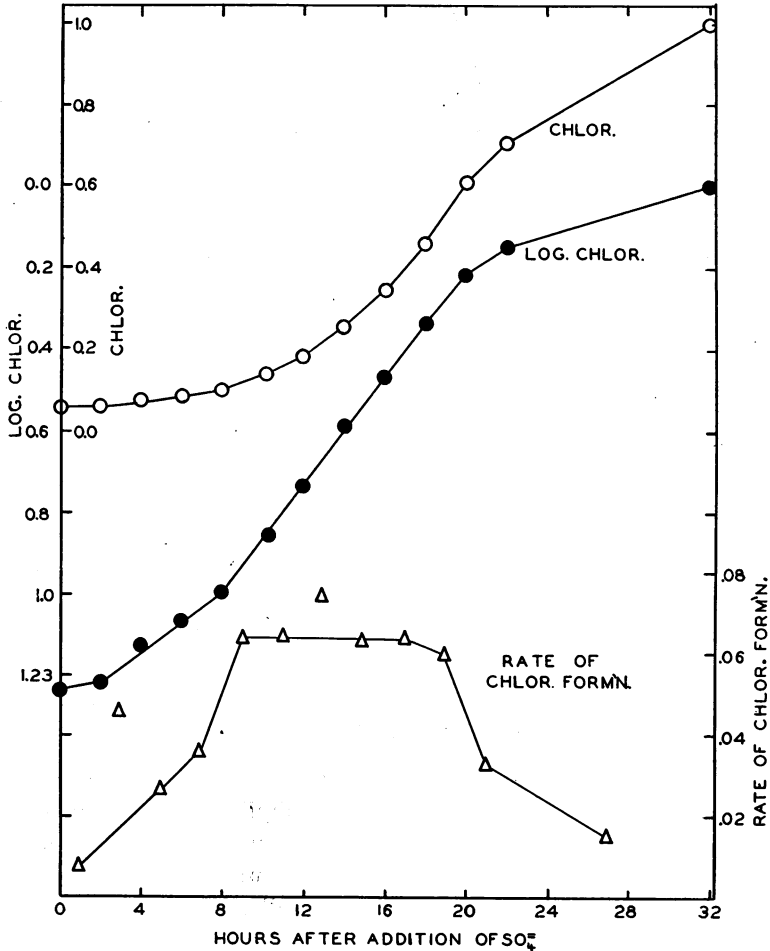


FIG. 2. Chlorophyll formation during recovery from sulphur deficiency. (2090 cells per  $\text{mm}^3$ ; 84 per cent. chlorosis; 1.67 p.p.m. of S as  $\text{SO}_4^{2-}$ ;  $25^\circ\text{C}$ .)

of sulphate. In a culture having a population of  $1500\text{ cells}/\text{mm}^3$ , formation of chlorophyll occurs at concentration of sulphate as low as  $3 \times 10^{-7}\text{ M}$  (0.01 p.p.m. of S). Recovery does not proceed sufficiently to permit cell division unless about 0.1 p.p.m. of S is added. If cell division is to occur there is a lag of about 24 hours before autospore formation is noted. Growth by increase in cell volume, as noted by microscopic observation, occurs before the cells divide (no quantitative measurements were made).

Typical data showing the formation of chlorophyll are presented in figure 2. The process of chlorophyll formation during recovery can be separated into four phases:

1. A lag period of a few hours (usually about four hours) between addition of sulphate and chlorophyll formation.
2. A period of acceleration in chlorophyll synthesis during which the rate of formation increases from zero to a maximum which is attained at about the tenth hour.
3. A period of logarithmic increase in chlorophyll. It should be noted that the maximum rate of synthesis which is maintained during this period (about 10 hours) is considerably in excess of the rate of chlorophyll formation during normal growth in full nutrient solution.

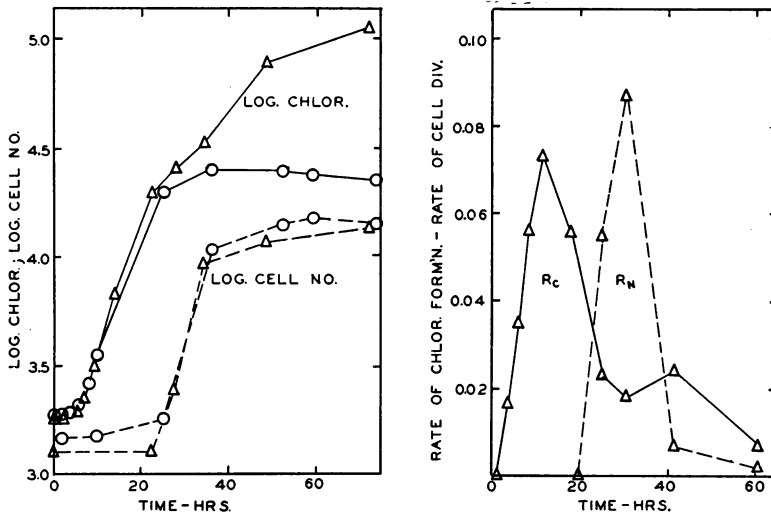


FIG. 3. Chlorophyll formation and cell division during recovery. (1.93 p.p.m. of S  $\circ$ ; 10.0 p.p.m.  $\Delta$ ; broken line—log. cell no.; solid line—log. chlorophyll) (70 per cent. initial chlorosis; 25° C.)

FIG. 4. Rate of chlorophyll formation ( $R_c$ ) and rate of cell division ( $R_N$ ) during recovery in 10 p.p.m. of S culture.

4. A period of decrease in rate of synthesis. In the culture under consideration here, sufficient sulphate for continued recovery was not present so that the rate decreases to zero.

Data showing recovery with respect to both chlorophyll formation and cell division in two comparable cultures at different concentrations of sulphate are given in figures 3 and 4. There is a rapid increase in chlorophyll in both cultures. The cells, initially about 70 per cent. chlorotic, contain several times the normal chlorophyll complement at the time cell division is initiated. At a concentration of 1.93 p.p.m. of S there was insufficient sulphate for continued recovery. Thus there is only a slight increase in chlorophyll after the inception of cell division. A rapid decrease in the amount

of chlorophyll per cell is found during the spurt in cell division (from 8.66 to 1.88  $\mu$  moles/ $10^9$  cells). Subsequently there is a gradual decrease due first to cell division and finally to decomposition. Thus the cells have returned to a condition of sulphur deficiency.

At the concentration of 10 p.p.m. further recovery was possible, synthesis of chlorophyll continuing after the initial spurt in cell division. The sudden decrease in slope of the log chlorophyll curve at the time cell division starts should be noted (fig. 4).

It should be observed that while there is a relation between the amount of chlorophyll formed and the amount of sulphate added, there is none with respect to increase in cell number. The chlorophyll yield will be considered in more detail later.

Since a detailed analysis of the process of cell multiplication during recovery is not directly pertinent to the scope of this paper, a brief summary will be adequate. Although chlorophyll formation can be detected when as little as 0.01 p.p.m. of S is added to a suspension of chlorotic cells, at least ten times this amount must be added before recovery proceeds to a stage where cell division will occur (in a culture of 1500 cells/mm.<sup>3</sup>). With this minimum amount of sulphate there will be a nine-fold increase in cell number. Increasing the sulphate concentration ten times above the minimum for cell division will still result in the same increase in cell number. If still greater amounts of sulphate are added then there will be an initial nine-fold increase in cell number followed by a short lag before further cell division continues. Thus at least at low concentrations of sulphate the increase in cell number is not proportional to the amount of sulphate added.

#### FACTORS INFLUENCING CHLOROPHYLL FORMATION DURING RECOVERY

**EFFECT OF INITIAL DEGREE OF CHLOROSIS.**—To study the effect of length of exposure of cells to a condition of sulphur deficiency upon subsequent recovery, a culture containing 8 l. of nutrient solution was used. Samples were removed aseptically at intervals corresponding to 6.83, 8.88, and 12.21 days after inoculation. Chlorophyll formation was studied after adding 1.0 p.p.m. of S as  $\text{SO}_4^-$ . Thus recovery was followed using portions of the same suspension of cells having chlorophyll complements equivalent to 22, 28.5 and 45 per cent. chlorosis. Although the first sample of cells was only 22 per cent. chlorotic, cell division had ceased. This is contrary to the preceding data in which cell division ceased when the cells were about 65 per cent. chlorotic. This anomalous behavior was probably due to inadequate provision for gas exchange during the development of the culture. Presumably the low  $\text{O}_2$  tension suppressed the amount of growth by cell division while chlorophyll formation did not suffer similar interference. The results reported below have been substantiated using "normal" sulphur deficient cells.

The data are summarized in figures 5 and 6. During the first few hours after addition of sulphate there is a decrease in chlorophyll. The extent of

this decomposition is greatest in the slightly chlorotic cells and decreases progressively in the two subsequent series. The interpretation of this phenomenon is not clear. The rate is too great to be normal decomposition.

The recovery curves (fig. 5) show that despite the relatively large differences in initial chlorophyll content the maxima reached are practically the same in all three series. Thus the number of moles of chlorophyll formed per mole of sulphate added is determined to a slight extent by the initial degree of chlorosis, the exact figures being 0.307, 0.356, and 0.360 for the three series.

Consideration of the rate of chlorophyll synthesis in the three series brings out another interesting relation (fig. 6). The curves show that the maximum rate of synthesis attained in each of the series is higher as the initial degree of chlorosis increases.

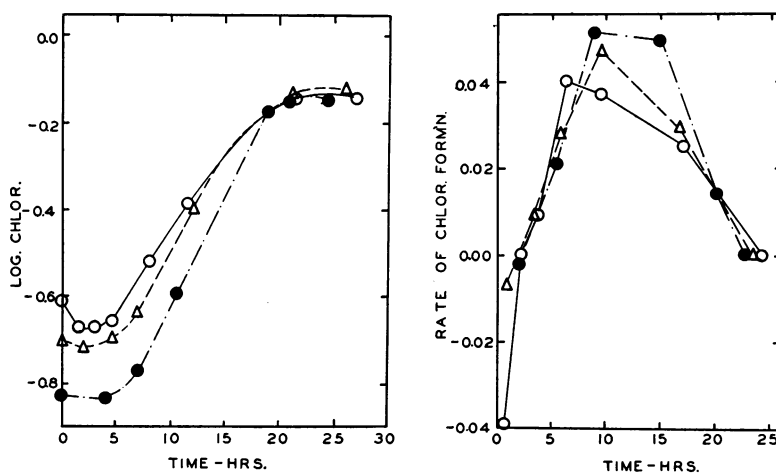


FIG. 5 and 6. Effect of initial degree of chlorosis on chlorophyll formation during recovery. (○ — 22 per cent.; △ — 28.5 per cent.; ● — 45 per cent. chlorosis.) (1.0 p.p.m. of S; 1520 cells 1 mm.<sup>3</sup>; 25° C.)

**EFFECT OF SULPHATE CONCENTRATION ON THE RATE OF CHLOROPHYLL SYNTHESIS.**—Eight liters of a suspension of sulphur deficient cells were divided into equal portions to which different amounts of sulphate were added. The vessels were then placed in the growth chamber under a condition of darkness to minimize chlorophyll decomposition. Since identical suspensions of cells were used, sulphate concentration was the only variable. Data showing the changes in rates of synthesis in the cultures are summarized in figure 7. Two effects are to be observed. First, the effect on the acceleration of chlorophyll synthesis—increasing the concentration up to about 0.5 p.p.m. of S results in a more rapid increase in rate of synthesis. Greater increments have little or no effect (see also fig. 3). Secondly, it is seen that the maximum rate of synthesis attained increases as sulphate concentration is raised to about 0.5 p.p.m. of S. This relation is seen more exactly in figure 8. From 0.05 to 0.5 p.p.m. the relation  $R_c = K \log [SO_4^{=}]$  obtains (where  $R_c = \text{maxi-}$

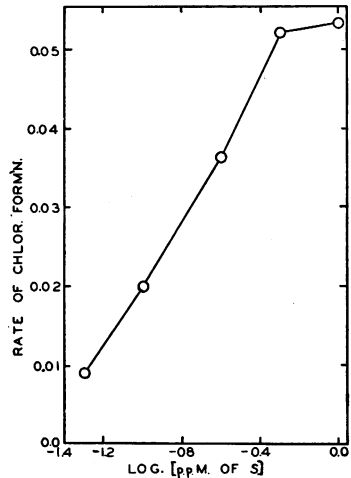
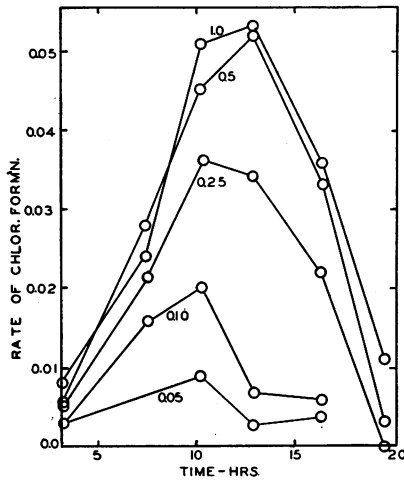


FIG. 7. Effect of sulphate concentration (figures in p.p.m. of S) on chlorophyll formation during recovery.

FIG. 8. Maximum rate of chlorophyll synthesis attained at different concentrations of sulphate during recovery.

imum rate of chlorophyll formation). At concentrations greater than 0.5 p.p.m. there is little or no effect on the maximum rate (see also fig. 3).

**EFFECT OF LIGHT.**—One of two similar cultures was darkened by enclosing it in several layers of black cloth with black rubberized cloth on the outside. Both cultures were then placed in the light chamber after adding sulphate. Any temperature differences were not considered as being significant. The results are shown in figures 9 and 10. Although the differences between the

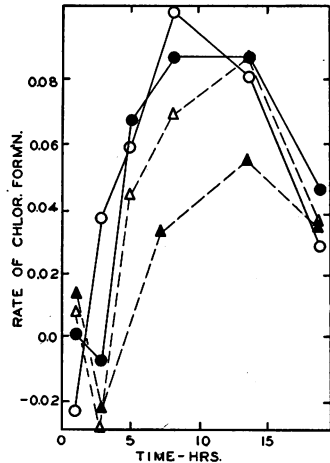
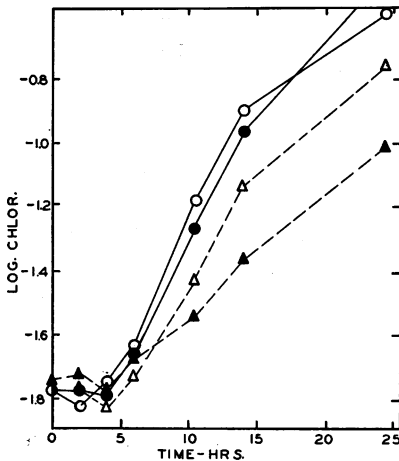


FIG. 9. Effect of light and source of sulphur on chlorophyll formation during recovery. (○ —  $\text{SO}_4$  in light; ● —  $\text{SO}_4$  in dark; △ —  $\text{H}_2\text{S}$  in light; ▲ —  $\text{H}_2\text{S}$  in dark.)

FIG. 10. Effect of light and source of sulphur on the rate of chlorophyll formation. (Symbols same as in fig. 9.)



two treatments are not very great, it should be noted that there is a slightly longer lag in the dark and also that the maximum rate of chlorophyll synthesis attained is slightly less in the dark. Another effect is brought out in table I which shows the chlorophyll yield to be slightly greater in the dark. Limited data show that during recovery the rate of cell division is retarded to a much greater extent in the absence of light than is synthesis of chlorophyll.

SOURCES OF SULPHUR AVAILABLE.—Some qualitative studies were made to test the availability of different sulphur compounds for recovery from

TABLE I  
MOLES CHLOROPHYLL FORMED\* DURING RECOVERY PER MOLE OF SULFATE ADDED

SULPHATE CONCENTRATION	MOLES $\text{SO}_4^{=}$ ADDED/10 <sup>9</sup> CELLS	INITIAL CHLOROSIS	CELL DIVISION	MOLES CHLOR. FORMED/MOLE $\text{SO}_4^{=}$ ADDED
		%		
Light 1.93 p.p.m. of S	42.0	70.5	+	0.310
1.0	20.4	22.0	+	0.307
1.0	20.4	28.5	+	0.356
1.0	20.4	45.0	+	0.360
1.0	24.4	68.6	+	0.394
0.5	10.2	22.0	+	0.314
0.5	10.2	28.5	+	0.357
0.5	10.2	45.0	+	0.415
0.167	2.74	85.4	—	0.291
0.10	2.44	68.6	—	0.321
0.01	0.244	68.6	—	0.321
Dark 1.0	20.4	52.0	?	0.280
0.5	10.2	51.8	?	0.405
0.25	5.1	51.8	?	0.483
0.10	2.04	51.8	?	0.483
0.05	1.02	51.8	?	0.420
0.05	1.02	51.8	?	0.385
0.025	0.51	51.8	?	0.456

\* Amount of chlorophyll formed = max. chlorophyll during recovery - initial chlorophyll.

Average yield in light—0.34 moles chlor./mole sulphate.

“ “ “ dark—0.42 “ “ “ “

sulphur deficiency. All the organic compounds tested were unavailable (cysteine, glutathione, methionine, thioglycollic acid, thiamin, potassium thiocyanate). The possibility that toxic concentrations of these substances were used is unlikely since recovery occurred in control cultures to which both sulphate and the various compounds were added. All of the inorganic compounds tried were available for recovery (hydrogen sulphide, thiosulphate, pyrosulphate, sulphite, persulphate). Tests also showed that sulphate could not be replaced by tellurate or selenate.

While it would be very interesting to have quantitative data showing the length of the lag period, the rate of chlorophyll synthesis and the chlorophyll yield during recovery when different sulphur compounds were used, comparative studies were made only with  $\text{H}_2\text{S}$  and  $\text{SO}_4^{=}$ . The results of this study are summarized in figures 9 and 10. The curves show a longer lag

when sulphur is supplied as  $H_2S$  than as sulphate and also a slower rate of synthesis, these effects being more pronounced in the dark.

**EFFECT OF TEMPERATURE.**—No detailed study was made of the effect of temperature on chlorophyll formation during recovery. Data are available, however, to show that: (1) The maximum rate of synthesis attained at  $30^\circ C$ . is significantly higher than at  $25^\circ C$ . (2) Recovery does not occur (chlorophyll formation or cell division) at  $10^\circ C$ . or lower, although the cells are not permanently injured by this treatment.

**NECESSITY OF OXYGEN.**—Results of several experiments showed that no recovery occurs if the atmosphere in equilibrium with the culture medium was unpurified tank nitrogen (probably containing about 0.5 per cent.  $O_2$ ).

**CHLOROPHYLL YIELD DURING RECOVERY.**—It is obvious that the extent of recovery must be some function of the amount of sulphate added and data presented already have anticipated the conclusion that the amount of chlorophyll formed shows some proportionality to the amount of sulphate added. Results from several different experiments showing the amount of chlorophyll formed during recovery per mole of sulphate added are summarized in table I. A consideration of the data obtained during recovery in the light shows that the chlorophyll yield is more or less constant over a wide range in concentration of sulphate—from 0.01 to 1.93 p.p.m. of S. About 0.34 moles of chlorophyll is formed per mole of sulphate added. In the dark, the yield is slightly greater, being 0.42 moles of chlorophyll per mole of sulphate added.

### Discussion

Presumably the pattern of development of chlorosis is distinct with deficiencies of various essential elements which result in chlorosis. In the case of sulphur deficiency, chlorosis develops in two stages. The first phase is due to the development of a differential between the rate of chlorophyll formation and the rate of cell division, chlorophyll synthesis decreasing before cell division. The second phase is due to decomposition of chlorophyll in the cells and is initiated at about the time cell division ceases. Attention should be directed to the fact that both cell division and chlorophyll synthesis do not stop abruptly as the supply of sulphur becomes limiting. There is a gradual decrease in rate. This can be interpreted in two ways. It is conceivable that this is not real but is only an apparent, statistical effect; the individual cells in a culture being of different ages would not all show deficiency symptoms at the same time. Microscopic observation reveals this to be at least partially true. On the other hand, it is possible that the phenomenon is real—that as the concentration of available sulphur decreases to some minimum value, the rate of chlorophyll synthesis, etc., gradually decreases. If this is true, it follows that the rate of chlorophyll synthesis and other metabolic processes are determined, at least partially, by the concentration of sulphate when it becomes limiting. In the author's opinion the true interpretation of the gradual decrease in rate is to be found by combining these two explanations. Assuming that the rate of chlorophyll syn-

thesis and other metabolic processes decrease gradually as sulphate becomes limiting, it follows that the rates of these processes are functions of sulphate concentration under these conditions. In this connection it is significant to note that with a decreasing supply of sulphate there is a differential action upon at least some metabolic processes. More specifically, chlorophyll formation is more sensitive to a deficiency of sulphur than is cell division. Differential responses of this nature could be useful in studies of sulphur metabolism.

Further evidence illustrating a disturbance of cell metabolism in sulphur-deficient cells is the observation that these chlorotic cells contain large quantities of fat, and little or no starch. This fat accumulation also occurs when the cells are grown autotrophically. (Starch is the normal storage product in *Chlorella*, very little or no fat being found.)

Addition of sulphate to sulphur-deficient cells was found to result in rapid recovery from chlorosis. When sulphate is added in concentrations of 0.5 p.p.m. of S, or greater, the recovery curve showing the formation of chlorophyll can be separated into four phases: (1) a lag period; (2) a period of increase in rate of synthesis to a maximum; (3) a period of exponential increase in chlorophyll at the maximum rate; and (4) a decrease in rate of synthesis to zero or to normal depending upon the amount of sulphate available.

It is conceivable that the initial lag is due to slow penetration of sulphate. This would appear very unlikely. If slow penetration were responsible there should be some direct relation between concentration of sulphate and length of the lag period since, according to Fick's law, the rate of diffusion is directly proportional to the concentration gradient. No such relation was found. If this lag is a real phenomenon, then it is to be inferred that sulphate bears no simple, direct relation to the synthesis of chlorophyll. It is possible that sulphate is metabolized during the lag period, these anabolic processes culminating or being involved in the synthesis of chlorophyll. On the other hand, it is conceivable that free sulphate initiates or activates a series of reactions leading to chlorophyll formation. It would seem that the first of these postulates is more reasonable in view of the stoichiometric relation between sulphate added and chlorophyll formed.

The initial degree of chlorosis—that is, the degree of sulphur starvation—has some bearing on the behavior during the lag period. The effect cannot be stated in any more definite terms because of insufficient data. It should be pointed out, however, that significant decomposition of chlorophyll occurs during the first few hours after addition of sulphate to slightly chlorotic cells.

An interesting phase of the formation of chlorophyll during recovery is the exponential increase in chlorophyll. The slope of the line showing the logarithmic increase in chlorophyll is several times (2.6) greater during recovery than during normal growth in full nutrient solution. It would appear from this that the factor (or factors) normally limiting the rate of

chlorophyll synthesis is not in operation during recovery from chlorosis. It should be pointed out in this connection, that with increased initial chlorosis the rate of logarithmic formation of chlorophyll during recovery was found to be greater. Although several interpretations of the behavior are possible the information available is inadequate to make discussion profitable.

The relation between the amount of sulphate added to a suspension of chlorotic cells and the amount of chlorophyll formed is significant. Summaries of data from several experiments reveal that about 0.34 moles of chlorophyll are formed per mole of sulphate added over a concentration range of 0.01–1.93 p.p.m. of S. This independence of the chlorophyll yield upon concentration of sulphate would indicate complete absorption of sulphate. The existence of this stoichiometric relation would seem to preclude any direct or indirect catalytic action of sulphate.

With greater initial chlorosis the chlorophyll yield is slightly increased. It should be mentioned that the increased yield is the same at different concentrations of sulphate and is equal to the differences in the initial amount of chlorophyll present in the cells. Thus if a given amount of sulphate is added to suspensions of cells of different degrees of chlorosis the maximum chlorophyll content which is found in the different suspensions is the same. An obvious hypothesis to interpret this behavior would be that decomposition products accumulate in the cells when the degree of chlorosis increases due to chlorophyll decomposition and that synthesis of chlorophyll from these products can occur during recovery without utilization of sulphate. This hypothesis must be discarded, however, since more rigid consideration of the data do not lend support.

For many years, it has been known that *Chlorella* can form chlorophyll when grown in the dark. It has also been recognized that when cultured in the dark on glucose, *Chlorella* grows much more slowly than in the light. It is interesting to note in this connection that light has very little effect on chlorophyll formation during recovery from sulphur deficiency, the rate of synthesis being only slightly greater in the light. On the other hand, the amount of chlorophyll formed in the dark per mole of sulphate is significantly greater. This increase in yield would seem too great to be explained on the basis of more rapid decomposition in cells in the light. With respect to initiation of cell division during recovery there is a very pronounced effect of light—cell division being many times slower in the dark. It might be inferred that more sulphate is available for the processes resulting in chlorophyll formation when recovery occurs in the dark.

An analysis of the effect of various sulphur compounds on recovery could be used to advantage in studies of sulphur metabolism. While the data presented here which relate to sulphur metabolism are rather meager, brief discussion will be profitable. Since cysteine hydrochloride, dl-methionine, glutathione, thioglycollic acid and potassium thiocyanate were all found to be unavailable for recovery, it would appear that the course of sulphur metabolism does not proceed through one of these compounds. If

it does, then we must assume that the metabolism proceeds in several directions and that these reactions are not readily reversible. An alternative interpretation is impermeability of the cell to these compounds. This would not seem to be the case with cysteine since LUDWIG (9) has shown that it can be used as a source of nitrogen by this same strain of *Chlorella*.

All the inorganic compounds tested were available for recovery—thio-sulphate, pyrosulphate, sulphite, persulphate, and hydrogen sulphide. When sulphur is supplied as  $H_2S$  the lag period is slightly longer, the acceleration in chlorophyll synthesis is slightly lower and the maximum rate of synthesis attained is less than when sulphate is supplied. This would lead to the conclusion (if questions of toxicity and rate of penetration are neglected) that sulphur in the form of  $H_2S$  must undergo more radical changes than sulphate before participation in metabolism. It is conceivable that oxidation to sulphate takes place. The response to sulphide in the dark is significantly slower than in the light. Possibly the lower oxidation potential in the cells under a condition of darkness results in slower oxidation to sulphate.

Lack of quantitative data on the course of recovery with the other inorganic compounds precludes any postulation of their relation to sulphur metabolism other than that they are assimilable.

### Summary

1. *Chlorella* sp. was grown under controlled conditions in sulphur-deficient cultures. The development of chlorosis and the process of recovery from chlorosis have been analyzed. The chlorophyll deficit of the chlorotic cells has been put on a quantitative basis and expressed as a percentage of the normal chlorophyll content.

2. Chlorosis induced by sulphur deficiency develops in two phases due respectively to: (a) A differential between the rate of cell division and the rate of chlorophyll synthesis as they both decline to zero. This results in a decrease in the amount of chlorophyll per cell before cell division stops. (b) Slow decomposition of chlorophyll in the non-dividing cells.

3. Fat accumulates in the deficient cells (starch is the normal storage product).

4. Addition of sulphate to deficient cells results in rapid recovery from chlorosis. Although chlorophyll synthesis is evident within about 5 hours, cell division does not occur until about 24 hours after addition of sulphate. Chlorophyll formation will occur at a lower concentration of sulphate than will cell division.

5. The curve showing the formation of chlorophyll during recovery can be separated into four stages: (a) A lag period of about five hours between addition of sulphate and synthesis of chlorophyll. (b) A period of acceleration in synthesis of chlorophyll during which the rate of formation increases from zero to a maximum over a period of about five hours. (c) A period of logarithmic increase in chlorophyll at a rate faster than occurs during normal growth in full nutrient solution. (d) A period of decrease in rate of synthesis.

6. The effect of various factors on recovery was studied: light *vs.* darkness; initial degree of chlorosis; concentration of sulphate; sources of sulphur available (organic and inorganic); necessity of oxygen.

7. The chlorophyll yield during recovery is constant over the concentration range 0.01–1.93 p.p.m. of S as sulphate, about 0.34 moles of chlorophyll being formed per mole of sulphate added. It is inferred that complete absorption of sulphate takes place.

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