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INHIBITION OF GROWTH AND METABOLISM OF *CHLORELLA* AND SOME OTHER PLANT TYPES BY CALCIUM DIPICRYLAMINE AND OTHER POISONS

by

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CHAPTER I

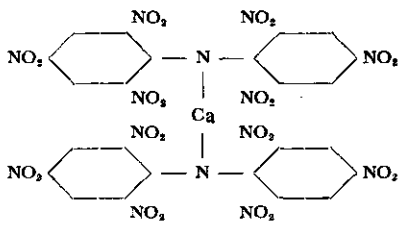
INTRODUCTION

§ 1. SCOPE OF THE PRESENT INVESTIGATION

Hexanitrodiphenylamine or dipicrylamine (abbreviated as D) is a weak acid, which has a low solubility in water (0.1 mg/l.). Salts of dipicrylamine are easily soluble in water with the exception of the potassium salt. The solutions have an intensely red colour, while sodium and ammonium salts have been widely used as dyes for leather and silk and for light filters for photographic purposes under the name „Aurantia". On account of the selective insolubility of the potassium salt, dipicrylamine can be used as a reagent for potassium in spot tests (FEIGL [15]). Industrial application of D has started with the separation of potassium out of seawater (Kielland, Norwegian patents). In connection with research by the N.V. Mekog, the author has made experiments on the influence of D on some physiological processes, especially photosynthesis, in relation to possible consequences of pollution of potassium fertilizers by D.

Only few indications of toxic action of D were available from literature. It has been shown that D in waste waters is toxic for fishes already at extremely low concentrations (59). GAVAUDAN *et al.* (24) reported a strong distortion of cell division by D, even at lower concentrations than is found for colchicine.

The work reported here considers especially the effects of D on photosynthesis, respiration and growth in *Chlorella*, which was used as a simple test plant. Our experiments were performed with the calcium salt of dipicrylamine, CaD_2, since this salt is readily soluble and could be maintained in solutions even for a long time without an appreciable decrease in concentration. The experiments are mainly concerned with inhibition of various metabolic processes by D under different conditions of temperature, light intensity, pH, etc. Moreover, experiments were made in which the effects of CaD_2 were compared with those of other, well-known inhibitors (Chapters III and IV).



Other plant species have also been tested as to their reactions upon D in order to find out if their responses are similar (Chapter V).

Since D will always be applied together with potassium, the potassium metabolism of the plant may be important in this respect. However, the low concentration of D at which inhibition of growth and photosynthesis already was observed, and the different effects of potassium starvation and D inhibition observed in *Lemna* (Chapter V, see also [5]) decrease the possibility of a direct connection between D-effects and potassium metabolism. The latter, therefore, has not been studied in greater detail.

A brief review of some literature on inhibition of metabolism in plants will be given in section 2 of this chapter.

§ 2. SOME REMARKS ON LITERATURE ON INHIBITION, WITH SPECIAL REFERENCE TO PHOTOSYNTHESIS

Inhibitors affect many processes in plants; the nature of the response does not only depend on the concentration applied, but also on the different sensitivity of various parts of the plants. It must be born in mind that on application of a poison to intact plants or isolated tissues, an overall effect is measured involving the movement and partition of the substance between the point of application and various sites of toxic action. The response to 2,4-dinitrophenoxyacetic acid, for instance, has been shown on photosynthesis (FREELAND [19]), water relations (BROWN [11]), stomatal behaviour (BRADBURRY and ENNIS [8]) and ion absorption and accumulation (NANCE [40]), SMITH *et al.* [63]). It was, therefore, felt as important to use a simple test plant like *Chlorella* which is easier for inhibition treatments because of little differentiation, while mass cultures are easily obtained in simple nutrient solutions.

In representing the percentage inhibition against the logarithm of the dosis, an approximately linear relationship is obtained in several cases (AUDUS [3], BROWN and WEINTRAUB [12], WARBURG [71]) which is in accordance with the adsorption theory of FREUNDLICH (20). In many cases deviations from this relationship occur at threshold and near complete inhibition so that a more or less sigmoid curve results. TAMIYA (68), studying inhibition phenomena in photosynthesis under different conditions, derived a functional relationship between the degree of inhibition (H) and the concentration of the inhibitor (G), according to the formula:

$$H = \frac{G^n}{\Theta^n + G^n}$$ (n and Θ are constants representing the order of inhibitory reaction and the concentration causing 50 % inhibition respectively). However, deviations from this formula still occur, which may be ascribed to differences in behaviour of the individual cells, or may depend on the nature of the inhibitor. Comparisons between compounds only based on a single dose check may give misleading results, since, as stated above, for a large number of biological effects the relationship between the effect and the dose is represented by a sigmoid curve. The concentration giving 50 % inhibition or mortality established by applying a range of concentrations, therefore, gives the most reliable information in spite of the fact that threshold and complete inhibition are sometimes more important data for practical purposes. BLACKMAN (7), TAMIYA (68) and many others, express the relation in terms of equieffective doses.

Photosynthesis is generally more affected by inhibitors than respiration, the latter usually being reduced only at higher concentrations. GAFFRON (21), however, reported the reverse in *Scenedesmus* strain D, which probably is the only case hitherto known. The same author (23) compared the inhibition of photosynthesis with that of photoreduction in order to find out whether an inhibitor of photosynthesis affects the oxygen liberating system only.

WARBURG (71) already distinguished two types of inhibitors, *viz.*, specific enzyme poisons and narcotics. Enzyme poisons act only on certain specific enzyme surfaces, while narcotics indiscriminately block all enzyme surfaces. Several specific poisons have been found which only affect the light saturation level of photosynthesis while narcotics decrease both this level and the initial slope of the photosynthesis versus light intensity curve. However, the distinction between these two types is not always clear. WELLER and FRANCK (80), *e.g.*, observed a decrease with hydroxyl amine under light limitation, while GAFFRON (23) clearly showed a specific enzyme inhibition with this poison in experiments on photoreduction in *Scenedesmus*.

If the initial slope in a light intensity versus photosynthesis curve is changed upon addition of an inhibitor, a change in the initial slope of fluorescence, either increasing or decreasing, may be expected since interference with energy transfer may then be assumed to occur. A decrease in fluorescence yield will be observed when the inhibitor itself has a high degree of energy acceptance. Considerations along these lines have been brought about in extensive studies with *Chlorella* by FRANCK *et al.* (16, 17, 18) and with *Chlorella*, *Chromatium* and diatoms by WASSINK *et al.* (75, 76, 77).

The effect of D on fluorescence, therefore, has been studied at different light intensities in order to find out whether the inhibition with D observed at light limitation, also changes the initial slope of the fluorescence versus light intensity curve. In addition to this some flashing light experiments were made, in order to sustain the information obtained in continuous light.

TAMIYA (68), using various inhibitors, has observed an increase as well as a decrease in inhibition of photosynthesis with a decrease in temperature. Comparing the results of cyanide inhibition on photosynthesis and catalase activity, he noticed, however, that the temperature effect observed in the last mentioned inhibition does not always appear in photosynthesis. He explained this by assuming that the reaction step which is suppressed by cyanide, becomes less rate determining for the overall photosynthetic process at lower temparatures.

A temperature effect on applying sprays to insects has been reviewed by BROWN (9), who ascribed this phenomenon to:

1. the rate at which, and the extent to which the poison is absorbed by an organism and reacts on its cells and tissues.
2. the rate at which, and the extent to which the poison is eliminated by decomposition or detoxication, excretion or washing out.

With rapidly acting poisons killing may be faster at higher temperatures, with less rapidly acting poisons the reverse may occur; a recovery in general will be slower at lower temperatures. Experiments by POTTER and GILHAM (48) and by TAMMES (66) on animals point to such relationships.

In connection hereto the attention should be drawn to the surveys of LIPSCHITZ (37) and HEUBNER (28) on decomposition of aromatic nitro- and amino compounds. According to LIPSCHITZ (37) these compounds are metabolized in animal tissues according to:

$$R.NO_2 \xrightarrow{H_2} R.NO \underset{O}{\overset{H_2}{\rightleftarrows}} R.NH.OH \underset{O}{\longleftarrow} R.NH_2$$

in which toxicity may be indicated as o + +++ o

In this case some of the intermediate products, especially the hydroxyl amine type, are toxic.

CHAPTER II

MATERIAL AND METHODS

§ 1. METHOD OF CULTIVATION OF THE ALGAE

Chlorella, strain A, was used throughout the assimilation and respiration experiments.

The algae were kept as sterile stockcultures in tubes with 1.5% agar and a medium which per litre contained:

a. $FeSO_4$	0.03 g.	b. glucose	15 g.
$MgSO_4$	2.46 g.		
Na-citrate	1.00 g.		
KNO_3	1.26 g.		
KH_2PO_4	1.22 g.		

Solutions a. and b. were sterilized separately and mixed afterwards. The tubes were in a light cabinet after VAN NIEL, in which a continuous illumination was obtained by a vertical row of incandescent lamps. Ventilation of the cabinet resulted in a temperature of about 20 °C, being the most favourable temperature for growth of *Chlorella*.

The cultures for the experiments were prepared as follows: The algae were inoculated with a platinum wire into 100 cc flasks containing 50 cc liquid medium of the same composition as the agar medium. These cultures were also incubated in the light cabinet for several days. In this way, by the stimulating effect of glucose, a rapid growth was obtained. From these cultures, 5 cc inoculum were transferred to a one litre erlenmeyer containing 500 cc of WARBURG culture medium. This medium per litre contained:

a. $Ca(NO_3)_2$	1.00 g.	b. KH_2PO_4	0.25 g.
KNO_3	0.25 g.		
NaCl	0.15 g.		
c. $MgSO_4$	0.50 g.	d. $FeSO_4$	0.006 g.

In addition 2 cc of an A 4 and B 7 solution of trace elements according to ARNON (1) were supplied. Concentrated stock solutions of the salts mentioned above, were sterilized separately (b, c, and d), or in a mixture of suitable matched quantities (a).

The erlenmeyer flasks were placed on a shaking device and illuminated from below by 4 daylight fluorescent tubes at light intensities from 3500–4500 lux on the bottom of the erlenmeyers placed on the machine. The agitation served to prevent precipitation of the cells and to ensure a homogeneous illumination. The suspension was flushed with a stream of air containing 5% CO_2 through a capillary tube closed by a cotton plug. The average room temperature was 23 °C, the temperature of the suspension, however, was 2–3 degrees higher dependent on duration and intensity of illumination.

Studies on inhibition of photosynthesis and growth in various other plant types are presented in Chapter V. The preparation of the cultures for these experiments is discussed in that chapter.

§ 2. DESCRIPTION OF THE WARBURG APPARATUS

Photosynthesis and respiration measurements were made in a WARBURG apparatus.

The apparatus was of the usual type (figure 1), and had a thermostat bath of 100 × 30 cm, which could be kept at temperatures between 15 and 35 °C with an accuracy of 0.05 °C by an electric heater controlled by a thermo relay and a cooling system consisting of a continuous flow of tap water through a copper spiral. Two „Philora SO 1000" sodium lamps under the glass bottom of the thermostat were mounted in a white reflector; the lamps were cooled by two small (25W) fans. A shaking frame on which 12 manometers could be placed, was mounted in front of the thermostat. This frame is moved via an excentric at a rate of 150 revolutions per min. with an amplitude of about 5 mm. The stirring of the waterbath was achieved by a horizontal stirrer with 6 blades. The conical vessels with flat bottoms had a volume of about 20 cc, including two side arms of about 3–4 cc.

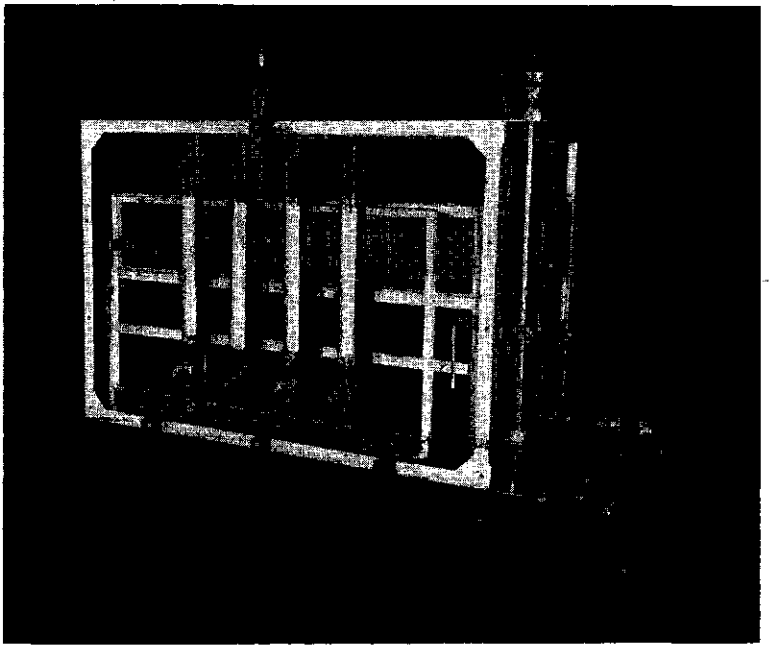


FIG. 1. Front view of the WARBURG apparatus.

Different light intensities were obtained by filters, placed in metal holders fixed onto the vessels. The light intensities were measured by a photocell which was calibrated against a large surface thermopile (KIPP en Zn., Delft). The position of the photocell was such that its sensitive surface could be placed at the same level as the bottom of each vessel. The highest light intensity obtainable in the arrangement was about 60,000 ergs/cm² sec.

§ 3. MEASUREMENT OF THE GAS EXCHANGE

Measurements with the WARBURG apparatus are based on the manometric principle, which implies that only those reactions can be observed, in which gases are formed or disappear. From the overall equation of photosynthesis:

$$CO_2 + H_2O \rightarrow CH_2O + O_2$$

it follows, however, that for each CO_2 molecule consumed, one molecule of O_2 is released which implies that no net gas exchange at all would be apparent. This difficulty can be overcome by various methods, which all have their advantages and disadvantages. This will be discussed in some detail below.

I. Measurements in which CO_2 is kept at a constant tension by a buffer mixture.

It is readily understood that the change in manometric reading (h) is due solely to oxygen evolution or uptake if the partial pressure of carbon dioxide in the gas space of the reaction vessel is maintained constant. Mixtures of carbonates and bicarbonates have been introduced by WARBURG (72) for keeping constant the partial pressure of CO_2. A large uptake of carbon dioxide from these mixtures, however, decreases the carbon dioxide pressure, especially if only small amounts of bicarbonate are present. Lower suspension densities were used or short time experiments were performed if such a decrease could be predicted. Usually mixture no. 9, consisting of 0.015 mol. carbonate and 0.085 mol. bicarbonate (pH 9.08), was used in experiments on photosynthesis.

Buffer no. 9 is in equilibrium with a carbon dioxide concentration of 9.1×10^{-5} M at 25°C. This concentration is not a limiting factor in photosynthesis of *Chlorella* as was already observed by WARBURG (72), who reported a strong decrease in rate of photosynthesis only at CO_2 concentrations lower than one tenth of that maintained by mixture no. 9. However, the pH and the cation concentration of the buffer may have an effect on photosynthesis as well.

Figure 2 represents the rate of photosynthesis at different light intensities measured in mixtures no. 9 and no. 2 (pH 10.3, 1.0×10^{-6} M CO_2 at 25°C). It shows that the lower rate of photosynthesis in buffer no. 2, with the lower carbon dioxide pressure, also occurs under light limitation, which would not be expected theoretically. This points to some disturbing effect of high pH, apart from a possible limitation of photosynthesis by the low carbon dioxide concentration.

In order to retain the advantage of a constant CO_2 pressure provided by the WARBURG buffer, whilst reducing the troublesome pH-effect, *Chlorella* was suspended in distilled water while 0.2 ml. buffer was placed in the side arms. The slow diffusion rate of CO_2 from the buffer to the suspension in the main part of the vessel, however, made it impossible to use normal suspension

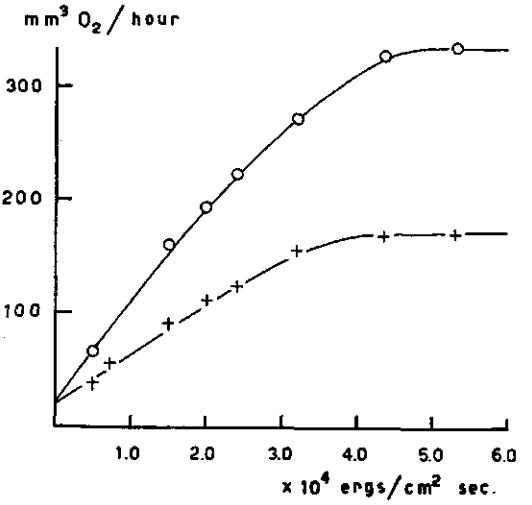


FIG. 2. Rate of photosynthesis of *Chlorella* in WARBURG buffer no. 2 (+ — +) and no. 9 (o—o), at different light intensities, at 25 °C (one out of 3 experiments).

densities. No limitation by the low rate of carbon dioxide diffusion was observed when not more than 1–2 mm³ of cells were used per flask. Now, under conditions of light limitation, the same rate of photosynthesis is found in buffers no. 9 and no. 2, as is shown in figure 3. Though this statement depends on one point only, the consistency of the results of 3 different experiments justify this deduction. Thus, the lower rate of photosynthesis observed at light limitation may be attributed to an unfavourable effect of the high pH of buffer no. 2, in case *Chlorella* is suspended directly in the buffer.

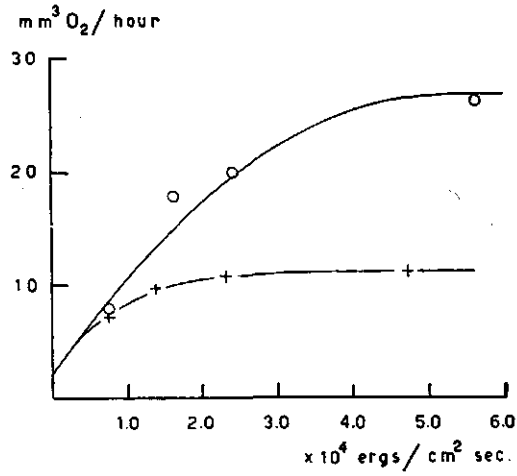


FIG. 3. Rate of photosynthesis of *Chlorella* in tap water at different light intensities with WARBURG buffer no. 2 (+ — +) and no. 9 (o — o) in the side arms (one out of 3 experiments).

PRATT (51) observed that sodium carbonate bicarbonate mixtures had a depressive effect on photosynthesis in long term experiments, while potassium mixtures had an accelerating effect. In a solution in which the molar ratio of sodium to potassium was 65/35, the increasing and decreasing actions of the potassium and sodium salts respectively were balanced, and according to PRATT the initial rate of photosynthesis was virtually constant for fifteen hours. In our experiments, we have only used sodium carbonate and bicarbonate mixtures in order to avoid a possible precipitation of D by potassium.

The gas exchange (x) was calculated in the well-known way by multiplying the measured pressure change (h) by the vessel constant (Vc)

$$x = h \times Vc$$

$$\text{while } Vc = \frac{Vg \times \frac{273}{T} + Vf\alpha}{10{,}000}$$

Herein Vg represents the volume of the gas space in the vessel, the capillary tube and the manometer, Vf the volume of the liquid phase and α the absorption coefficient for CO_2 at temperature T.

Usually, 2 cc of algal suspension were used in experiments on respiration and photosynthesis. As the total content of the vessels was about 20 ml., the vessel constant for oxygen was near 1.65 at 25 °C. One or two of the manometer vessels were provided with suspension liquid only, and served as thermo-barometers.

II. Simultaneous measurements of CO_2 and O_2 were made by using the two vessel method.

Identical samples of algae suspended either in 2 or in 5 cc of the same suspension liquid under identical conditions of light intensity and temperature, may be assumed to have equal gas exchange. This, however, will cause unequal pressure changes in both manometers, owing to the different solubilities of CO_2 and O_2 in the suspension liquid. From the pressure changes of the two manometers, the gas exchange (mm^3) can be computed by the formula

$$x_{O_2} = \frac{hk_{CO_2} - HK_{CO_2}}{\frac{k_{CO_2}}{k_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}} \qquad x_{CO_2} = \frac{hk_{O_2} - HK_{O_2}}{\frac{k_{O_2}}{k_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}}$$

in which

h is the manometric reading of the 2 cc vessel in mm,

H is the manometric reading of the 5 cc vessel in mm,

k_{CO_2} and K_{CO_2} are the vessel constants for CO_2 (p. 8) for the 2 cc and 5 cc vessel respectively,

k_{O_2} and K_{O_2} are the vessel constants for O_2 for the 2 cc and 5 cc vessel respectively.

In order to provide sufficient amounts of CO_2 for photosynthesis, the suspension and the gas phase were saturated with a mixture of 95% air and 5% CO_2 by flushing the WARBURG vessels with the mixture in the dark for half an hour. During this period, the vessels were shaken in order to attain equilibrium between gas phase and liquid. The suspension had been aerated previously with air and 5% CO_2 during a quarter of an hour.

III. Measurements with the one vessel method if the photosynthetic quotient $P = -O_2/CO_2$ is known.

This is in fact an experimental simplification of the foregoing method. The gas exchange is determined according to the formula

$$x_{CO_2} = h \times Vc = \frac{hk_{O_2} \times k_{CO_2}}{pk_{CO_2} - k_{O_2}}$$

In figure 4, Vc has been computed for 2, 4 and 6 cc fluid/flask at different assumed photosynthetic quotients for a flask with a total volume of 20 cc at 25 °C. From this it is clear, that an erroneous value of p has a smaller influence with 6 cc liquid than with 4 or 2 cc. In our experiments, when using this method, 5 cc was taken as the most suitable quantity, since the danger of disturbing effects due to mixing of the fluid in the main flask and that in the side arm then still was very small.

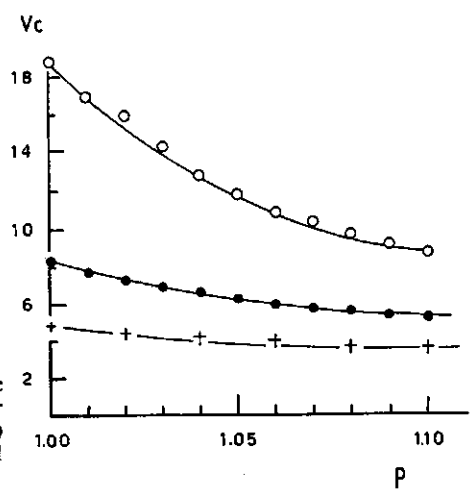


FIG. 4. The influence of the photosynthetic quotient p at 25 °C on the change in Vc for 2 cc (o—o), 4 cc (● — ●) and 6 cc (+ — +) fluid per vessel (total volume of the vessel 20 cc).

Measurements with the one and the two vessel method have been made in tap water and in phosphate buffers. Phosphate buffers may show carbon dioxide fixation or retention according to: $Na_2HPO_4 + CO_2 + H_2O \rightleftarrows NaH_2PO_4 + NaHCO_3$, which retention appears above pH 5, and increases exponentially with increasing pH. This may result in large errors of the computed gas exchange. These errors can be eliminated by direct determination of the retention. For this purpose an amount of a strong acid is added from the side bulb at the start of the experiment and in another vessel at the end of the experiment, which acid will release the carbon dioxide from the buffer.

JOHNSON (31) has suggested a method of correcting CO_2 retention, without the addition of acid. Since at high pH values HCO_3^- as well as CO_2 is retained in the solution, the effective value of α (which will be designated as α_1) will be larger than the theoretical value. If the vessel constant is calculated by the use of α_1, instead of α, retention of CO_2 is corrected automatically. As α_1, increases rapidly with increasing pH, the retention correction becomes very large at pH values above 7, and the accuracy of the CO_2 measurements suffers accordingly. The extent of the correction is computed according to the formula:

$$\frac{\alpha_1}{\alpha} = \text{antilog (pH} - 6.382) + 1$$

Figure 5 represents the decrease in Vc (corrected with α_1) with increase in pH of the buffer, computed for one vessel. It is clear, that large errors occur if no correction for α is applied.

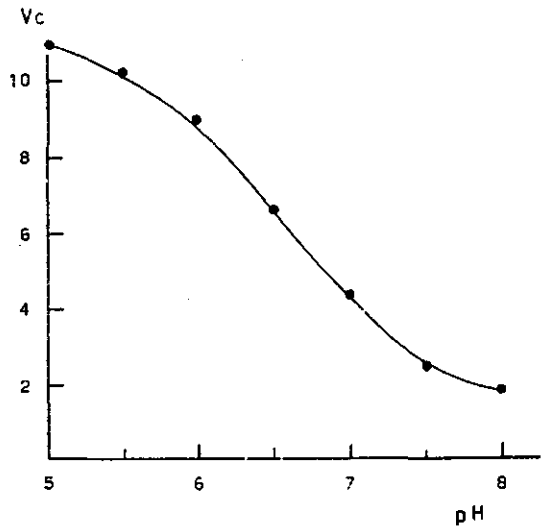


FIG. 5. The computed decrease in Vc versus increasing pH of phosphate buffers for 2 cc fluid/vessel (p = 1.06, total volume = 20 cc, at 25 °C).

To check the validity of these considerations for our experimental conditions, experiments were made at pH 7.2, 6.5, and 5.9. The gas exchange was computed without correction, by adding acid, and also by using the above equation. The results are summarized in table 1, averaging three experiments.

This table shows that without applying the suggested correction large errors in the computation of the gas exchange occur, especially at high pH values. Comparing the acid addition method with the method of theoretical correction, the last method yielded lower values at pH 7.2 and higher ones at pH 6.5 and 5.9. These differences may be due to uncertainties in the pH measurements which were made at the end of the experiments only. For a fully critical check measurements to within 0.1 pH unit should be made during the experiments.

TABLE I. The rate of photosynthesis in *Chlorella* (mm^3 O_2/hr.) in phosphate buffers at different pH values, as an average of 3 experiments, at 25 °C, under light saturation ($5–6 \times 10^4$ ergs/cm^2 sec.).

pH	Without correction	Applying JOHNSON's formula	Measured by addition of acid
7.2	1210	375	435
6.5	835	455	415
5.9	540	450	390

§ 4. MEASUREMENT OF THE INHIBITION

Standard solutions of the inhibitors were made; 0.05 to 0.2 ml. of which were pipetted into the side arm of a manometer vessel, ultimately ascertaining various inhibition percentages. Usually, one or two control vessels received 0.2 ml. of the solvent only.

The procedure of each experiment included measurement of the non-inhibited photosynthesis in all flasks during half an hour. Only series from which the computed gas exchange in all vessels showed less than 5 % variation were used for inhibition experiments. After half an hour different concentrations of the inhibitor were tipped into the main space of the vessels. After 10 minutes, when temperature equilibrium was re-established, the measurements were continued for one or more hours.

Dependent on the rate of pressure change, manometer readings were taken at intervals of 10 or more minutes. The inhibition is expressed by indicating the decrease in rate as a percentage of the control. Usually, for the sake of simplicity, the standard errors have only been indicated at the calculated inhibition percentages, while the absolute values have been reported without reference to the standard deviation.

A comment has to be made on the ageing effect, which occurs in cultures of unicellular algae (VAN HILLE [29], WASSINK and KATZ [75], PRATT [50]) and which according to PRATT (49, 50) is due to a production of a growth inhibiting factor, which should act like a catalist poison rather than like a narcotic (PRATT [49], WASSINK and KATZ [75]).

Figure 6a represents the decline of photosynthesis in *Chlorella* with age, under light saturation, each point representing an average of 5 experiments. The results are in general agreement with those of WASSINK and KATZ and of PRATT. It is clear from figure 6b that the inhibition percentage depends on the age of the culture, especially at higher concentrations of the inhibitor. Therefore, throughout the experiments described furtheron in this paper, only cultures of 3 and 4 days old were used in photosynthesis experiments, unless indicated otherwise.

In table II, the rate of photosynthesis and the inhibition percentage at concentrations of 1.0, 2.83, 5.2, and 10.0×10^{-6} M CaD_2 are presented as a function of different densities of suspension. The rate of photosynthesis is linear up to 20 mm^3 cells/flask and there is no significant influence of density of the suspension upon the degree of inhibition.

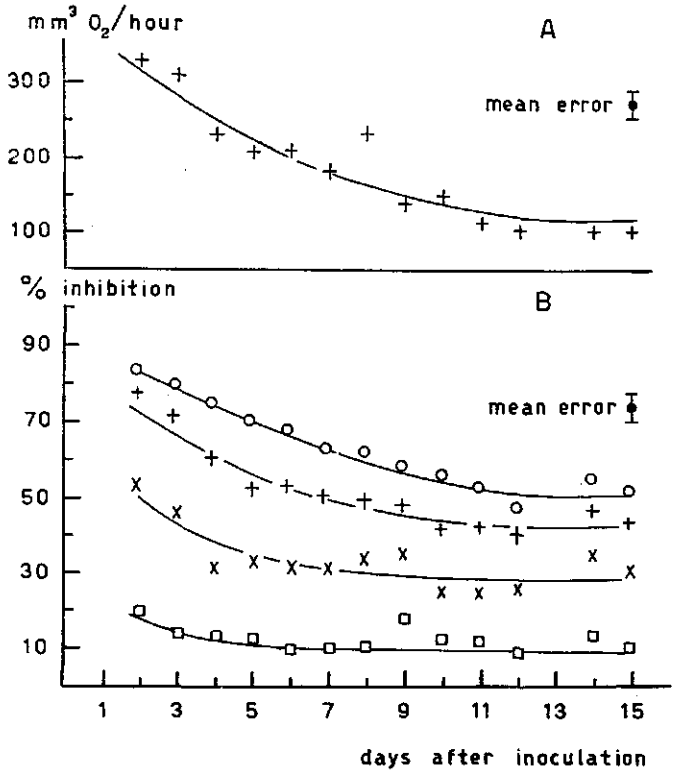


FIG. 6a. The effect of age on the rate of photosynthesis of *Chlorella* at 25 °C, under light saturation (5.0 – 6.0 × 10^4 ergs/cm² sec.) in WARBURG buffer no. 9.

FIG. 6b. The effect of age on the inhibition of photosynthesis by CaD_2 (o — o = 1.0 × 10^{-5}M, + — + = 5.2 × 10^{-6}M, × — × = 2.8 × 10^{-6}M, and □ — □ = 1.0 × 10^{-6}M CaD_2).

TABLE II. The inhibition of photosynthesis in *Chlorella*, at different concentrations of CaD_2, with different suspension densities, as an average of 4 experiments, at 25 °C, under light saturation (5–6 × 10^4 ergs/cm² sec.), in WARBURG buffer no. 9.

mm³ Cells/ vessel	Rate of photo-synthesis of the control (mm³ O_2/hr.)	Per cent inhibition at different concentrations of CaD_2 (10^{-6} M)			
		1.0	2.83	5.2	10.0
2.5	41	14 ± 5	50 ± 5	70 ± 3	78 ± 3
5	81	7 ± 2	38 ± 4	58 ± 3	71 ± 3
10	163	11 ± 2	41 ± 1	57 ± 4	73 ± 4
15	243	13 ± 2	36 ± 1	58 ± 4	74 ± 2
20	315	8 ± 2	37 ± 4	52 ± 4	72 ± 4

CHAPTER III

INHIBITION OF PHOTOSYNTHESIS WITH CaD_2 AS COMPARED WITH OTHER INHIBITORS IN *CHLORELLA*

§ 1. INTRODUCTION

As has been set forward in Chapter I, various authors have studied inhibition of photosynthesis in *Chlorella* and other small algae. Different opinions have been expressed regarding the mode of action of poisons, and the results obtained with any special poison do not always agree. Especially, the distinction between the type of action of „narcotics" and „specific poisons" is not always clear. Moreover, differences in sensitivity towards the same inhibitor have been frequently observed, also in experiments with the same species.

The cultivation of the algae may play an important part in the observed differences in inhibition. The age of the culture also influences inhibition (29, 50, 75; fig. 6b). It may be assumed that the method of cultivation either increases or decreases the amount of certain catalists, or causes a change in the structure of the photosynthetic apparatus or in the permeability of the protoplast. All such changes will affect the inhibition of photosynthesis upon administration of a certain poison.

The possible influence of cultivation induced us to compare the inhibition of photosynthesis in *Chlorella* with CaD_2 with that of other inhibitors. This also may seem the most reliable method to find out whether the action is specific, owing to affinities to certain enzymes of the photosynthetic apparatus, or whether it is a result of surface activity only.

§ 2. INFLUENCE OF INHIBITOR CONCENTRATION, AT LIGHT SATURATION

2.1. *Calcium dipicrylamine*

The inhibition of photosynthesis by calcium dipicrylamine (CaD_2), is shown in figure 7a. The inhibition is represented as a percentage of the corresponding

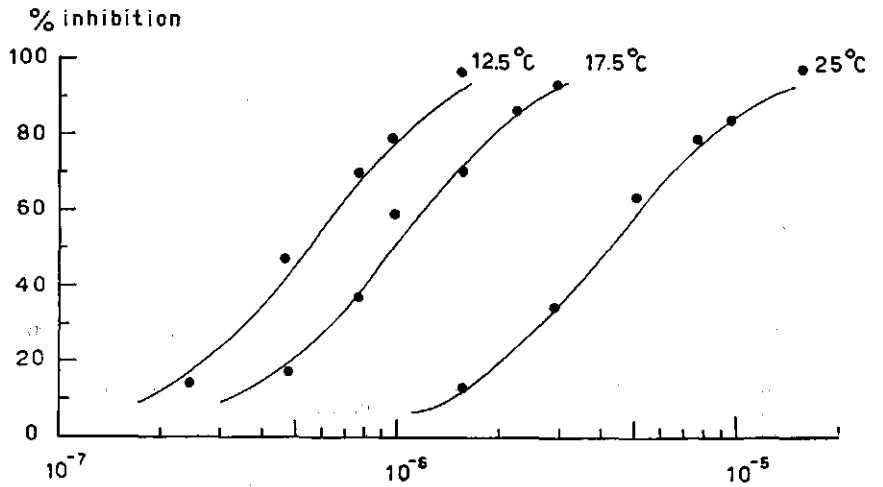


Fig. 7a. For explanation, see p. 14.

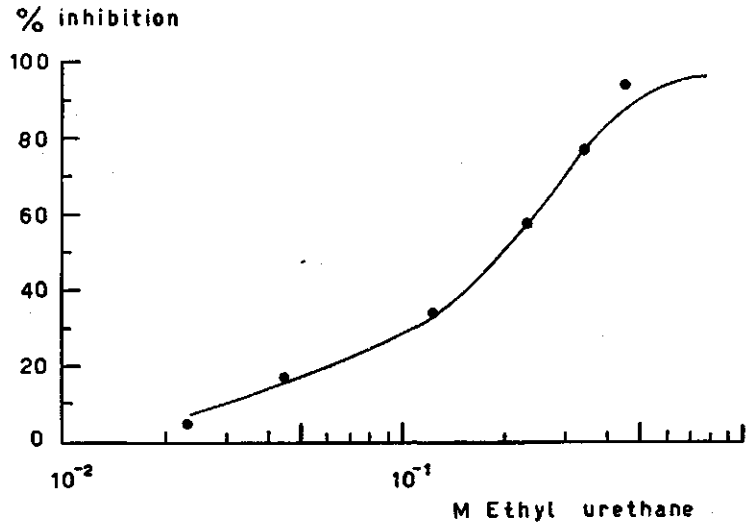


Fig. 7b.

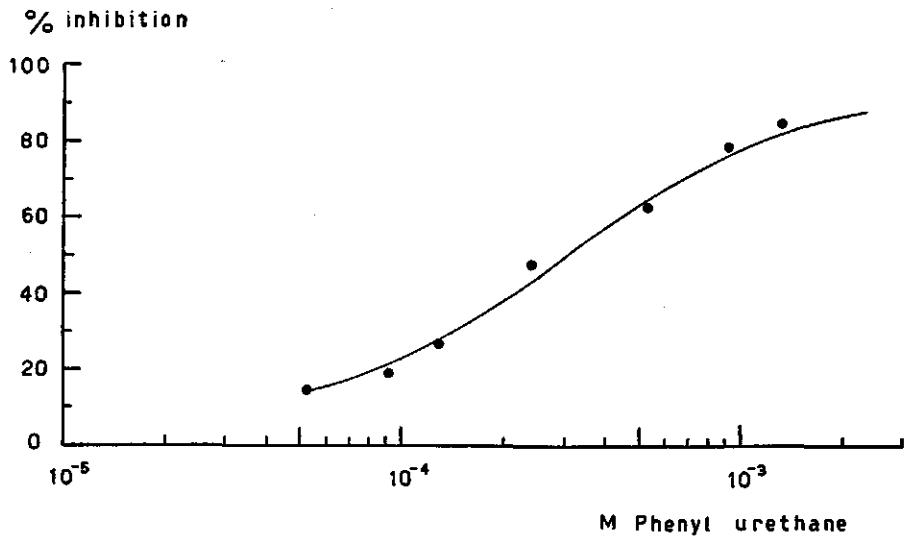


Fig. 7c.

FIG. 7. The inhibition of photosynthesis of *Chlorella* under light saturation ($5.0 - 6.0 \times 10^4$ ergs/cm² sec.) in WARBURG buffer no. 9 with:

a. CaD_2 at 25°, 17.5° and 12.5 °C,
b. ethyl urethane at 25 °C,
c. phenyl urethane at 25 °C,
d. hydroxyl amine at 25 °C,
e. 2,4-dinitrophenol at 25 °C,
f. sodium azide at 25 °C.

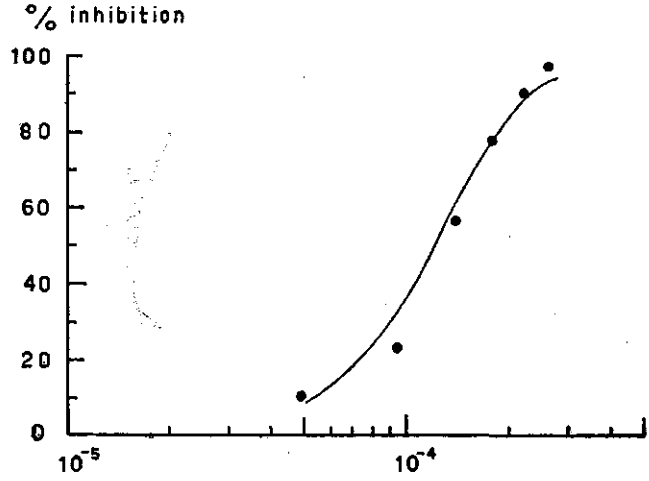


Fig. 7d.

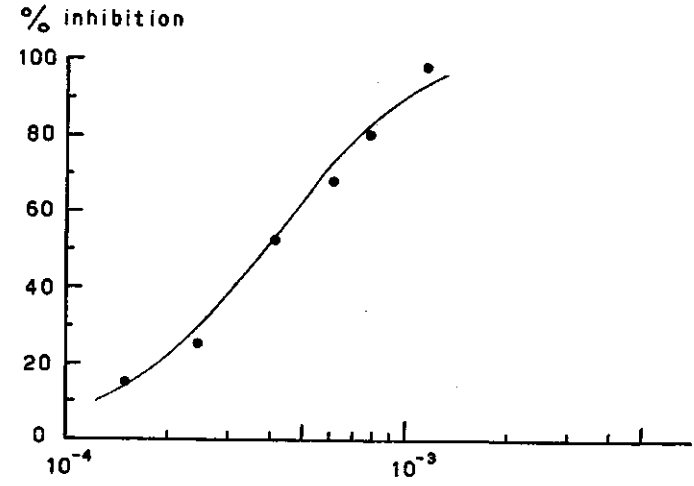


Fig. 7e.

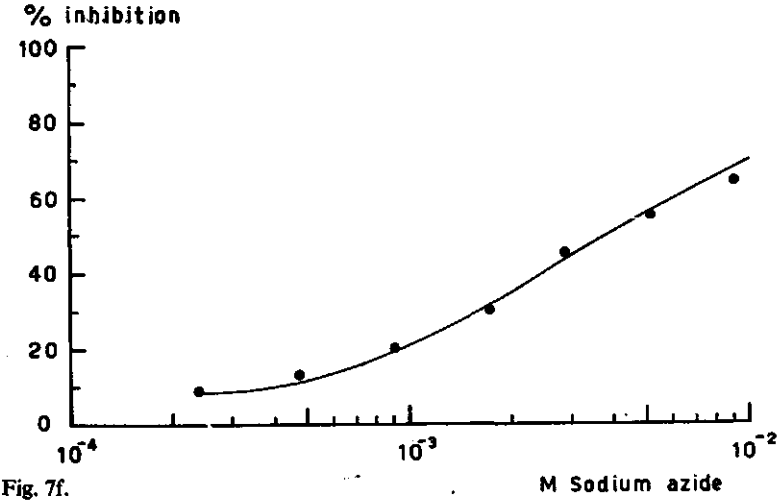


Fig. 7f.

control against the molar concentration of CaD_2 on a logarithmic scale, as an average of 5 experiments. The measurements have been made in the carbonate bicarbonate buffer no. 9 at light saturation (48,000–55,000 ergs/cm^2 sec.), and at 25°C. Further details of experimental conditions have already been described in Chapter II. The inhibition increases regularly with an increase in concentration. The results are in accordance with the adsorption theory of FREUNDLICH (20) since nearly straight curves were obtained if the concentration is plotted on a logarithmic scale. The threshold of inhibition is at about 10^{-6} M. Complete inhibition shifts to lower concentrations with increasing duration of exposure to the inhibitor. A stationary state of inhibition was hardly observed after 2 hours especially at the higher concentrations. This fact will be discussed in greater detail in section 3 of this chapter. The results represented in figure 7a are those in which approximately a stationary state was achieved. Complete inhibition occurred at about 2×10^{-5} M CaD_2, as may be seen from the figure.

In higher CaD_2 concentrations, negative readings were observed, exceeding those of the control in the dark. Consequently, the release of O_2 was reversed in an uptake in the light. It seems likely that this is due to a photooxidation. Anyhow, according to WARBURG this phenomenon may be taken as „ein sicheres Zeichen der definitiven Zerstörung".

2.2. *Ethyl urethane*

The urethanes have been introduced as inhibitors by WARBURG (71), who reported an increase in inhibition with the increase in length of the C-chain according to TRAUBE's rule. TRAUBE (69) observed a complete correlation between the last mentioned increase and the increase in capillary activity. Experiments by WARBURG (71) and by WASSINK *et al.* (78) pointed to the same inhibition under light limitation as well as under light saturation.

In figure 7b, the inhibition of photosynthesis with ethyl urethane ($C_2H_5NHCOOC_2H_5$) is given in per cent of the corresponding control. The inhibition occurs in the same range as was reported by WARBURG (71) and by WASSINK *et al.* (78) with a threshold at 2.0×10^{-2} M and complete inhibition at 5.0×10^{-1} M. The inhibition becomes stationary within 20 minutes and remains so for at least 2 hours after addition of the inhibitor. There is a direct relationship between log concentration and percentage of inhibition, the curve, however, shows a peculiar bend between 0.1 and 0.2 M. It is possible that in this range shrinking of the protoplasm owing to a desiccating effect by the osmotic value of the solution at these high concentrations also plays a role in the inhibition. This is supported by GREENFIELD's observation (27) that the rate of photosynthesis in strong light is inhibited by sucrose at concentrations of 0.1 to 0.3 M.

2.3. *Phenyl urethane*

Phenyl urethane ($C_6H_5NHCOOC_2H_5$) is not soluble in water in concentrations at which inhibition occurs. The use of 0.2 % ethanol as a solvent was sufficient to dissolve phenyl urethane in such concentrations as to obtain 100 % inhibition of photosynthesis. No effect of 0.2 % ethanol on photosynthesis was observed as may be seen from table III.

TABLE III. The inhibition of photosynthesis in *Chlorella* with ethanol as an average of 3 experiments, at 25 °C, under light saturation ($5–6 \times 10^4$ ergs/cm² sec.) in WARBURG buffer no. 9.

Concentration of ethanol in per cent	Rate of photosynthesis in mm^3 O_2/hr.	Per cent inhibition
0	225	–
0.095	228	–1
0.18	220	2
0.95	206	8
1.8	191	15

The inhibition with phenyl urethane is represented by figure 7c as an average of 4 experiments. The inhibition becomes constant within 20 minutes while threshold and complete inhibition are at 4×10^{-5} and 3×10^{-3} M respectively. These results are in agreement with those of WARBURG (71) and WASSINK *et al.* (78).

2.4. *Hydroxyl amine*

Hydroxyl amine ($NH_2OH.HCl$) is an inhibitor strongly affecting photosynthesis as was shown for the first time by SHIBATA and YAKUSHII (60). Similar results were obtained by NAKAMURA (39), GAFFRON (23), WELLER and FRANCK (80), and TAMIYA (68). In his experiments with hydrogen adapted algae, GAFFRON (23) observed a much smaller sensitivity of photoreduction. He concluded from this observation that the mode of action of hydroxyl amine was as an enzyme poison rather than as a narcotic. Since phenyl urethane and ethyl urethane are known to behave like narcotics (WARBURG [71], WASSINK *et al.* [78]), hydroxyl amine therefore was also tested.

The inhibition is represented by figure 7d as an average of 4 experiments. It is clear that the curve is steeper than that obtained with ethyl urethane and phenyl urethane. In this case the straight relationship between log concentration and percentage of inhibition is only evident between 20 and 80 per cent inhibition. Beyond these values the curve approaches assymptotically 0 and 100 per cent inhibition. A constant inhibition was observed after 30 to 60 minutes. The range at which inhibition of photosynthesis (in our experiments 50 % at 1.1×10^{-4}M) was manifest, was similar to that reported by TAMIYA (68).

2.5. 2,4-*Dinitrophenol*

Dinitrophenol ($(NO_2)_2C_6H_3OH$) is of interest in the present investigation, because more is known of this inhibitor in relation to photosynthesis (GAFFRON [23], WINTERMANS [83]), and because its structure more or less corresponds to that of CaD_2.

As this inhibitor is more soluble in alkaline solutions, standard solutions were made using WARBURG's carbonate bicarbonate buffer no. 9 (pH 9.2) as a solvent instead of water. According to WINTERMANS (83) the inhibition is less pronounced in alkaline solutions, due to the fact that its activity is ascribed to the undissociated molecules, because only those are able to penetrate into the cells.

The inhibition with 2,4-dinitrophenol in WARBURG buffer no. 9 (pH 9.2) is represented in figure 7e, as an average of 4 experiments. The curve is steeper

than that for phenyl urethane and ethyl urethane, and resembles more that for CaD_2 and hydroxyl amine. It is, therefore, more difficult to indicate the concentrations which represent threshold and complete inhibition. As may be seen from the figure, the concentration at which 50 per cent inhibition occurs is 3.8×10^{-4}M.

2.6. *Sodium azide*

The inhibition of photosynthesis with sodium azide (NaN_3) is represented by figure 7f, as an average of 4 experiments. The curve is less steep than that for 2,4-dinitrophenol, CaD_2 and hydroxyl amine. Threshold and 100 per cent inhibition are observed at 1×10^{-4} M and 5×10^{-2} M respectively.

§ 3. Influence of time after addition of the inhibitor, at light saturation

From the preceding experiments it is clear that, though there was a slight decrease with time in the rate of photosynthesis also in the blank, after addition of CaD_2 this rate dropped much more clearly with time, especially at higher concentrations. Figure 8 gives another example of this phenomenon. The lower concentrations showed a tendency to remain constant around 1.55×10^{-6}M CaD_2; below that concentration a slight decrease in inhibition with time was manifest.

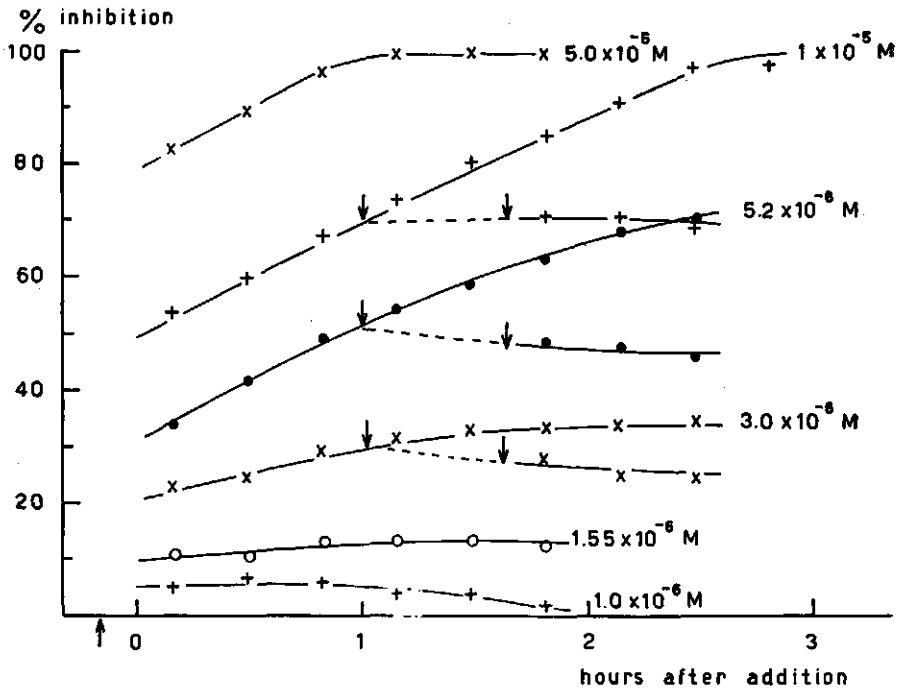


FIG. 8. The inhibition of photosynthesis of *Chlorella* versus time after addition of different CaD_2 concentrations at 25 °C, under light saturation ($5.0 - 6.0 \times 10^4$ ergs/cm² sec.) in WARBURG buffer no. 9, and the effect of washing on the inhibition (↑ addition of CaD_2, ↓ ---- ↓ time of washing).

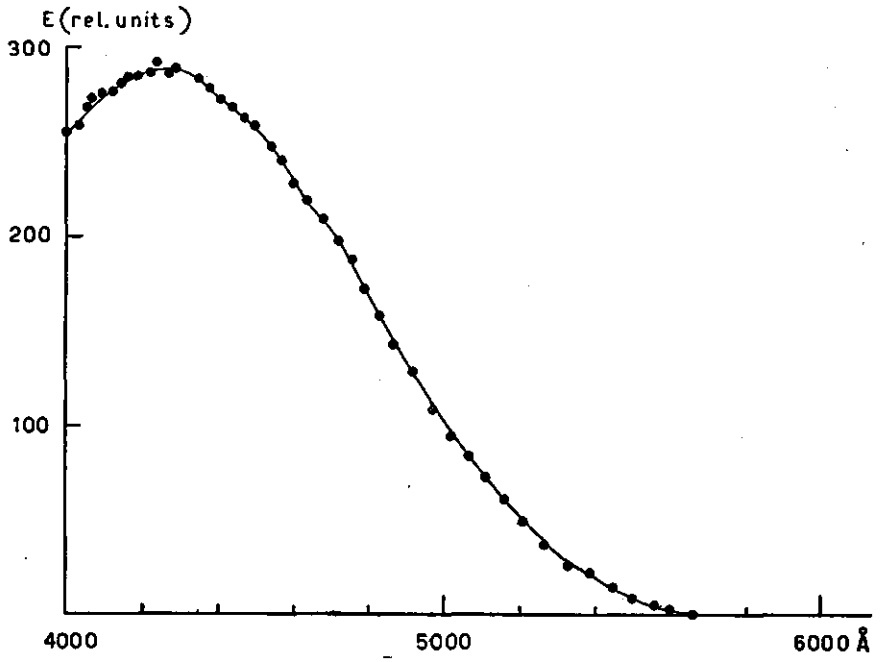


FIG. 9. The absorption spectrum of calcium dipicrylamine in relative units.

At least two different explanations of the increase in the inhibition versus time after addition of CaD_2 might be suggested, one of which could be along the line of a photodynamic action. An absorption spectrum of CaD_2, therefore, was measured with a double monochromator (fig. 9). The data for the emission of the sodium lamp were taken from ORANJE (43) and are represented in table IV.

TABLE IV. The emission of a sodium tube in different spectral areas in relative units (data from ORANJE [43]). The line drawn between 5149/54 and 5683/88 represents the cut off by the OG2 filter. The wavelengths above this line are completely absorbed by the filter.

Wavelength (Å)	Relative units
4391/94	0.2
4494/4500	0.7
4543/46	0.2
4665/69	1.15
4748/52	0.5
4979/83	2.76
5149/54	1.6
	OG2
5683/88	6.33
5890/96	100
6154/64	4.1

Except for the yellow lines at 5893–96 Å, the emission from the sodium lamp is fairly strong in the infra-red and very weak in the blue-green spectral

region (table IV). CaD_2 shows no absorption at all in the yellow and infra-red, but a high absorption in the blue-green spectral area (fig. 9). There is a slight possibility, therefore, that the weak blue-green emission from the sodium lamp had a photodynamic effect on the cells, owing to absorption by CaD_2.

Some experiments have been made in which the inhibition upon 9.9×10^{-6}M CaD_2 was measured at the same time in different flasks. In some of these flasks the blue-green radiation was filtered out by means of an OG2 filter which absorbs the blue-green rays completely, as represented in table IV. The average inhibition during the first hour after addition of CaD_2 in five different experiments with 9.9×10^{-6}M CaD_2 was:

with OG2	$56\% \pm 2$
without filter	$58\% \pm 2$

The inhibition was nearly the same in both cases. Moreover, the inhibition increased in the same way with and without the OG2 filter. We may conclude, therefore, that no photodynamic action can be responsible for the observed time effect.

The second supposition is that after addition of CaD_2 no immediate equilibrium is established between the outside concentration and that in the cell, but that there is a continuous uptake of CaD_2. However, the independence of the inhibition of the glucose-stimulated respiration from time (Chapter IV, p. 32, figure 16) indicates that this supposition is probably not true.

Washing experiments were made with three different concentrations in order to obtain more data on this phenomenon. These experiments consisted in measuring the inhibition in several flasks with the same concentration for one hour. Half the number of flasks were then centrifuged and the algae washed twice with the suspension liquid (WARBURG buffer no. 9) and then measured again in the WARBURG apparatus. It appears (fig. 8) that after washing no further increase of inhibition occurred while such increase was observed in the flasks in which the CaD_2 solution had not been removed by washing. The inhibition in the washed algae, however, did not decrease. Comparable results were obtained in other experiments in which different concentrations (3.0×10^{-6}, 5.2×10^{-6} and 1.0×10^{-5}M) of CaD_2 were used.

Two important conclusions may be drawn from these washing experiments:

a) The increase in inhibition with time is due to a continuous uptake of CaD_2.
b) The inhibition is not reversible.

It is remarkable that the first conclusion is not supported by the observation that the inhibition of the glucose-stimulated respiration remains unchanged with time (Chapter IV). In the latter case a constant degree of inhibition was reached within 20 minutes after addition of CaD_2 (fig. 16), which time was more or less the same as that observed in inhibition of photosynthesis with the urethanes and hydroxyl amine. It is possible that CaD_2 is continuously reduced, only in light, to a product inhibiting photosynthesis, while CaD_2 uptake can proceed only as long as this reduction takes place.

This consideration was tested, taking a concentration of CaD_2 giving complete inhibition after a long time (fig. 8, 5.2×10^{-6}M CaD_2). This concentration was tipped at the same time into the suspension in different WARBURG vessels. Hereafter, the inhibition of photosynthesis under light saturation was measured, whereas some vessels remained in the dark and were illuminated one and two hours later (figure 10).

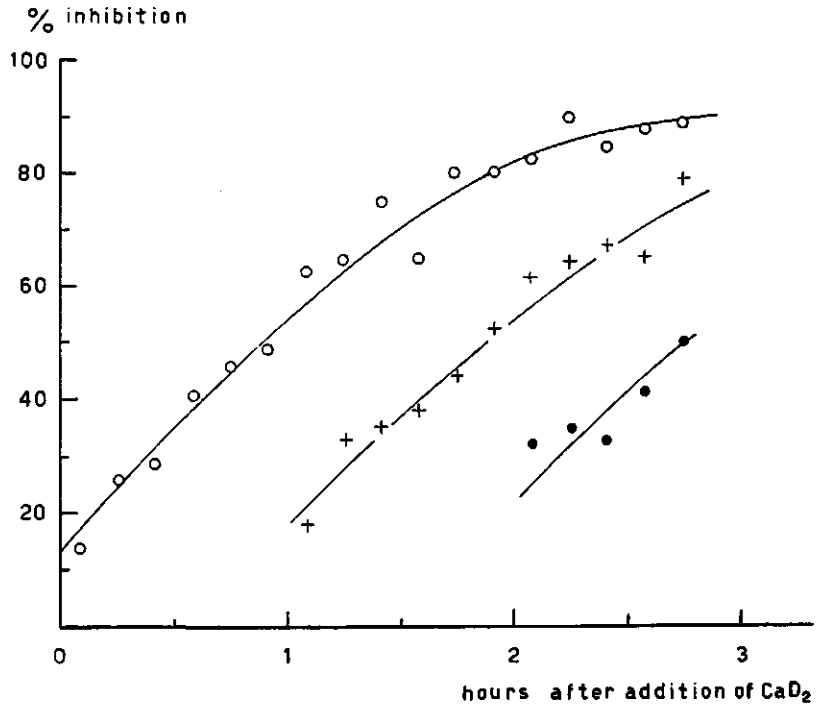


FIG. 10. Inhibition of photosynthesis of *Chlorella* versus time after addition of 5.2×10^{-6} M CaD_2 at 25 °C, under light saturation ($5.0 - 6.0 \times 10^4$ ergs/cm² sec.) in WARBURG buffer no. 9 (o — o = 3 hours in the light, + — + = one hour in the dark followed by 2 hours in the light, ● — ● = two hours in the dark followed by one hour in the light).

The inhibition increases with time, as was already mentioned. It is clear that, after three hours, the inhibition of photosynthesis was larger, when the time of illumination was longer. Approximately, concurrent curves were obtained in this way. This fact strongly supports a continuous uptake of CaD_2 in light only. These results will be discussed with those on the inhibition of glucose-stimulated respiration in Chapter VI.

§ 4. EFFECT OF TEMPERATURE, AT LIGHT SATURATION

In some incidental experiments we found that the inhibition of photosynthesis with CaD_2 increases at lower temperatures. Consequently, lower concentrations had to be used to obtain the same range of inhibitions. TAMIYA (68) reported an increase in the inhibition of photosynthesis with sodium azide and a decrease with hydroxyl amine at lower temperatures. Therefore, our results with CaD_2 obtained at 25 °C were extended to other temperatures. The effect of temperature was investigated with other inhibitors also.

In order to obtain results of the best possible comparability, the observations at different temperatures were made, if possible, with the same culture in one

day, so that differences in age of the algae could be neglected. The temperature coefficient, Q_{10} in our experiments was 1.8 in the range from 12.5 °C to 25 °C. The inhibition of photosynthesis with CaD_2, ethyl urethane, phenyl urethane and hydroxyl amine is represented in table V as an average of 4–6 experiments. The results with CaD_2 (table V) have also been plotted in figure 7a.

Though there is a considerable increase in inhibition with CaD_2 at lower temperatures, the slope of the inhibition curve against log concentration remains the same (figure 7a). The observed increase in inhibition is probably not effected by a higher uptake of CaD_2 at lower temperatures, as these figures were obtained at equilibrium. Besides, the time to attain equilibrium was nearly the same at all temperatures. The influence of temperature on the inhibition is given in figure 11. The concentration producing 50 % inhibition is represented on a logarithmic scale. In this way approximately a straight line relation is obtained between temperature and concentration.

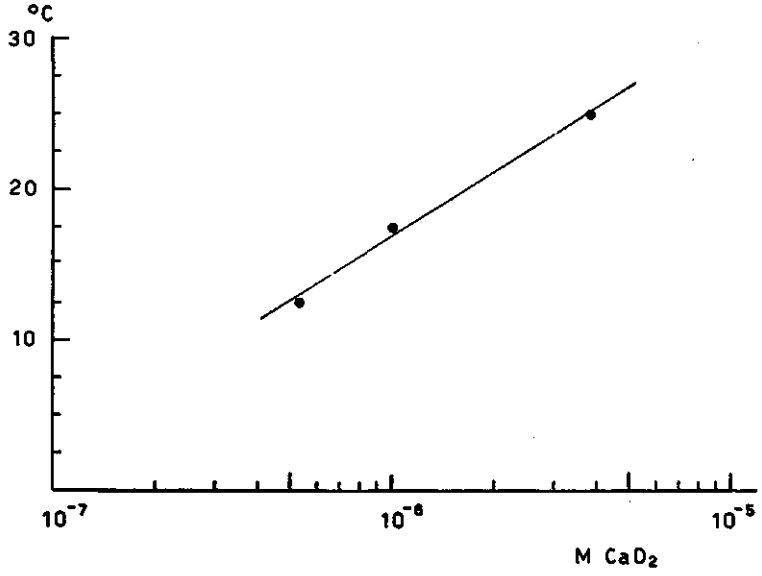


FIG. 11. The CaD_2 concentration causing 50 % inhibition of photosynthesis versus temperature.

With hydroxyl amine, phenyl urethane and ethyl urethane on the other hand, neither a significant increase nor decrease of the inhibition with temperature were observed.

§ 5. INFLUENCE OF LIGHT INTENSITY

In previous years a comprehensive study (52, 53) has been made on the influence of light intensity and temperature on photosynthesis. Generally, a linear relation between light intensity and photosynthesis was observed at low light intensities, which relationship is independent of temperature. At high light intensities (light saturation) the rate of assimilation of carbon dioxide does not increase with an increase in light intensity. In this range, however, the increase is dependent on temperature. The transition from light limitation to light

TABLE V. The inhibition of photosynthesis in *Chlorella* with CaD_2, ethyl urethane, phenyl urethane, and hydroxyl amine, at different temperatures, under light saturation (5–6 × 10^4 ergs/cm² sec.) in WARBURG buffer no. 9.

Inhibitor	Concentration in M	25°C		17.5°C		12.5°C	
		Rate of photosynthesis in mm^3O_2/hr.	Inhibition in per cent	Rate of photosynthesis in mm^3O_2/hr.	Inhibition in per cent	Rate of photosynthesis in mm^3O_2/hr.	Inhibition in per cent
CaD_2	0	320	–	210	–	140	–
	2.44×10^{-7}					121	13 ± 3
	4.7			175	17 ± 4	74.0	47 ± 5
	7.75			134	36 ± 4	41.8	70 ± 6
	9.9	318	1 ± 1	86.0	59 ± 4	29.4	79 ± 6
	1.56×10^{-6}	279	13 ± 2	62.8	70 ± 9	5.6	96 ± 5
	2.27			27.3	87 ± 6		
	2.98	211	34 ± 2	14.7	93 ± 8		
	5.2	119	63 ± 3	4.2	98 ± 5		
	7.75	67.5	79 ± 2				
	9.9	20.8	84 ± 2				
	1.56×10^{-5}	9.6	97 ± 1				
ethyl urethane	0	350	–	220	–		
	2.39×10^{-2}	330	5 ± 2	210	4 ± 2		
	3.5	310	11 ± 1	195	11 ± 1		
	4.55	290	17 ± 2	185	16 ± 1		
	1.22×10^{-1}	230	34 ± 2	148	33 ± 2		
	2.39	146	58 ± 4	73.0	67 ± 4		
	3.5	77.0	78 ± 2	44.0	80 ± 2		
	4.55	21.0	94 ± 1	8.8	96 ± 1		
phenyl urethane	0	350	–	220	–		
	5.3×10^{-5}	300	14 ± 3	192	13 ± 1		
	9.1	282	19 ± 5	183	17 ± 1		
	1.3×10^{-4}	255	27 ± 4	160	27 ± 2		
	2.4	181	48 ± 4	116	47 ± 4		
	5.3	132	62 ± 3	83.5	62 ± 4		
	9.1	73.0	79 ± 2	52.8	76 ± 3		
	1.3×10^{-3}	52.5	85 ± 2	35.1	84 ± 5		
hydroxyl amine	0	365	–	225	–	155	–
	4.9×10^{-5}	330	10 ± 3	206	8 ± 3	140	9 ± 2
	9.5	280	23 ± 4	154	31 ± 4	120	22 ± 6
	1.4×10^{-4}	156	57 ± 6	94.0	58 ± 6	68.0	56 ± 6
	1.8	80.0	78 ± 6	43.0	81 ± 5	35.5	77 ± 6
	2.2	36.5	90 ± 4	20.6	92 ± 4	23.2	85 ± 6
	2.6	29.0	97 ± 4	11.1	95 ± 4	14.0	91 ± 6

saturation depends on the density of the suspension. Therefore, in our experiments always the same density was used.

In figure 12a the rate of photosynthesis against light intensity is represented at 25°C and 17.5°C. Saturation occurs at about 38,000 ergs/cm² sec. at 25°C, and at 25,000 ergs/cm² sec. at 17.5°C. The figure is very similar to those obtained by other investigators.

So far, we have studied the inhibition of photosynthesis by poisons only at light saturation. It seems important to know, therefore, whether the inhibition under light limitation is the same as that under light saturation, or whether it is smaller. The latter situation would be likely to arise in case of specific interactions of the poison with definite catalists.

In figure 12b, which represents one out of 5 experiments, the inhibition is given at different light intensities, in three concentrations of CaD_2, *viz.*, 1.2×10^{-5}; 5.2×10^{-6}; 3.0×10^{-6}M at 25°C. The corresponding figure 12c shows the inhibition at 17.5°C with 3.0×10^{-6} and 1.18×10^{-6}M. It is clear, that at both temperatures roughly the same inhibition occurs at light limitation and at light saturation. However, the inhibition is much more pronounced at 17.5°C than at 25°C, so that lower concentrations of CaD_2 had to be used to obtain the same percentage of inhibition. This confirms the observation reported in the preceding section.

In table VI a and b, the inhibition percentage, obtained in these experiments, is calculated for light limitation and light saturation. The percentages of inhibition in the light limiting range and in the light saturation range are the same within the experimental error.

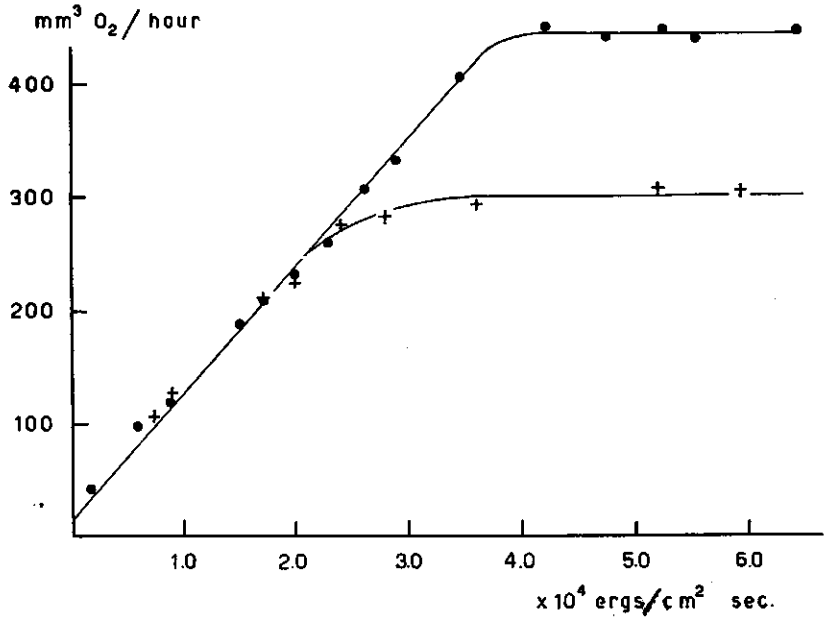


FIG. 12a. The rate of photosynthesis of *Chlorella* in WARBURG buffer no. 9 versus light intensity, at 25 °C (● — ●) and 17.5 °C (+ — +); one out of 5 experiments.

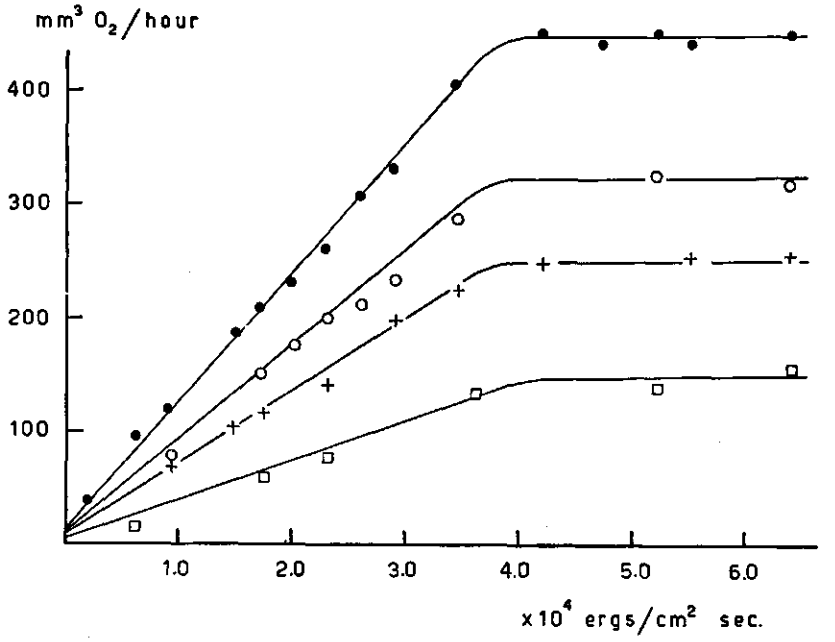


FIG. 12b. The rate of photosynthesis of *Chlorella* in WARBURG buffer no. 9 during the first hour after addition of CaD_2, versus light intensity, at 25 °C (● — ● = control, o — o = 3.0×10^{-6}M, + — + = 5.2×10^{-6}M, and □ — □ = 1.2×10^{-5}M CaD_2).

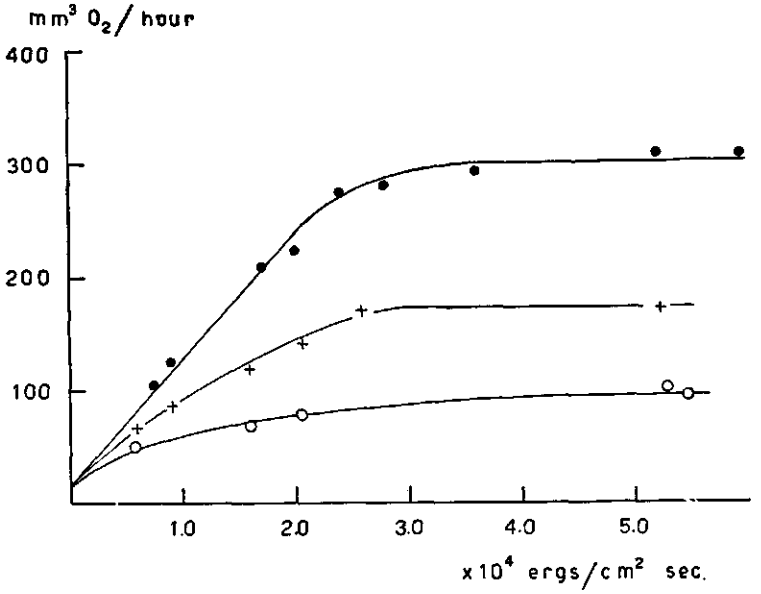


FIG. 12c. The rate of photosynthesis of *Chlorella* in WARBURG buffer no. 9 during the first hour after addition of CaD_2, versus light intensity, at 17.5 °C (● — ● = control, + — + = 1.18×10^{-6}M, and o — o = 3.0×10^{-6}M CaD_2.

TABLE VIa. The inhibition of photosynthesis in *Chlorella* during the first hour after addition of the inhibitor at light intensities of 20,000 and 55,000 ergs/cm² sec., at 4 concentrations of CaD_2, at 25 °C, as an average of 5 experiments.

Concentration of CaD_2 (10^{-6} M)	20,000 ergs/cm² sec.		55,000 ergs/cm² sec.	
	Rate of photosynthesis in mm³/hr.	Inhibition in per cent.	Rate of photosynthesis in mm³/hr.	Inhibition in per cent.
0	225	–	425	–
2.96	162	27 ± 1	305	28 ± 1
5.2	132	41 ± 2	245	42 ± 3
10.0	96.0	57 ± 1	174	59 ± 3
12.1	67.0	70 ± 2	131	69 ± 2

TABLE VIb. The inhibition of photosynthesis in *Chlorella* during the first hour after addition of the inhibitor at light intensities of 10,000 and 55,000 ergs/cm² sec., at 5 concentrations of CaD_2, at 17.5 °C.

Concentration of CaD_2 (10^{-6} M)	10,000 ergs/cm² sec.		55,000 ergs/cm² sec.	
	Rate of photosynthesis in mm³/hr.	Inhibition in per cent.	Rate of photosynthesis in mm³/hr.	Inhibition in per cent.
0	125	–	300	–
0.81	78.5	37	207	31
1.18	68.5	45	147	51
1.97	50.0	60	122	59
3.0	38.7	69	84.0	72
5.2	28.7	77	54.0	82

The fact that the inhibition is the same at light saturation and under light limitation may lead to the supposition that there is a narcotic action of CaD_2 on photosynthesis. Narcotic poisoning is generally less specific than enzymatic poisoning, both with regard to the molecular structure of the poison and to the constitution of the catalytic systems affected by it. However, the very low concentration of CaD_2 (10^{-6} to 10^{-5}M) in which inhibition occurs, is likely to be due to specific interactions with definite catalists rather than to an indifferent mechanism of surface blocking. In addition, the irreversible inhibition of photosynthesis with CaD_2 (fig. 8) seems to contradict the assumption of a narcotic poisoning. The conclusion seems to lay at hand that CaD_2 interferes with a type of enzyme present in low concentrations. It is possible, however, that in our experiments the exposure to CaD_2 was too long and the pH too high, which may have led to irreversible injury.

GAFFRON (23) found photoreduction in hydrogen adapted algae less sensitive towards hydroxyl amine than photosynthesis. He concluded that hydroxyl amine only participates in the oxygen liberating reaction of photosynthesis, in which it acts as an enzyme inhibitor. WELLER and FRANCK (80), however, found that the inhibition of photosynthesis by hydroxyl amine was independent of light intensity. It is evident that in our experiments CaD_2 yields curves similar to those WELLER and FRANCK (80) obtained with hydroxyl amine. In this connection it may be remarked that LIPSCHITZ (37) observed that aromatic nitro compounds in blood are reduced to hydroxyl amines, and then to nontoxic products. The first reduction, however, proceeds faster than the second, so that accumulation of hydroxyl amine may occur. It is possible that the

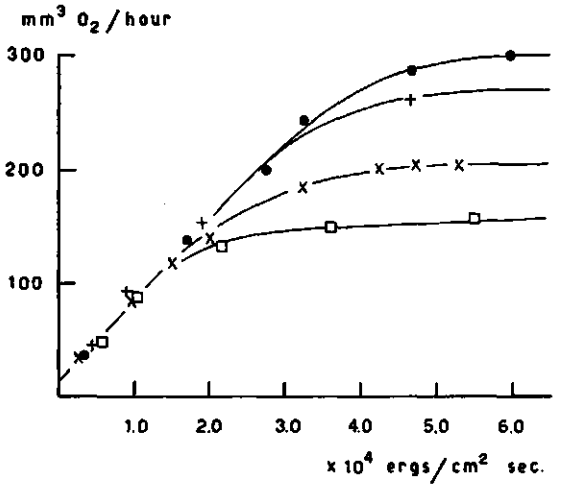


FIG. 13. The inhibition of photosynthesis of *Chlorella* with hydroxyl amine versus light intensity, at 25 °C, in WARBURG buffer no. 9 (● — ● = control, + — + = 0.95×10^{-4}M, × — × = 1.16×10^{-4}M, □ — □ = 1.4×10^{-4}M $NH_4OH.HCl$).

inhibition of photosynthesis with CaD_2 proceeds through a reduction to a hydroxyl amine like compound.

It seemed important, therefore, to repeat the experiments of WELLER and FRANCK (80) with hydroxyl amine and also to measure the inhibition of photoreduction with CaD_2 (for the latter see Chapter V). In figure 13 the inhibition of photosynthesis with hydroxyl amine at different light intensities at 25 °C is represented in one out of 7 experiments with similar results. We thus cannot confirm the results of WELLER and FRANCK (80). Though in our experiments lower concentrations of hydroxyl amine (0.95, 1.16 and 1.4×10^{-4}M) have been used than those applied by WELLER and FRANCK (2.5×10^{-4}M), our results suggest that even in higher concentrations, inhibition is much stronger in the light saturation range than in the light limiting range.

§ 6. DISCUSSION

A linear relationship between the percentage of inhibition and the concentration for various inhibitors (on a logarithmic scale) was observed between 20–80 % inhibition at light saturation (figs. 7a, 7c, 7d, 7e, 7f). Beyond the mentioned degrees of inhibition the curves approach threshold and complete inhibition more or less asymptotically. In some cases, therefore, only the concentration causing 50 % inhibition was given. With ethyl urethane (fig. 7b), however, a bend in the curve occurred at 0.1–0.2 M, which was ascribed to a desiccating effect by the osmotic value of the inhibitor at these high concentrations (p. 16).

A relationship between the inhibition and the concentration of the inhibitor was derived by TAMIYA (68) and rendered in the formula:

$$H = \frac{G^n}{\Theta^n + G^n}$$

in which H = degree of inhibition (per cent inhibition in our experiments, divided by 100).
G = the concentration of the inhibitor.
Θ = a constant, corresponding to the concentration causing 50 % inhibition.
n = a constant, representing the order of inhibitory action.

A simpler formula can be derived from that given above, *viz.* $\log G = {}^1/n \log \frac{H}{1-H} + \log \Theta$. By representing log G versus $\log \frac{H}{1-H}$ straight curves will result from which the slope n and the intercept Θ, can be calculated. From these constants inhibition curves were calculated and drawn in the figs. 7a, 7b, 7c, 7d, 7e, 7f. The calculated curves fit the observed values well, which emphasizes the validity of the formula. The value n is characteristic for each inhibitor, and as a rule remains constant under different conditions, *e.g.*, with CaD_2 at different temperatures. The value Θ (50 % inhibition) may vary, *e.g.*, with temperature, pH (Chapter IV) and light intensity. The results represented here confirm those obtained by TAMIYA (68). However, with hydroxyl amine no definite decrease in inhibition was observed at lower temperatures (table V). His experiments, however, were carried out at 25° and 4°C, and at a lower pH of the medium.

TABLE VII. Computed values of n (order of inhibitory reaction) and Θ (concentration in M giving 50 % inhibition) for various inhibitors with respect to photosynthesis in *Chlorella*.

Inhibitor	I		II		III		IV	
	n	Θ	n	Θ	n	Θ	n	Θ
CaD_2	2	4.2×10^{-6}						
ethyl urethane	1–2	1.95×10^{-1}	2	1.6×10^{-1}				2.2×10^{-1}
phenyl urethane	1	3.05×10^{-4}	1	6.3×10^{-4}			1	5.0×10^{-4}
hydroxyl amine	3	1.15×10^{-4}	2	1.0×10^{-4}				
2,4-dinitro-phenol	2	3.75×10^{-4}			2	2.0×10^{-6} (6cc) 3.7×10^{-6} (3cc)		
sodium azide	1	3.8×10^{-3}	2	1.6×10^{-4}	2	6.5×10^{-6}		

I = our experiments II = TAMIYA (68) III = WINTERMANS (83)
IV = WARBURG (71)

In table VII computed values for n and Θ are represented for various inhibitors, also from experiments carried out by other authors (TAMIYA [68], WARBURG [71], WINTERMANS [83]). There is a general agreement by these authors in the value of Θ (50 % inhibition) with the same inhibitor, as may be seen from the table. It is again clear that an exceptionally low concentration of CaD_2 already inhibits photosynthesis. Only the observations of WINTERMANS (83), concerning inhibition with 2,4-dinitrophenol (at pH 4 to 5) are in the same concentration range as those with CaD_2. In addition to this, HOLZER (30) demonstrated a far smaller inhibition (10^{-4}–10^{-3}M being required) with 2,4-dinitrophenol (at pH 8 to 9), which agrees with our results (at pH 9.2). This increase in inhibition with a decrease in the pH, which also may occur with sodium azide, has been discussed by WINTERMANS (83) and by SIMON and BEEVERS (61,62). The computed order of the inhibitory reaction n was the same in these cases.

Though in our experiments the value for Θ with hydroxyl amine agrees with that obtained by TAMIYA (68), a different order of the inhibitory reaction was observed. It is not yet understood, why in our case an order of 3 was found. This hardly can have a real significance, so that, probably, the validity of the formula under discussion, is not sufficiently general to be applied to all sorts of inhibitors. In connection herewith, also the different behaviour in inhibition of photosynthesis at light limitation in our experiments and in those of WELLER and FRANCK (80) is not clear. Possibly, the formula applied by TAMIYA (68) is not applicable to all inhibitors, whilst preparation of the cultures certainly affects the results, as was already mentioned in the introduction of this Chapter.

A large increase in inhibition of photosynthesis with decrease of temperature was found only with CaD_2. To obtain the same inhibition percentage, lower concentrations had to be used at lower temperatures, which amounted roughly to a Q_{10} (conc. $[T + 10]$ °C/conc. T° for 50 per cent inhibition) of 5 (fig. 11). It is not yet understood, why such a high Q_{10} occurs. This fact is possibly not due to higher uptake of CaD_2, since at all temperatures a constant inhibition was attained after the same time interval elapsed since the addition of the inhibitor.

CHAPTER IV

INHIBITION OF PHOTOSYNTHESIS WITH CaD_2 AS COMPARED WITH THE INHIBITION OF OTHER METABOLIC PROCESSES IN *CHLORELLA*

§ 1. INTRODUCTION

In the preceding chapter we have compared the influence of CaD_2 on *Chlorella* photosynthesis with that of other inhibitors. Under complete inhibition of photosynthesis by CaD_2 we have often observed negative manometer readings which even excelled those of the dark respiration of the non-inhibited control (Ch. III). In connection herewith we have studied respiration in the presence and absence of glucose with different concentrations of CaD_2. These experiments will be discussed in this chapter.

Moreover, the inhibition of photosynthesis with CaD_2 was investigated at different pH of the medium and in flashing light. In addition fluorescence measurements were made.

Finally, experiments regarding the inhibition by CaD_2 of autotrophic and heterotrophic growth of *Chlorella* in long term experiments will be presented.

§ 2. INHIBITION OF ENDOGENOUS AND GLUCOSE-STIMULATED RESPIRATION

Respiration was also measured in the WARBURG apparatus. *Chlorella* was suspended in distilled water, and 0.2 cc 1 N KOH was pipetted into one of the side arms of the vessel, which was sufficient to absorb all carbon dioxide produced in respiration. Consequently, the negative change in the manometric reading is due to oxygen uptake. Sometimes, when very small readings could be expected, the suspension was made up to a total quantity of 30 mm^3 of

cells per vessel. For reasons of comparison these results have been converted to 20 mm^3, the amount normally used. In the experiments with the glucose-stimulated respiration the cells were washed and suspended in distilled water with 0.5 % glucose. This quantity of glucose was sufficient to obtain a constant rate of respiration for at least 4 hours.

Experiments on endogenous respiration

Measurements were made in a range from 0 to 1×10^{-3} M CaD_2, for 2 hours after addition of the inhibitor. The influence of CaD_2 on respiration is shown in figure 14 as an average of eight experiments at 25 °C. The relatively high mean error in these experiments results from the small readings, the rate of respiration being low (rate of the control 15 mm^3 O_2/hour). In our experiments, however, the respiration of the control never exceeded that of cells with CaD_2. On the contrary, there is a stimulation of respiration which increases with increase of the CaD_2 concentration up to about 2.2×10^{-4}M. This high rate of respiration was maintained even at 1×10^{-3}M CaD_2. The ultimate stimulation at this concentration ranged from 35 to 100 % and remains at that level for at least three hours. The reversibility of this stimulation has not been tested by washing experiments.

Experiments on glucose-stimulated respiration

Because of the low rate of respiration, it may be assumed that not all active places on the enzyme systems are occupied, which probably explains the relative insensitivity to CaD_2. By adding 0.5 % glucose to the *Chlorella* suspension, the rate of respiration is increased about 4–8 fold.

The same range of concentrations and the same procedure of measurement have been used as for endogenous respiration. Usually, glucose was added one hour before the experiments started in order to avoid interference of the adaptation from endogenous to glucose respiration, which takes place during the first hour. After that, a constant rate was observed for at least 4 hours.

The inhibition of glucose-stimulated respiration is shown in figure 15 as an average of 17 experiments at 25 °C. The inhibition increases steadily with the increase of the CaD_2 concentration, but the oxygen uptake still was 45 % of the control at the highest concentration. From the course of the curve a further increase in inhibition may not be expected at higher concentrations. This, however, has not been tested, since 1×10^{-3}M CaD_2 was the highest concentration available. At this concentration, the rate of glucose respiration was still higher than the stimulated endogenous respiration at the same concentration of the poison (see previous section).

Washing experiments were performed at 5 concentration levels. After a 2 hours treatment with CaD_2, *Chlorella* was centrifuged, washed three times and measured again after being suspended in 0.5 % glucose. It is clear that the inhibiting effect on respiration is at least partly reversible, as may be seen from figure 15.

This reversibility contradicts the results obtained on photosynthesis. Another difference is that, unlike in photosynthesis, the inhibition in the glucose respiration remains constant at any concentration. The inhibition reaches a constant value within 20 minutes, as may be seen from figure 16. This fact will be discussed lateron in this paper (Chapter VI).

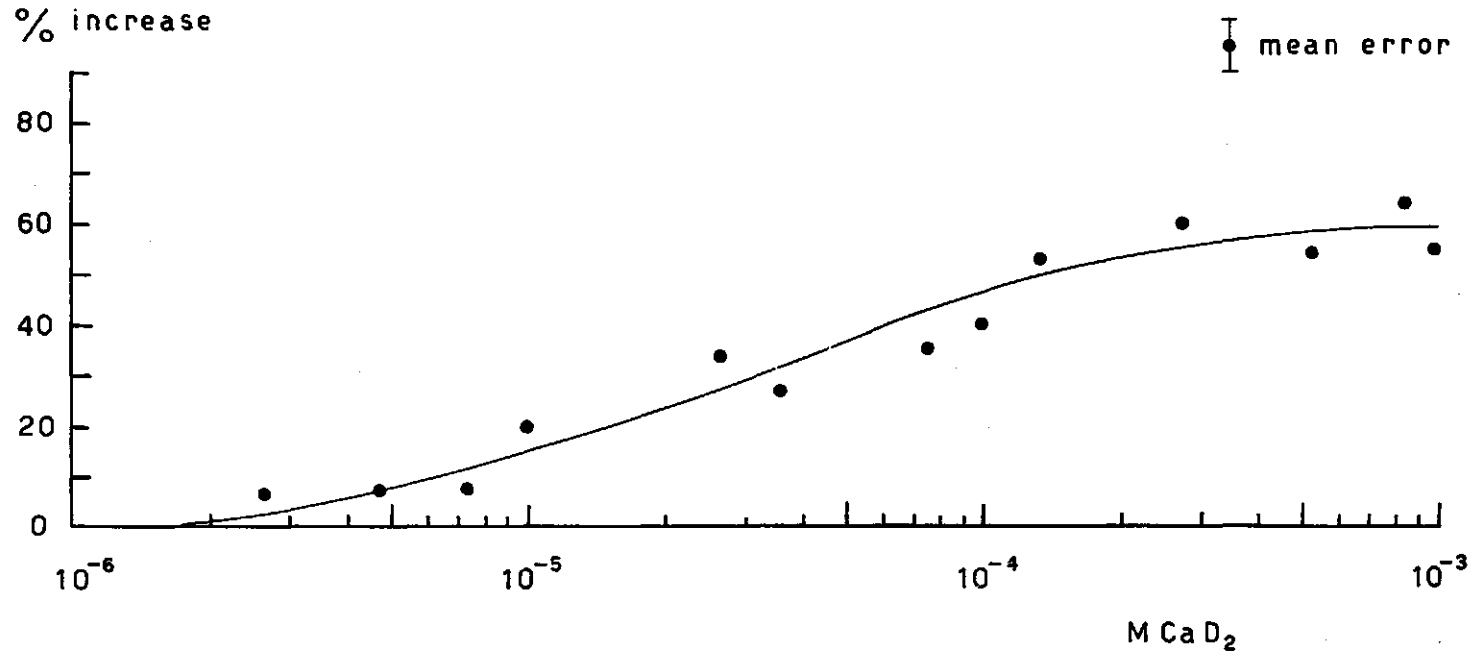


FIG. 14. The increase of endogenous respiration of *Chlorella* suspended in tap water with 0.2cc 1 N KOH in the side arm of the WARBURG vessel, at 25 °C.

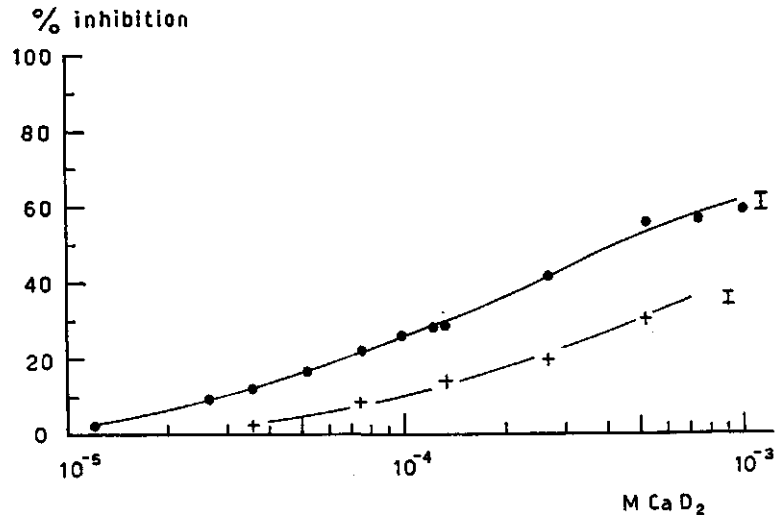


FIG. 15. The inhibition of the glucose-stimulated respiration at different concentrations of CaD_2 (● — ●) and the effect of washing on the inhibition (+ — +) at 25 °C (*Chlorella* suspended in 0.5% glucose solution with 0.2 cc 1 N KOH in the side arm of the WARBURG vessel).

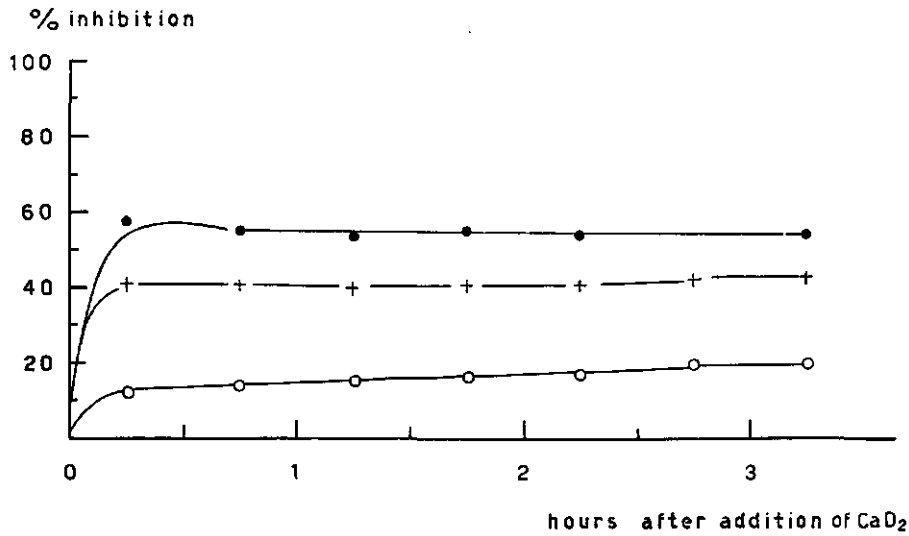


FIG. 16. The inhibition of the glucose-stimulated respiration of *Chlorella* versus time after addition of the inhibitor at 3 concentrations of CaD_2 (● — ● = 5.2×10^{-4}M, + — + = 2.65×10^{-4}M, o — o = 7.6×10^{-5}M CaD_2).

§ 3. INHIBITION OF PHOTOSYNTHESIS AT DIFFERENT pH VALUES OF THE MEDIUM

So far, measurements have been made in WARBURG buffer no. 9, consequently the inhibition of CaD_2 has always been measured as a change in O_2 (Method I). In order to find out whether the uptake of CO_2 was inhibited to the same degree, the photosynthetic quotient $p = -O_2/CO_2$ was calculated by measuring the inhibition at pH 5.8 and pH 7.2, using the two vessel method. From the two manometric readings, the uptake of CO_2 and the release of O_2 can be computed as already described (p. 9, Method II). The same quantity of cells was suspended in 2 and 5 cc phosphate buffer respectively. The measurements were made at light saturation, at 25 °C.

The photosynthetic quotient was determined without CaD_2 and with 1.56×10^{-6}M CaD_2. The results are shown in table VIII.

TABLE VIII. The photosynthetic quotient of *Chlorella* at pH 5.8 and pH 7.2 (sodium phosphate buffer) with and without addition of CaD_2. Each quotient representing the average of 5 experiments at 25 °C.

pH	Control		1.56×10^{-6} M CaD_2	
	Average	Extreme values	Average	Extreme values
5.8	1.03 ± 0.02	(0.97–1.09)	1.05 ± 0.02	(1.00–1.10)
7.2	1.07 ± 0.01	(1.00–1.15)	1.07 ± 0.01	(1.04–1.10)

Within the limits of error, no differences in the photosynthetic quotient occur between the control and the suspension containing 1.56×10^{-6}M CaD_2, neither at pH 5.8 nor at pH 7.2. The photosynthetic quotients in the control and in the presence of CaD_2 at pH 7.2 are in general somewhat higher than those at pH 5.8. The differences, however, are not significant.

As there was no effect at all on the photosynthetic quotient (p), the inhibition of photosynthesis at different pH values has been measured in phosphate buffers using the one vessel method (Method III) in order to enable a wider variety of treatments. The computations in Chapter II show that the influence of erroneous p-values becomes smaller with larger volumes of fluid (lower Vc values). Also the resulting manometric readings are higher, which is another advantage of using as much fluid as possible. In our measurements, therefore, we used 5 cc, in which disturbing effects due to mixing of the fluid in the main part of the vessel and in the side arm do not yet occur.

The influence of pH on the inhibition of photosynthesis by CaD_2 is given in table IX. The measurements have been made in phosphate buffers (pH 5.9 and 7.2) or in carbonate bicarbonate buffers (pH 9.2 and 10.4). In the latter case the calculations proceed according to Method I (Chapter II).

It was mentioned in Chapter II that considerable errors in the computed rate of gas exchange occur if no correction for CO_2 retention is applied. Since this only affects the overall vessel constant, it has no influence if the inhibition is represented as a percentage of the control (see columns 3 and 5). No pH effect was observed in the phosphate buffers (columns 3 and 7). In the carbonate bicarbonate buffers the inhibition at pH 9.2 was less than at pH 10.4. It is possible, however, that in the latter case an unfavourable effect of the high pH occurs, which was also found in control experiments (Chapter II). The concentrations of CaD_2 yielding 50 % inhibition were not entirely the same

TABLE IX. The inhibition of photosynthesis in *Chlorella* by CaD_2 at different pH of the medium (pH 5.9 and 7.2 in phosphate buffers, pH 9.2 and 10.4 in carbonate bicarbonate buffers), at 25 °C.

Concentration of the inhibitor (10^{-6}M)	pH 5.9		pH 5.9 [1])		pH 7.2		pH 9.2 (fig. 7a)		pH 10.4	
	Rate of photosynthesis in mm^3/hr.	Per cent inhibition	Rate of photosynthesis in mm^3/hr.	Per cent inhibition	Rate of photosynthesis in mm^3/hr.	Per cent inhibition	Rate of photosynthesis in mm^3/hr.	Per cent inhibition	Rate of photosynthesis in mm^3/hr.	Per cent inhibition
0	630		520		514		243		82.0	
0.263	592	6 ± 4	490	6 ± 4	495	4 ± 2				
0.524	550	13 ± 3	455	13 ± 3	452	12 ± 5				
0.99	492	22 ± 4	409	21 ± 4	380	26 ± 8	226	7 ± 3	55.8	32 ± 3
1.58	360	43 ± 8	298	43 ± 8	320	38 ± 7	211	13 ± 3		
2.98	234	63 ± 5	193	63 ± 5	206	60 ± 4	160	34 ± 4	27.0	67 ± 4
5.35	190	70 ± 5	157	70 ± 5	165	68 ± 8	92.0	62 ± 3	20.4	75 ± 3
6.95	120	81 ± 7	100	81 ± 7	82.5	84 ± 8	65.5	73 ± 2		
50% inhibit.		2.5×10^{-6}		2.5×10^{-6}		2.5×10^{-6}		4.2×10^{-6}		1.8×10^{-6}
Order of inhibition reaction		2		2		2		2		2

[1]) Per cent inhibition after application of JOHNSON's formula (Chapter II).

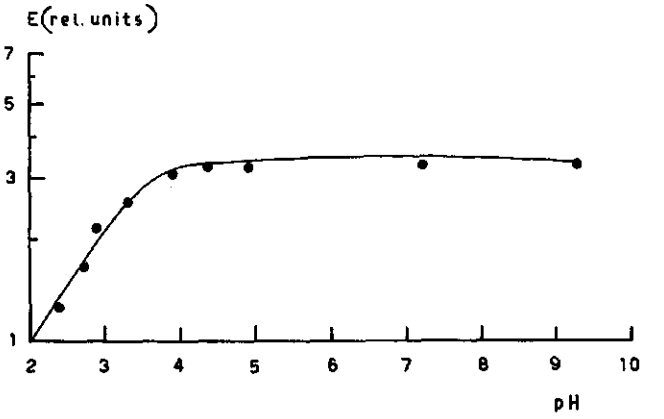


FIG. 17. The extinction of CaD_2 measured in relative units versus pH (buffers of different pH, measurements with 5.8×10^{-6} M CaD_2).

in phosphate buffers and in carbonate buffers. However, the experiments were made on different data, which may, at least in part, account for this difference. The order of the inhibitory reaction was 2 in all cases, calculated as indicated on p. 28.

If only undissociated molecules penetrate into the cell, a decrease in pH will increase the formation of HD molecules. As only the D-ion is intensely coloured, the variation in extinction of a solution of CaD_2 with pH may furnish a measure of the dissociation. In figure 17, the extinction, on a logarithmic scale, is plotted against pH for 5.8×10^{-6}M CaD_2, giving about 75 % inhibition of photosynthesis at pH 9.2. From this figure it is clear that a decrease in extinction only occurs at pH values lower than 4.0. It is probable, therefore, that stronger inhibition of photosynthesis is only perceptible below this value. However, measurements of the rate of photosynthesis at pH values below pH 4.0 are unreliable under these non-physiological conditions. The internal pH of the cell, on the other hand, is to a high degree independent of the pH of the outside medium.

§ 4. THE INHIBITION OF PHOTOSYNTHESIS IN CONNECTION WITH CHLOROPHYLL FLUORESCENCE

Excited chlorophyll may dissipate energy into fluorescence, heat or as potential energy to another molecule during collision. Only a small fraction of the absorbed quanta is dissipated as fluorescence. However, the intensity of fluorescence is a direct measure of the number of excited chlorophyll molecules present. Inhibitors changing the initial slope in photosynthesis will usually affect fluorescence, whereas those only influencing the saturation level will have no effect on fluorescence or affect it only indirectly. Measurements have been reported by KAUTSKY and HIRSCH (32), KAUTSKY and SPOHN (33), and lateron by FRANCK *et al.* (17) with *Chlorella*, and by WASSINK *et al.* (75, 76, 77) with *Chlorella*, Diatoms and *Chromatium*.

Fluorescence measurements were made in small rectangular vessels ($3 \times 2 \times 1$ cm). A high pressure mercury vapour lamp was used as a light source in these

experiments, because the relatively small amount of red light could easily be removed with a 6 cm half saturated copper sulphate solution removing all the infra-red, red and orange components of the light. Since fluorescence directly depends on the incident light intensity, the mercury lamp was fed via an a.c. stabilizer. We used the same equipment as SPRUIT and WASSINK (65), measuring the fluorescence light with a caesium photo-electric cell connected with an a.c. amplifier. The experiments were performed at room temperature (about 19 °C). Different light intensities were obtained by using copper wire screens of different mess width.

In figure 18, the fluorescence is represented in relative units at different light intensities and at different CaD_2 concentrations. There is a linear relation up to a light intensity of $2.0 - 3.0 \times 10^4$ ergs/cm^2 sec. after which the slope increases. The transition range agrees closely with the reach of light saturation in photosynthesis (fig. 12a). With 1.0×10^{-6}M giving nearly complete inhibition of photosynthesis, the fluorescence is smaller, both at high and low light intensities. At still higher CaD_2 concentrations (2.6×10^{-5}M) a further decrease in fluorescence was observed, especially at high light intensities, resulting in straightening the curve. At lower concentrations of CaD_2, giving 50 % inhibition of photosynthesis, a decrease in fluorescence was also observed.

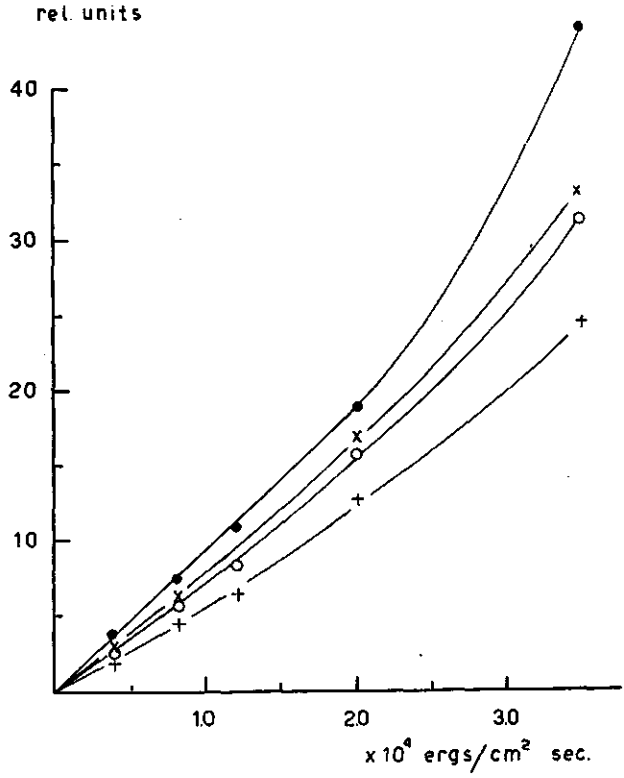


FIG. 18. The effect of CaD_2 on fluorescence of *Chlorella* versus light intensity at different concentrations (● — ● = control, × — × = 5.2×10^{-6}M, o — o = 1×10^{-5}M, + — + = 2.6×10^{-5}M CaD_2).

The results indicate that CaD_2 accepts the energy transferred from the excited chlorophyll and does so even with a higher probability than the normal energy acceptor, since fluorescence is decreased. It is known that nitrophenols generally accept energy quite easily, with which our results are in accordance.

§ 5. INHIBITION OF PHOTOSYNTHESIS WITH CaD_2 IN FLASHING LIGHT

Intermittent light was already applied in the study of photosynthesis by BROWN and ESCOMBE (10), and later by WARBURG (71). In the classical studies of EMERSON and ARNOLD (13), improved methods for the study of flash light were used. By variation of the dark period, they found that, after the addition of cyanide, causing 60 % inhibition in continuous light, the yield of photosynthesis per flash increases with increasing dark period and was the same as the control if the dark period was sufficiently long. The inhibition of photosynthesis with phenyl urethane, on the other hand, was not counterbalanced by a longer dark period. Lateron, inhibition experiments of photosynthesis in connection with flash light were made by WELLER and FRANCK (80) and TAMIYA (68).

Our experiments were performed at 30 °C, with *Chlorella* suspended in 0.2 M WARBURG buffer no. 10 (pH 8.7). CaD_2 was added to the suspension before the latter was pipetted into the reaction vessel. The yield of photosynthesis was measured with a recording volumeter, the design of which has been described in full detail by KOK (35). Different light intensities were obtained by using copper screens of different mess width. By varying the width of the slit in the rotating disc and the rotation speed, different dark periods (0.05–0.8 sec.) could be combined with the same flash time (0.032 sec.). With the aid of this apparatus light intensity curves could be made in continuous light as well as in flash light. In the intervals between the flash light parts of the experiment, dark periods were intercalated in which respiration has been measured. In these experiments an increased respiration up to 60 % was observed in the dark with increasing CaD_2 concentrations. The high respiration rate may give rise to sources of error in the computation of the yield of photosynthesis especially in case of large dark intervals between the separate flashes. Especially so, because the possibility of an influence of the flashes upon the respiration in the dark intervals cannot be excluded *a priori*.

The results obtained in continuous light confirm those obtained with the WARBURG apparatus (figs. 12b and 12c) and are not recorded here again. In figure 19, the per cent inhibition at saturation is plotted against the length of the dark period at different concentrations of CaD_2. It is clear that neither at 6.9×10^{-6}M CaD_2 nor at 4.7×10^{-6}M CaD_2 a decrease in the inhibition of photosynthesis is observed even with dark periods of 0.8 sec. These results are very similar to those obtained with phenyl urethane in flashing light experiments (13).

§ 6. INHIBITION OF AUTOTROPHIC AND HETEROTROPHIC GROWTH

The growth experiments were performed in 300 cc erlenmeyer flasks containing 150 cc of WARBURG culture medium. The flasks were inoculated with the same amount of cells *i.e.* 15 mm^3 cells/flask and then placed on the shaking device. By interchanging the places of the erlenmeyers every day, differences in light intensity on the shaking device were eliminated.

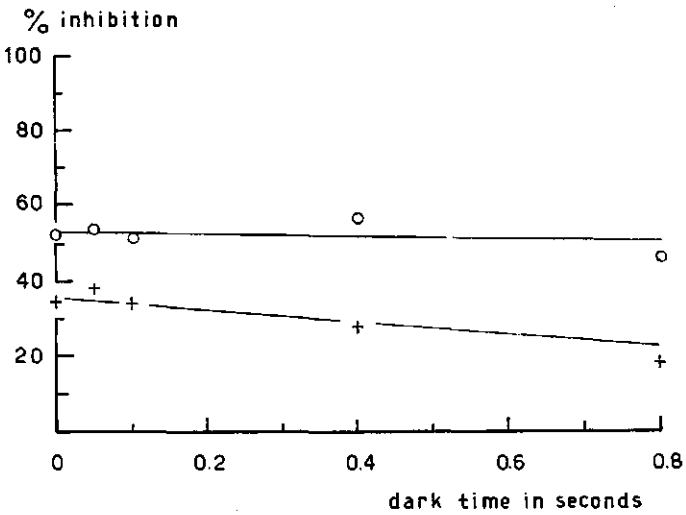


FIG. 19. Per cent inhibition of photosynthesis of *Chlorella*, in continuous and flashing light versus length of dark period at different CaD_2 concentrations (+ — + = 4.7×10^{-6}M, and o — o = 6.9×10^{-6}M CaD_2).

Growth has been measured by centrifuging a fixed volume in a TROMMSDORFF tube for 11 minutes at a speed of 2250 revolutions per minute. The height of the column in the capillary of the TROMMSDORFF tube has been taken as a measure of the density of the culture.

The uptake of CaD_2 in the growing culture was determined by measuring the transmission of the supernatant in the TROMMSDORFF tube at 480μ in a photo-electric colorimeter. The concentration of CaD_2 could be read from a calibration graph. It sometimes happened that the cultures became turbid, and the supernatant did not become clear even after centrifuging for a long period of time. This tends to suggest a higher concentration of CaD_2 than is actually present. The turbidity is probably due to calcium compounds in collodial suspension. Assuming that the turbidity occurs in the same way in a culture without CaD_2, one can take the supernatant of the control instead of distilled water for comparison, as has been done in the first experiments. However, as growth in these controls is stronger than in cultures with CaD_2, higher turbidities often occur in the controls, resulting in too low estimations of the CaD_2 concentration. Better results were obtained by diluting the supernatant with distilled water and shaking it with a small volume of methyl-isobutyl ketone. The ketone phase collects at the top and contains all CaD_2, whereas most of the turbidity remains in the water phase.

Autotrophic growth

Each growth experiment consisted of a range of 8 erlenmeyer flasks with CaD_2 concentrations from 0 to 6.6×10^{-6}M. The concentrations of CaD_2 were measured directly after inoculation. After 5 days, the growth of *Chlorella* and the concentration of CaD_2 were measured. In table X, the number of cells is given after 5 days as an average of 6 experiments (column 2). The concentration of CaD_2 after inoculation, the inhibition of growth and the concentration of CaD_2 at the end of the experiments are shown in columns 1,3, and 4 respectively.

The inhibition of growth increases with the increase in the concentration of CaD_2 (column 3). Comparing these results with those on photosynthesis (figure 7a, 25°C) it may be seen that the range of inhibition is about the same. The absorption of CaD_2 tends to increase with the concentration (column 5);

TABLE X. The inhibition of autotrophic growth in *Chlorella* with different concentrations of CaD_2 as an average of 6 experiments.

1. Concentration of CaD_2 (10^{-6}M) directly after inoculation	2. Quantity of cells in mm^3/10 cc after 5 days[1])	3. Rel. growth rate[2])		4. Concentration of CaD_2 (10^{-6}M) in supernatant after 5 days	5. Absorption of CaD_2 (10^{-6}M) from 10 cc suspension by the cells in 5 days
		Numer. value[2])	Per cent inhibition		
0	40.0 ± 2	0.320	–	–	–
0.66 ± 0.06	44.4 ± 4	0.329	–3	0.11 ± 0.06	0.55
1.54 ± 0.06	31.2 ± 6	0.299	7	0.82 ± 0.06	0.72
2.4 ± 0.06	30.0 ± 9	0.295	8	1.2 ± 0.06	1.2
3.4 ± 0.06	12.0 ± 2	0.216	33	2.42 ± 0.06	1.08
4.5 ± 0.06	10.0 ± 5	0.200	38	3.2 ± 0.06	1.2
5.5 ± 0.06	5.2 ± 2	0.143	55	4.05 ± 0.06	1.45
6.5 ± 0.06	2.8 ± 1	0.089	72	4.95 ± 0.06	1.55

[1]) Initial density 1 mm^3 cells in 10 cc.
[2]) Relative growth rate: log. increase of cell density per day.

this is even more so per cell, because of the stronger inhibition of growth. We have investigated whether the decrease in concentration of CaD_2 might be merely due to the presence of potassium in the medium. That this could not be the explanation was demonstrated by the observations that no decrease in concentration occurred at 8.8×10^{-6}M CaD_2 in erlenmeyer flasks with water or a WARBURG medium without algae, neither in dark nor in light, after 5 days.

Heterotrophic growth

Each experiment consisted of a range of concentrations from 0 to 1.73×10^{-5}M CaD_2. The inoculation and the measurements have been made in the same way as already described above in the section on autotrophic growth. These experiments were performed in the light (3500–4500 lux), in the same way as those on autotrophic growth, 15 gr. glucose/l. having been added to the medium. The results are represented in table XI.

TABLE XI. The inhibition of heterotrophic growth in *Chlorella* with different concentrations of CaD_2 as an average of 5 experiments.

Concentration of CaD_2 (10^{-6}M) directly after inoculation	Quantity of cells in mm^3/10cc after 5 days[1])	Rel. growth rate[2])	
		Numerical[2]) value	Per cent inhibition
0	100 ± 10	0.400	–
1.53 ± 0.06	120 ± 20	0.416	–4
3.5 ± 0.06	100 ± 10	0.400	0
5.25 ± 0.06	120 ± 10	0.416	–4
7.1 ± 0.06	100 ± 10	0.400	0
8.2 ± 0.06	70 ± 25	0.369	8
8.9 ± 0.06	40 ± 30	0.320	20
13.5 ± 0.06	6 ± 6	0.156	61
17.3 ± 0.06	3 ± 3	0.095	76

[1]) Initial density 1 mm^3 cells in 10 cc.
[2]) Relative growth rate = log. increase of cell density per day.

No definite measurements of CaD_2 could be made at the end of these experiments, because of the high turbidity of the glucose cultures. The extraction method of D with methyl-isobutyl ketone was not followed, since it was not yet known to us during these experiments. Visually, the uptake of CaD_2 was higher than in the autotrophic series owing to the higher CaD_2 concentrations, and the higher density of the suspension, which was 40.0 and 100.0 mm^3 in the control of the autotrophic and heterotrophic growth series respectively. It may be seen from tables X and XI that the inhibition of heterotrophic growth is smaller. The concentration at which complete inhibition of autotrophic growth occurs hardly reaches the threshold for inhibition of heterotrophic growth.

§ 7. DISCUSSION

Generally, inhibition of respiration is found at higher concentrations of the inhibitor than that of photosynthesis. Up to the present the reverse has only been reported for cyanide inhibition in *Scenedesmus* (GAFFRON [21]). Aromatic nitro-compounds usually increase the rate of respiration to a high extent in concentrations in which synthetic processes are decreased or completely inhibited. Inhibition of respiration was observed at higher concentrations (BEEVERS, [4], KELLY and AVERY [34]). In our experiments an increase of respiration from 30 to 100 % was observed with increasing CaD_2 concentrations up to 10^{-3}M in the dark. Inhibition may well occur at still higher concentrations, but this could not be tested since 10^{-3}M CaD_2 was the highest concentration available. The absence of a clear inhibition of respiration may well be due to its low rate. Addition of glucose results in an increase of respiration up to 6–8 times the value of the endogenous respiration. An inhibition up to 60 % of the glucose-stimulated respiration was observed at 10^{-3}M. Yet the rate still was nearly 2 times that of the stimulated endogenous respiration at the same CaD_2 concentration. It may be seen from figure 15 that the inhibition of glucose respiration tends to increase more slowly in the region of 10^{-3}M. The data contain no indication that the rate of respiration at still higher concentrations of CaD_2 should be below that of the CaD_2 stimulated endogenous respiration.

Heterotrophic growth was found to be less sensitive to CaD_2 than autotrophic growth. It may be seen from table X and figure 7a that in autrotrophic growth and photosynthesis the threshold concentrations of CaD_2 for inhibition are almost the same. However, the concentration at which complete inhibition occurs is higher in photosynthesis. It must be mentioned that the growth experiments covered a period of 5 days, whereas the inhibition of photosynthesis was measured for 3 hours after addition of CaD_2. A progressive increase in inhibition of photosynthesis was observed (figure 8). It may be concluded that inhibition of photosynthesis and autotrophic growth present nearly the same characteristics. As threshold and complete inhibition of heterotrophic growth are much higher, it is probable that the inhibition of autotrophic growth is due to the inhibition of photosynthesis, resulting in a shortage of assimilates, which becomes even worse by the increase in respiration. The observations of SEEL (58), that nitro-compounds in lower concentrations are sometimes more toxic than in higher concentrations, may also be due to a shortage of assimilates since respiration increased especially at lower concentrations. It is known that D is an even stronger inhibitor for cell division

than colchicine (GAVAUDAN *et al.* [24]). Nevertheless, this process, still may be less sensitive than photosynthesis.

The observations on fluorescence and flashing light emphasize the evidence already obtained in Chapter III, that CaD_2 inhibits equally the energy transferring part and some of the dark chemical parts of the photosynthetic process. For, if a poison inhibits photosynthesis in the light limiting range, a decrease or an increase of fluorescence may be expected, dependent on whether the poison has a higher or a lower energy accepting capacity than the energy acceptor involved in photosynthesis.

In our case a decrease in fluorescence was observed with CaD_2 in the light limiting as well as in the light saturation range. This indicates that CaD_2 has a high energy accepting capacity. Since it acts, as found, in low molar concentrations this fact may suggest some chemical similarity to the normal primary energy acceptor in photosynthesis.

If a poison inhibits photosynthesis in the light limiting range no decrease in inhibition percentage will be observed in flashing light, contrary to what is found for a poison preferably inhibitory in the light saturation range (13). With CaD_2 we found no decrease of inhibition in flashing light as compared with continuous light. Though these results represent a strong evidence for a narcotic action of CaD_2, the low concentration at which inhibition already occurs and the non-reversibility point to a specific bond to an enzyme rather than to a non-specific narcotic action.

The results obtained at different pH will be discussed with those in *Scenedesmus* lateron in this paper.

CHAPTER V

INHIBITION OF PHOTOSYNTHESIS AND GROWTH BY CaD_2 IN VARIOUS PLANT TYPES AS COMPARED WITH *CHLORELLA*

§ 1. INTRODUCTION

So far we have studied the effect of CaD_2 on various metabolic processes in *Chlorella* only. It could be expected that the range of concentrations at which growth and photosynthesis are affected by CaD_2 differs in various plant species. The inhibition in other respects as, *e.g.*, regarding chlorophyll content, dry weight, root length, may differ as well.

Strains of *Lemna minor*, *Lemna gibba*, *Lemna arrhiza*, *Scenedesmus*, *Chromatium spec.*, *Nostoc species*, *Rhodospirillum rubrum*, *Oscillatoria spec.*, and *Chlamydomonas* are kept in this laboratory in sterile stock cultures on agar or in solution, the latter especially for *Lemna* and *Oscillatoria*. The organisms were transferred repeatedly to fresh agar slants, or fresh culture solutions at short intervals in order to activate the cultures. Hereafter, they were inoculated into 300 cc erlenmeyer flasks containing 100–150 cc of a nutrient solution. During cultivation the nutrient solution was flushed with a stream of air, enriched with 5% CO_2. The erlenmeyer flasks were placed between fluorescent tubes („day light" type) at a light intensity of 3000–5000 lux, and incubated at a temperature of 20° ± 2 °C.
Growth was measured by centrifugation in TROMMSDORFF tubes for various algae and purple bacteria, by counting fronds (*Lemna*) or by visual estimation of growth (*Oscillatoria*). Photosynthesis and respiration were measured in the WARBURG apparatus. For specific details about culture methods and measurements we refer to the sections dealing with each species separately.

§ 2. INHIBITION IN *Lemna minor*, *Lemna gibba* AND *Lemna arrhiza*

Cation deficiencies in *Lemna* have been studied frequently, *e.g.*, by ASHBY and OXLEY (2), WHITE (82), PIRSON and SEIDEL (46), BIERHUIZEN (5); phosphate deficiency was studied by PIRSON *et al.* (47) and LINDEMAN (36). To the author's knowledge, however, few experiments were made on the effect of inhibitors on growth and photosynthesis.

Details of culturing are as follows:

The solution was based on that of GORHAM (26), containing 5×10^{-3} mol. $Ca(NO_3)_2$, 4 H_2O; 2×10^{-3} mol. $MgSO_4$, 7 H_2O; 5×10^{-3} mol. KNO_3; 1×10^{-3} mol. KH_2PO_4 and 0.005 gr. ferritartrate per litre with the following addition of micro elements: 2.86 mg. H_3BO_3; 1.82 mg. $MnCl_2$, 4 H_2O; 0.22 mg. $ZnSO_4$, 7 H_2O; 0.07 mg. MoO_3; 0.08 mg. $CaSO_4$, 5 H_2O. Unless indicated otherwise, 5–10 fronds were inoculated per erlenmeyer. Flushing of the cultures with air enriched with 5% CO_2 for three 2-hours periods per day was sufficient to obtain exponential growth. Growth was measured daily, by counting the numbers of fronds. In order to minimize the effect of different stages of development in *Lemna minor* and *Lemna gibba*, a young frond was rated as $\frac{1}{4}$, a medium one as $\frac{1}{2}$, an older one as $\frac{3}{4}$, and a mature frond as 1.

Frond area was measured from photographic contact prints of the fronds by means of a planimeter. Dry weight was measured by ovendrying (at 105 °C) 100–200 fronds in small weighing bottles during 2 days. Chlorophyll determinations were made by extracting the fronds with 90% ethanol. The extraction was repeated three times in a waterbath (50–60 °C) which was sufficient for total extraction. The chlorophyll concentration was measured in a BLEEKER colorimeter at 6650 Å. Photosynthesis was measured in the WARBURG apparatus with 10 to 25 fronds in 5 cc tap water per flask (pH 5.5) in the case of *Lemna minor* and with 100–150 fronds in that of *Lemna arrhiza*.

The increase in frond number of *Lemna minor*, *Lemna gibba* and *Lemna arrhiza* is represented in figure 20, in which the frond number is plotted on a logarithmic scale. The straight lines indicate a constant growth rate throughout the experiment, with the highest rate in *Lemna minor* and the lowest in *Lemna arrhiza*.

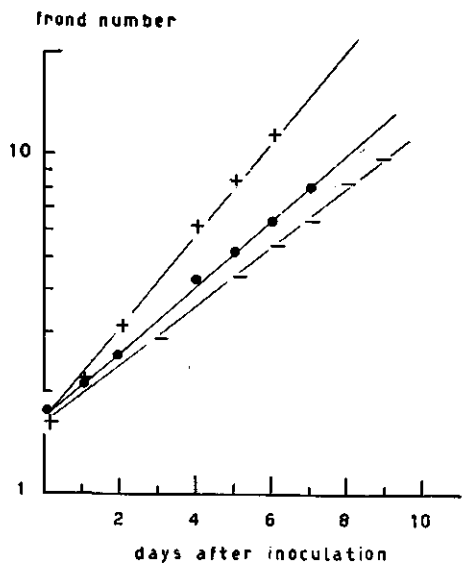


FIG. 20. The increase in frond number (on a logarithmic scale) versus time (in days after inoculation) *Lemna minor* (+ — +), *Lemna gibba* (● — ●), and *Lemna arrhiza* (– — –).

Experiments with *Lemna minor* and *Lemna arrhiza* were made at different concentrations of CaD_2. The results are represented in table XIIa and b as an average of 4 experiments.

TABLE XIIa. The effect of different concentrations of CaD_2 on the rate of growth, on dry weight, on frond area, on chlorophyll content and on root length in *Lemna minor*, as an average of 4 experiments.

Concentration of CaD_2 (10^{-6}M)	Rel. growth rate [1]		Dry weight per 100 fronds		Frond area		Chlorophyll [2] content per 15 fronds		Root length	
	Numerical value [1]	Per cent inhibition	mg	Per cent decrease	mm²	Per cent decrease	Numerical value [2]	Per cent decrease	cm	Per cent decrease
0	0.169	–	12.0	–	2.34	–	0.119	–	2	–
1.97	0.150	11	8.9	26	3.05	–30	0.120	–1	0.4	80
4.70	0.112	34	8.6	28	2.17	7	0.090	24	0	100
8.50	0.073	57	7.6	37	1.83	22	0.059	53	0	100

[1]) Rel. growth rate = log. increase in frond number per day.

[2]) measured as log. $\frac{E_o - E}{E_o}$

TABLE XIIb. The effect of different concentrations of CaD_2 on the rate of growth and on dry weight in *Lemna arrhiza*, as an average of 4 experiments.

Concentration of CaD_2 (10^{-6}M)	Relative growth rate [1]		Dry weight per 100 fronds	
	Numerical [1] value	per cent inhibition	mg	Per cent decrease
0	0.119	–	3.10	–
0.55	0.111	7	2.50	19
1.45	0.100	16	1.81	41
1.80	0.086	28	1.84	40
1.97	0.065	45	1.84	40
4.7	0	100	–	–
8.5	0	100	–	–

[1]) Relative growth rate = log. increase in frond number per day.

The inhibition of growth is represented as a percentage of the control. The table includes also the influence of CaD_2 on dry weight, leaf area, chlorophyll content and root length, the inhibition expressed as percentages of the respective controls. *Lemna arrhiza* is the more sensitive one. Complete inhibition of growth occurs already with 4.7×10^{-6}M, whereas in *Lemna minor* at the same concentration the growth rate still is 66 % of that of the control. Complete inhibition of *Lemna minor* only occurs at concentrations above 8.5×10^{-6}M. The same relation was observed in dry weight/frond. The chlorophyll content in *Lemna minor* decreases to about the same extent as the rate of growth; leaf area is less affected by addition of CaD_2 and decreases only in concentrations above 4.7×10^{-6}M CaD_2. The number and the length of the roots were reduced enormously, with 1.97×10^{-6}M already to about 20 % of the control, and with 4.7×10^{-6}M no roots at all were observed. These effects are shown in figure 21 which is a contact print of some fronds grown at the different concentrations (1 is the control, 2 represents 1.97, 3 represents 4.7, and 4 represents 8.5×10^{-6}M CaD_2).

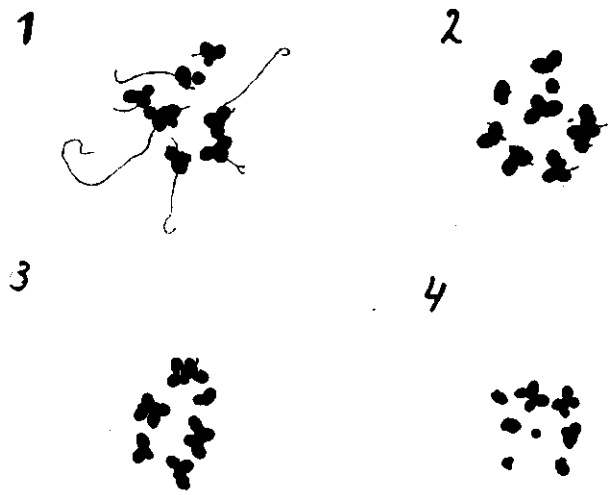


FIG. 21. The effect of CaD_2 on root formation of *Lemna minor* (1 = control, 2 = 1.97×10^{-6}M, 3 = 4.7×10^{-6}M, and 4 = 8.5×10^{-6}M CaD_2).

The inhibition of photosynthesis in *Lemna minor* is represented as per cent of the control, in figure 22, in short time experiments (0–2 hours after addition of CaD_2) and in long term experiments (after 24 hours). The inhibition in growth and photosynthesis in *Lemna minor* as compared with that in *Chlorella* indicates a slower uptake of CaD_2 in *Lemna minor*.

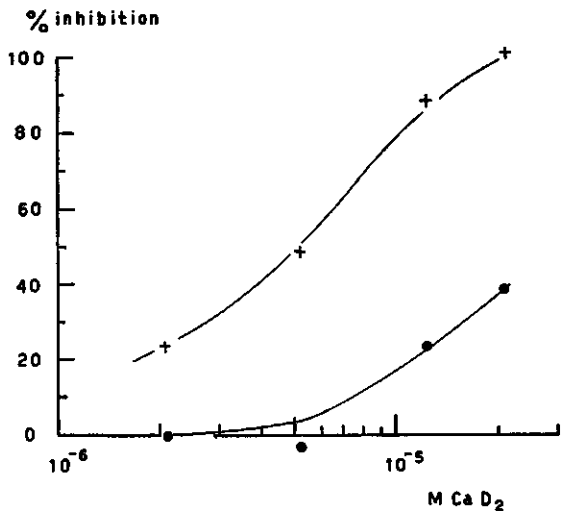


FIG. 22. The inhibition of photosynthesis of *Lemna minor*, with CaD_2, at 25 °C, under light saturation ($5.0 - 6.0 \times 10^4$ ergs/cm² sec.), in tapwater; 0–2 hours after addition of the inhibitor (● — ●) and 24 hours later (+ — +).

§ 3. INHIBITION IN *Scenedesmus*

Extensive inhibition experiments with *Scenedesmus* have been made by GAFFRON (23) and NAKAMURA (38). GAFFRON (23) reported for the first time a case in which respiration was suppressed by cyanide without injury to photosynthesis. It therefore seemed important to measure respiration also in connection with CaD_2.

The rate of respiration was measured in the dark in a concentration range up to 1×10^{-3}M CaD_2. No inhibition at all was observed at any of these concentrations. On the contrary, a stimulation of respiration occurs, which increases with increasing CaD_2 concentrations up to about 60% at 2.67×10^{-4}M CaD_2. This high rate of respiration was maintained even at 1×10^{-3}M. These results are in agreement with those obtained with *Chlorella*.

Growth experiments were made with *Scenedesmus* in a WARBURG medium (see Chapter II). The experiments were repeated 3 times with 5 different concentrations of CaD_2 (0, 0.55, 1.09, 2.19, 3.28 $\times 10^{-6}$M CaD_2). Inhibition of growth occurred already at 0.55×10^{-6}M (the growth rate being 75% of that of the control), and complete inhibition was observed at 2.19×10^{-6}M CaD_2. Inhibition of growth, therefore, occurs at somewhat lower concentrations than in *Chlorella*.

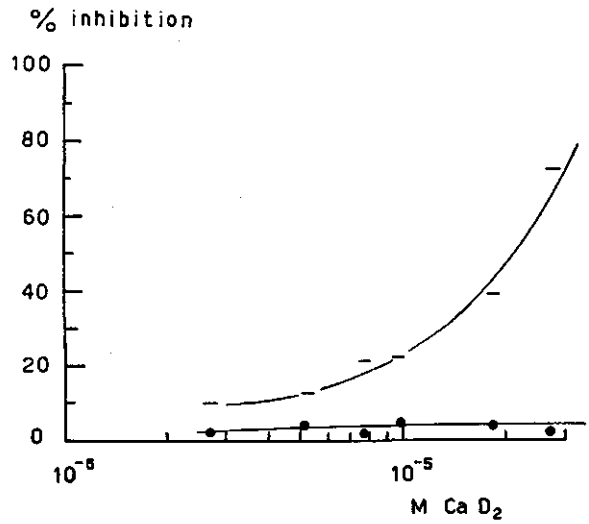


FIG. 23. The inhibition of photosynthesis of *Scenedesmus*, with CaD_2 at 25 °C, under light saturation ($5.0 - 6.0 \times 10^4$ ergs/cm² sec.), in WARBURG buffer no. 9; 1 (● — ●) and 3 hours (– — –) after addition of the inhibitor.

In addition hereto some measurements on photosynthesis were made at different concentrations (0, 2.63, 5.2, 7.65 $\times 10^{-6}$M, 1.0, 1.84, 2.7 $\times 10^{-5}$M CaD_2). In this concentration range no inhibition occurs in the first hour after addition (figure 23). Inhibition then became noticeable and increased gradually until after 3 hours a marked inhibition was manifest, which furtheron remained rather constant. These results show a marked difference with the strong inhibition of growth reported above, the more so since the inhibition of photo-

synthesis was less than that in *Chlorella*. This indicates, that growth is not inhibited solely by a shortage of photosynthates, as could be concluded to in the case of *Chlorella* in Chapter IV. The growth experiments were performed in a nutrient solution at pH 5.0 to 7.0 while the rate of photosynthesis was measured in WARBURG buffer no. 9 at pH 9.2. Therefore, simultaneous measurements of photosynthesis in WARBURG buffer and in tap water have been made, with the addition of 1.84×10^{-5}M CaD_2. The inhibition in the WARBURG buffer was found to be smaller than that in the phosphate buffer (table XIII). These results show that the observed discrepancy between the inhibition of growth and that of photosynthesis is at least in part due to a decrease in inhibition of the latter at high pH values. In *Chlorella*, on the contrary, no differences in inhibition were observed at different pH values.

TABLE XIII. The inhibition of photosynthesis in *Scenedesmus* at 25 °C, upon addition of 1.84×10^{-5}M CaD_2 in a carbonate bicarbonate buffer, and in tap water.

Time after addition	Carb.bicarb. buffer			Tap water		
	Rate of photosynthesis (mm^3/hr.)		Per cent inhibition by CaD_2	Rate of photosynthesis (mm^3/hr.)		Per cent inhibition by CaD_2
	No CaD_2	CaD_2		No CaD_2	CaD_2	
1 hour	173	159	8	142	30	70
2 hours	169	140	16	139	–9.5	100
3 hours	161	129	20	138	–9.2	100

The smaller inhibition of photosynthesis in the WARBURG buffer may be due to a more difficult uptake of CaD_2 into the cell from this buffer. Besides, it is known that *Scenedesmus* is more tolerant towards a high pH than *Chlorella*. This fact might explain the slight, but continuous decrease in the rate of photosynthesis observed in *Chlorella*, confirming PRATT's (51) results. Such a decrease is not observed with *Scenedesmus*, confirming ÖSTERLIND (44), who reported utilisation of HCO_3^- as substrate in *Scenedesmus quadricauda*, whereas this did not occur in *Chlorella pyrenoidosa*.

§ 4. INHIBITION IN *Chromatium*

Earlier experiments with purple sulphur bacteria have been carried out by GAFFRON (22), VAN NIEL (42), WASSINK *et al.* (76) and others. Our experiments were made with a pure culture of *Chromatium* indicated as „strain D" by ROELOFSEN (54).

The organism was maintained in a malate thiosulphate medium in 100 cc glass-stoppered bottles with a small jar over the stopper of the bottle in order to prevent contamination (EYMERS and WASSINK [14]).

As this species is anaerobic, all media had to be boiled and cooled before use. The culture medium was based on that of ROELOFSEN (54), as modified by EYMERS and WASSINK (14) containing: 2% NaCl, 0.1% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.02% $MgSO_4$ in tap water. It proved to be desirable to use the 2% NaCl partly in pure, partly in crude form; 1% of each was used. The pH was brought to 7.4 by addition of 1 N NaOH or 1 N HCl, before filtration and sterilization. Hereafter, 0.25 cc of a 10% Na_2S solution, 1.0 cc of a 10% Na_2CO_3 solution, one drop of a 10% H_3PO_4 solution, 3.75 cc of a 10% sodium malate solution, and 2.5 cc of a 10% thiosulphate solution were added to 162.5 cc of the solution. The species was repeatedly transferred to fresh cultures in short intervals, in order to activate the cells. Hereafter, 0.5 mm^3 of cells was pipetted into each of the glass-stoppered bottles.

The rate of photoreduction was measured in the WARBURG apparatus at 29 °C in a 1/15 M phosphate buffer with 0.6% NaCl (pH 7.6). Before use, one

day old cultures were centrifuged, resuspended in the same medium, without the addition of sodium malate, and shaken in the dark for a period of about 15 hours in order to use all the malate left in the cells. The vessels were attached to the manometers, one of the side tubes was provided with an outlet during the filling of the vessels ($\pm$ 3/4 hour). The gas mixture (N_2, 30 % H_2 and 5 % CO_2) was passed through an electrically heated reduction oven in order to remove all oxygen left in the mixture. The rate of photoreduction was computed by Method III. According to the formula $CO_2 + 2H_2 \rightarrow CH_2O + H_2O$, an uptake ratio $H_2/CO_2 = 2$ was used, which has been experimentally verified by WASSINK (74).

Growth experiments were made with CaD_2 at different concentrations (0, 2.19, 4.38, 6.55, 8.73 $\times$ 10^{-6}M, 1.09, 1.31, 1.96, 2.73 $\times$ 10^{-5}M) of CaD_2. The bottles were placed in the light cabinet at a light intensity of about 500 lux at 29 °C. Growth was measured after 1 or 2 days by centrifuging 5 cc suspension in TROMMSDORFF tubes. The actual activity of the bacteria after this treatment was determined by measuring the rate of photoreduction in the WARBURG apparatus. In this case, measurements were made directly, also in the blanks, without using the procedure of malate starvation. The results are summarized in table XIV as an average of 4 replicates.

TABLE XIV. The effect of CaD_2 on the rate of growth and the rate of photoreduction in *Chromatium*, as an average of 4 experiments.

Concentration of CaD_2 (10^{-6}M)	Quantity of cells in mm³/10 cc after 2 days[1])	Rel. growth rate[2])		Rate of photoreduction	
		Numerical value[2])	Per cent inhibition	mm³/hr.	Per cent inhibition
0	69.0	1.57	–	25.9	–
2.19	47.8	1.49	5	25.9	0
4.38	31.5	1.40	11	25.6	1
6.55	39.7	1.45	7	24.9	4
8.73	12.0	1.19	24	25.6	1
10.9	3.15	0.90	43	22.7	12
13.1	3.15	0.90	43	23.0	11
19.6	0.34	0.42	73	23.5	9
27.3	0.12	0.19	88	–	–

[1]) Initial density 0.05 mm³ cells in 10 cc.
[2]) Relative growth rate = log. increase of cell density per day.

It is clear, that the inhibition of growth, expressed as percentage of the control, increases with increasing CaD_2 concentrations up to 88 % inhibition at 2.73 $\times$ 10^{-5}M CaD_2. The inhibition of growth in *Chromatium* is smaller than that observed in other species, the sensitivity of which species is similar to that of *Chlorella* in heterotrophic growth. A comparison of the results of columns 4 and 6 in table XIV indicates that photoreduction is still less sensitive and is hardly inhibited at concentrations at which the growth rate is strongly decreased. These measurements were made by suspending the cells grown at the various CaD_2 concentrations in fresh phosphate buffer without CaD_2.

These findings were confirmed by measuring the inhibition of photoreduction in short time experiments upon addition of CaD_2 of *Chromatium* cells cultivated without CaD_2. The rate of photoreduction was measured during half an hour, after which different concentrations of CaD_2 were tipped into the suspension while the measurement was continued until 2–3 hours after addition (table XV).

TABLE XV. The inhibition of photoreduction of *Chromatium* upon addition of CaD_2 in phosphate buffer (pH 7.6), at 5–6 × 10^4 ergs/cm² sec., at 29 °C, as an average of 3 experiments.

Concentration in 10^{-5} M CaD_2	Rate of photoreduction	
	mm³/hr.	Per cent inhibition
0	26.0	–
2.08	27.5	–6
4.04	25.8	3
6.23	25.0	4
8.42	20.8	20
10.0	9.9	62
12.9	4.7	82

It is clear that the range from the threshold of inhibition to complete inhibition extends from 4.04 × 10^{-5} to 1.29 × 10^{-4}M CaD_2. Contrary to the results with *Chlorella*, inhibition in *Chromatium* was found to be constant within 20 minutes, and it remained constant for at least 3 hours. The concentration at which inhibition of photoreduction occurs is much higher than that for inhibition of photosynthesis in *Chlorella*.

§ 5. EXPERIMENTS WITH *Rhodospirillum rubrum, Oscillatoria species, Nostoc species, Chlamydomonas*

Rhodospirillum rubrum

Growth experiments with this species were performed in a NaCl-peptone medium, containing 1 % peptone, 0.5 % NaCl, the latter partly in pure, partly in crude form, 0.25 % of each being used. Growth was measured by centrifuging 5 cc suspension in TROMMSDORFF tubes.

No growth at all was observed at CaD_2 concentrations above 4.38 × 10^{-6}M CaD_2. At lower concentrations growth increases with decreasing CaD_2 concentrations and threshold was found somewhere between 0.55 × 10^{-6} and 1.09 × 10^{-6}M CaD_2. Some observations with a microscope revealed that decrease in growth was correlated with decrease in motility of the cells. No motility at all was observed in concentrations higher than 4.38 × 10^{-6}M CaD_2.

Oscillatoria species

Growth experiments with *Oscillatoria* were performed in a nutrient solution containing 0.1 % $Ca(NO_3)_2$, 0.02 % K_2HPO_4, 0.02 % $MgSO_4$, 6 × 10^{-4} % $FeSO_4$.

The inoculum was introduced by means of a platinum wire and usually sank to the bottom of the erlenmeyer flask. Growth occurred as long filaments at the surface of the culture medium, rapidly covering the whole area. It may be mentioned here that some calcium containing precipitate always occurred in the erlenmeyer flasks, in the control as well as in the flasks with different CaD_2 concentrations. Only after this precipitate has been dissolved by the growing culture, a vigorous growth at the surface was observed.

Growth was estimated in four degrees, *viz.* the same rate as the control, moderate growth, slow growth and total inhibition of growth. Experiments were performed with 6 concentrations of CaD_2, *viz.* 0, 0.55, 1.09, 2.19, 3.28,

4.38 × 10^{-6}M CaD_2. From these estimations the threshold of inhibition, 50 % inhibition, and complete inhibition could be determined with a reasonable accuracy. Inhibition began to be noticeable at 1.09 × 10^{-6}M and was complete at 3.28 × 10^{-6}M CaD_2. *Oscillatoria* was reddish brown to red with deformed cells in 3.28 and 4.38 × 10^{-6}M CaD_2.

Nostoc species

The same nutrient solution was used as for *Oscillatoria* species, except that 0.05 % $Ca(NO_3)_2$ was applied instead of 0.1 % $Ca(NO_3)_2$. Inhibition of growth again occurred between 1.09 and 3.28 × 10^{-6}M CaD_2.

Chlamydomonas

In these experiments a WARBURG medium was used as nutrient solution, the composition of which has been described in Chapter II. Inhibition occurred also here between 1.09 and 3.28 × 10^{-6}M CaD_2.

§ 6. DISCUSSION

A survey of inhibition of growth, respiration and photosynthesis by CaD_2 in various plant types as compared with *Chlorella* is represented in table XVI. It is clear that the inhibition of photosynthesis and that of growth show similar features. However, usually a somewhat stronger inhibition of growth was observed, which may be due to the long time of exposure to CaD_2 (4–8 days). Photosynthesis was usually measured in short time experiments, within one day (1–3 hours after addition). The inhibition range extends roughly between 10^{-6} and 10^{-5}M(~ 1–10 mg. CaD_2/l.) for all the species. *Lemna minor*, *Chromatium* and *Chlorella* in heterotrophic growth, are somewhat less sensitive. Photoreduction is far less inhibited than photosynthesis, while no inhibition at all was observed for endogenous respiration. The latter was stimulated up to 60 % even at 10^{-3}M CaD_2.

Growth experiments in sand-water cultures with maize and tomatoes by SCHRIJFSMA (56) and with barley by VERHAGEN (70) revealed that the threshold of inhibition and especially complete inhibition occur at higher concentrations than in *Chlorella*. According to TAMMES (67) and WEZENBERG (81) CaD_2 adheres strongly to soil particles. The latter reported increasing adsorption in the order: clay soil, sandy soil and peat moss, which increase was almost proportional to the percentage of organic material of the soil. These high adsorption values and the fact that inhibition in higher plants is more or less restricted to the root zone probably explains the smaller inhibition of growth in higher plants.

A few remarks may be made on the inhibition of other aspects of the metabolism of *Lemna minor*. Experiments in potassium deficient cultures (5) revealed that potassium deficiency yields an increase in dry weight/frond. A decrease in dry weight/frond, however, is observed upon addition of CaD_2. The inhibition by CaD_2 therefore is neither due to potassium removal nor acts in the same way as potassium deficiency. Also the low CaD_2 concentration at which inhibition already occurs does not support the idea of potassium removal. Another argument in this direction is given by the fact, that the threshold of KD solubility is roughly at 10^{-3}M, which is far higher than the concentrations at which inhibition occurs.

TABLE XVI. Survey of the inhibition of metabolic processes in various plant species by calcium dipicrylamine (CaD_2). Concentrations in M, giving threshold, 50% inhitition and complete inhibition.

Process studied, and Organism	Threshold[1])	50% Inhibition	Complete inhibition[1])
Photosynthesis at 12.5 °C in *Chlorella*	1.5×10^{-7}	5.1×10^{-7}	2.3×10^{-6}
17.5 °C in *Chlorella*	2.9×10^{-7}	1.2×10^{-6}	4.4×10^{-6}
25 °C in *Chlorella*	1.0×10^{-6}	4.0×10^{-6}	2.2×10^{-5}
Endogenous respiration in *Chlorella*		stimulated up to at least 10^{-3} M	
Glucose-stimulated respiration in *Chlorella*	1.0×10^{-5}	4.2×10^{-4}	no complete inhibition observed.
Autotrophic growth in *Chlorella*	1.0×10^{-6}	4.7×10^{-6}	9.9×10^{-6}
Heterotrophic growth in *Chlorella*	7.0×10^{-6}	1.1×10^{-5}	2.4×10^{-5}
Growth in *Chromatium*	5.0×10^{-6}	1.3×10^{-5}	4.0×10^{-5}
Photoreduction in *Chromatium*	5.4×10^{-5}	9.8×10^{-5}	1.8×10^{-4}
Growth in *Lemna minor*	1.4×10^{-6}	7.5×10^{-6}	1.8×10^{-5}
„ „ *Lemna arrhiza*	1.1×10^{-6}	2.4×10^{-6}	4.7×10^{-6}
„ „ *Rhodospirillum rubrum*	8.2×10^{-7}	1.9×10^{-6}	4.4×10^{-6}
„ „ *Oscillatoria, Nostoc species* and *Chlamydomonas*	1.1×10^{-6}	1.9×10^{-6}	3.8×10^{-6}
„ „ *Scenedesmus*	3.5×10^{-7}	9.9×10^{-7}	2.3×10^{-6}

[1]) Threshold and complete inhibition are approximations; the values presented in the table are those to which the curves seem to approach asymptotically.

The most striking feature in *Lemna minor* is the strong inhibition of growth and root formation (fig. 21). In connection herewith it may be mentioned that VERHAGEN (70) also observed the first symptoms of inhibition in the root system, and in barley reported a lack of formation of new side roots, a stunted appearance and a clavate swelling. SCHRIJFSMA (57) observed a swelling also, and a ramification of small maize roots, remaining short. She observed an abnormal anatomy in root slides and a yellow discolouring of cell wall and protoplast with CaD_2. The discolouring comes up to or just beyond the endodermis, therefore the enclosed central cylinder seems to be protected against penetration of CaD_2.

CHAPTER VI

DISCUSSION AND SUMMARY

§ 1. GENERAL DISCUSSION

This investigation is concerned with the effects of calcium dipicrylamine (CaD_2) on metabolic processes in various plant types as compared with the action of other inhibitors.

The threshold of the toxic action on growth and photosynthesis was roughly 10^{-6}M while complete inhibition was reached at 10^{-5}M CaD_2. This shows clearly that CaD_2 is a very powerful inhibitor, as could be expected, since aromatic nitro-compounds, including D, have been used as insecticides for a long time. Lower temperatures still increase considerably the inhibitory effect on photosynthesis in *Chlorella*, so that a certain inhibition was produced at 12.5°C by roughly ten times lower concentrations than those required at 25°C (figure 7a). The better known inhibitors of photosynthesis, both enzyme poisons and narcotics, are far less inhibitory (figures 7b, 7c, 7d, 7e, 7f). With 2,4-dinitrophenol WINTERMANS (83) observed an increase in inhibition of photosynthesis at lower pH values of the medium (coming finally into the same range as CaD_2). A similar effect was observed with sodium azide, which has been ascribed to the increase in the concentration of undissociated molecules (SIMON and BEEVERS [61, 62], WINTERMANS [83]). Our experiments were made in a carbonate bicarbonate buffer (pH 9.2). Under these conditions the values for 50 % inhibition (table VII) which are the most accurately determined ones, are in agreement with those obtained by other authors (68, 71, 83). The inhibitory effects of hydrogen sulfide (NEGELEIN [41]) and of copper sulphate (GREENFIELD [27]) appear to be in the same range as that of CaD_2. We have, so far, not studied these inhibitors.

Aside of *Chlorella* (Chapter III and IV), eight other plant species from various classes have been tested for their behaviour with regard to CaD_2 (Chapter V). The range of inhibition of growth in these species and that of autotrophic growth and photosynthesis in *Chlorella* are roughly the same (10^{-6} to 10^{-5}M). Heterotrophic growth of *Chlorella*, growth of *Chromatium* and of *Lemna minor* show a smaller sensitivity. Moreover, glucose respiration in *Chlorella* (figure 15), photoreduction in *Chromatium* (table XV) and photosynthesis in *Lemna minor* (figure 22) are less inhibited. These facts indicate

that growth generally is somewhat less sensitive than photosynthesis. It may be assumed therefore, that growth of all plant types is inhibited in more or less the same range, though exceptions may be possible under more unfavourable conditions shifting the inhibition to either higher or lower concentrations. In connection with the preceding considerations, the observation of WEZENBERG (81) with sugarbeets in water-cultures, of SCHRIJFSMA (56, 57) with maize and tomatoes and of VERHAGEN (70) with barley in sand-water cultures, may be mentioned. They all indicate more or less the same range of inhibiting concentrations, though complete inhibition occurred only at higher concentrations. In these experiments a strong distortion of the roots was observed (58, 70), which is in agreement with our results with *Lemna minor*, in which the length and the number of the roots were considerably more reduced than growth in general.

It is of some interest to mention here a few field studies regarding effects of CaD_2 on crop plants (6, 64, 81). An important aspect in this case is the high adsorption of CaD_2 in the soil (TAMMES [67], WEZENBERG [81]) which largely depends on the percentage of humus actually present. This fact makes a direct comparability with the results obtained in water-cultures rather difficult. A rough calculation may indicate, that the effects observed in crop yields in the field would occur to much the same extent in water-cultures.

According to TAMMES (66), the total amount of poison required to kill a fish is considerable, though fishes die in waste water contaminated with D already in 10–100 times lower concentrations than *Chlorella*. TAMMES (66) explained this extreme sensitivity by a strong absorption of D even from very low concentrations, which absorption according to this author takes place mainly through the gills. Moulds, on the other hand, are far less sensitive, and no fungistatic effects were observed at CaD_2 concentrations of the order of 10^{-4}M in 14 species (SCHOL–SCHWARZ [55]), except in one species which could stand only 10^{-6}M CaD_2. In 8 species even 6 hours exposures at 10^{-2}M were survived.

The inhibition with 2,4-dinitrophenol, as already mentioned, depends largely on the pH of the medium. Though the same effect was expected with CaD_2, no significant differences in inhibition of photosynthesis on *Chlorella* were observed at different pH in a range from pH 10.4 to 5.9. However, the pK value for CaD_2 is 2.85, whereas that of 2,4-dinitrophenol is 4.0 (WINTERMANS [83]). A decrease in extinction of a solution containing 5.8×10^{-6}M CaD_2, which gives about 75 % inhibition of photosynthesis occurs only below pH 4.0. Another difference with 2,4-dinitrophenol is, that in our experiments with CaD_2 no deviations were observed in the inhibition, using 2 and 5 cc suspension liquid, contrary to the results by WINTERMANS in which in 6 cc a stronger inhibition than in 3 cc was observed. It is probable, therefore, that a still stronger inhibition of photosynthesis with CaD_2 will only occur below pH 4.0. It is not clear at the moment, why a marked difference in inhibition was observed in *Scenedesmus* in WARBURG buffer as compared with tap water. In *Chlorella* a slight but continuous decrease of photosynthesis was observed in the control in WARBURG buffer, confirming PRATT's results, whereas no such decrease occurred in *Scenedesmus* under the same conditions, confirming ÖSTERLIND (44). The latter reported utilisation of HCO^-_3 in *Scenedesmus* photosynthesis, whereas this did not occur in *Chlorella*. It seems likely, therefore, that *Scenedesmus* is more adapted to high pH than *Chlorella*.

The inhibition of photosynthesis with CaD_2 is the same in the light limited

and in the light saturated region, the point of transition remaining the same as in the non-inhibited suspensions and occurring roughly at 3.8×10^4 ergs/cm^2 sec. and 2.5×10^4 ergs/cm^2 sec., at 25°C and 17.5°C respectively. This fact might point to a narcotic action of CaD_2 on photosynthesis. Narcotic poisoning is generally less specific than enzymatic poisoning both with regard to the molecular structure of the poison and the constitution of the catalytic systems affected by it. However, the very low concentration of CaD_2 (10^{-6} to 10^{-5}M) in which inhibition occurs points to specific interactions with definite catalists rather than to an indiscriminate surface blocking mechanism. Photoreduction in *Chromatium*, moreover, is far less inhibited than photosynthesis. Another feature against the assumption of narcotic type of poisoning is the irreversible inhibition of photosynthesis by CaD_2. However, it is possible that in these experiments the exposure to CaD_2 was too long or at too high pH values, which may well lead to irreversible injury. Besides this CaD_2 can hardly be removed (efficiently only with methyl-isobutyl ketone). True narcotization is characterized by reversibility. All together, the above considerations may well rule out narcotic poisoning.

A decrease in fluorescence was observed with CaD_2 in the light saturated as well as in the light limiting range, while no decrease of the CaD_2 inhibition was observed with an increase of the dark period in flashing light. These preliminary data suggest that the inhibition takes place primarily in the photochemical part of photosynthesis. The inhibition of photosynthesis at light saturation, however, implies that a dark system involved in photosynthesis is also affected in roughly the same percentage as that in the photochemical part. It has been mentioned already (Chapter III), that according to WELLER and FRANCK, the inhibition of photosynthesis with hydroxyl amine is the same at different light intensities, while GAFFRON's experiments with hydrogen adapted algae made it clear that hydroxyl amine is an enzyme poison rather than a narcotic. The inhibition with CaD_2 shows similar features, and it may be suggested that CaD_2 acts in the same way as hydroxyl amine. This view might even seem supported by LIPSCHITZ' suggestion (37) that aromatic nitro-compounds in general are reduced via the hydroxyl amine level to less poisonous substances. The reduction itself takes place through a sequence of steps. The decrease in inhibition of photosynthesis with increase in temperature might point to an increased reduction of CaD_2 to non-poisonous substances. With hydroxyl amine itself, however, a similar temperature effect was not found (table V). Regarding the differences in results, obtained with hydroxyl amine in this study, and those described by TAMIYA (68), and WELLER and FRANCK (80), see discussion Ch. III, § 6, p. 29.

We have observed that a considerable time elapsed before inhibition of photosynthesis by CaD_2 reaches a constant level (fig. 8). After washing no decrease in inhibition occurred. From these experiments we concluded to a continuous uptake of CaD_2, while, moreover, the inhibition was irreversible. On the contrary the inhibition of the glucose-stimulated respiration was constant within 20 minutes, and remained so hereafter, while the inhibition was at least partly reversible by washing. As CaD_2 is strongly absorbed (67, 81), washing may not remove the inhibitor completely, especially at the high concentrations which are required for inhibition of the glucose-stimulated respiration. It may be assumed, therefore, that the respiration inhibition, in principle is reversible. The observed time of 20 minutes, moreover, was nearly

the same as that for other inhibitors (ethyl urethane, phenyl urethane, hydroxyl amine) with regard to photosynthesis. From experiments on administration of CaD_2 in different flasks at the same time, but starting illumination at different moments thereafter (fig. 10), it was concluded that the increase in inhibition of photosynthesis by a continued uptake of CaD_2 occurs in light only. This may indicate the inhibition of an enzyme which is activated by light only. The increase in inhibition at lower temperatures may be ascribed to a decrease in the dark deactivation. Before more can be said definitely about this suggestion, exact measurements on CaD_2 uptake under different experimental conditions have to be made. Since inhibition appears already at extremely low concentrations, and the determination of CaD_2 by extraction with methyl-isobutyl ketone was available only later on in the course of this investigation, this fact has not yet been studied in greater detail.

The different aspects observed with CaD_2 compared with other inhibitors on photosynthesis and respiration, may induce us to a further investigation with this inhibitor.

§ 2. SUMMARY

Experiments are described in which the inhibitory effects of calcium dipicrylamine (CaD_2) and other inhibitors have been investigated in *Chlorella* and several other plant species belonging to different classes. The rates of photosynthesis, photoreduction and respiration have been measured with a WARBURG apparatus, and growth has been determined by means of centrifuging cell suspensions in TROMMSDORFF tubes, counting fronds in the case of *Lemna*, or by visual estimation in *Oscillatoria* and some other algae.

CaD_2 proved to be a much more effective inhibitor of photosynthesis in *Chlorella*, than ethyl urethane, phenyl urethane, hydroxyl amine, sodium azide, or 2, 4-dinitrophenol. The range of inhibition from threshold to complete inhibition is roughly between 10^{-6} and 10^{-5} M CaD_2 and, therefore, is more comparable to that reported for hydrogen sulphide and copper sulphate in literature. Inhibition of autotrophic growth in *Chlorella* was comparable to that of photosynthesis, while heterotrophic growth in *Chlorella*, and growth, and especially photoreduction in *Chromatium* are less inhibited. This probably means that autotrophic growth in *Chlorella* is limited by a shortage of assimilates. Contrary to the high sensitivity of growth and photosynthesis, no inhibition, but a stimulation up to 60% was found even at 10^{-2}M CaD_2 in respiration. Glucose-stimulated respiration was 50% inhibited at this concentration. This inhibition was at least partly reversible.

The same inhibition percentage was observed in *Chlorella* photosynthesis under conditions of light saturation as well as under those of light limitation, the inhibition was found to be non-reversible by washing. Photoreduction in *Chromatium* was considerably less sensitive than *Chlorella* photosynthesis. These facts do not support a purely narcotic action of this poison, but rather point to a specific type of inhibition. Some flash light experiments revealed that inhibition was scarcely influenced by longer dark periods. Fluorescence measurements clearly showed the change in yield at low as well as at high light intensities. The generally high acceptance of energy by aromatic nitrocompounds probably explains the decrease observed in yield of fluorescence upon addition of CaD_2.

A strong increase in the inhibition of photosynthesis was observed with CaD_2 at lower temperatures, which did neither occur with ethyl- and phenyl urethane nor with hydroxyl amine. For an explanation one could think of a decrease in the dark deactivation of the CaD_2 sensitive enzyme. Though the time required to obtain equilibrium was considerable, especially in carbonate bicarbonate buffer (1–2 hours), no definite differences in time required for reaching the equilibrium were observed at various temperatures.

The inhibition of growth of *Lemna arrhiza*, *Scenedesmus*, *Nostoc spec.*, *Rhodospirillum rubrum*, *Oscillatoria spec.*, and *Chlamydomonas* by CaD_2 occurred roughly in the same range of concentrations as that of autotrophic growth in *Chlorella*. *Chromatium* and *Lemna minor* are somewhat less sensitive. The roots of the latter, however, are a very sensitive indicator of CaD_2.

Though CaD_2 has a marked influence on growth and photosynthesis of species in water cultures, CaD_2 is much less harmful under field conditions owing to the high adsorption capacity of the soil. This has been confirmed in field experiments. Only at very high artificial increase of the contamination a decrease in yield was observed.

§ 3. SAMENVATTING

De werking van calcium dipicrylamine op planten is van belang in verband met de mogelijkheid van toepassing van deze stof bij bereiding van kaliumhoudende meststoffen.

De remming van verschillende stofwisselingsprocessen door calcium dipicrylamine (CaD_2) en andere remstoffen werd nagegaan bij *Chlorella*, *Lemna minor*, *Lemna gibba*, *Lemna arrhiza*, *Chromatium*, *Scenedesmus*, *Nostoc spec.*, *Rhodospirillum rubrum*, *Oscillatoria spec.*, en *Chlamydomonas*. De photosynthese snelheid, de photoreductie en de ademhaling werden gemeten in een WARBURG apparaat, de groeisnelheid door centrifugeren van cel suspensies in TROMMSDORFF buisjes, door telling van het aantal schijven (*Lemna*), of door een schatting van de toename (*Oscillatoria*, *Nostoc*).

CaD_2 remt de photosynthese reeds in zeer lage concentraties (10^{-6}–10^{-5}M, fig. 7a). Ethyl urethaan (fig. 7b), phenyl urethaan (fig. 7c), hydroxyl amine (fig. 7d), 2,4-dinitrophenol (fig. 7e) en natrium azide (fig. 7f) daarentegen zijn pas in hogere concentraties werkzaam.

De autotrophe groei van *Chlorella*, *Lemna arrhiza*, *Scenedesmus*, *Nostoc spec.*, *Rhodospirillum rubr.*, *Oscillatoria* en *Chlamydomonas* wordt min of meer bij dezelfde CaD_2 concentraties geremd als de photosynthese, tabel XVI geeft hiervan een overzicht. Overeenkomstige resultaten werden verkregen met bieten in water-cultures (81), mais, tomaten en gerst (57, 70) in zand-cultures. Vooral de wortelgroei was sterk geremd (58,70). In *Lemna minor* werd de wortelgroei volledig geremd bij CaD_2 concentraties, waarin andere processen nog nagenoeg geen vergiftigingssymptomen te zien gaven. De heterotrophe groei van *Chlorella* en *Chromatium*, maar vooral de photoreductie van *Chromatium* zijn minder gevoelig. In tegenstelling hiermee werd een stimulatie van de ademhaling waargenomen, welke zelfs bij 10^{-2}M CaD_2 nog 60 % bedroeg.

Bij de glucose-ademhaling werd bij deze concentratie een remming van 50 % waargenomen, welke in tegenstelling tot die van de photosynthese gedeeltelijk reversibel is na uitwassen. Over het algemeen werd, met uitzondering van CaD_2, reeds 20 minuten na toevoeging van de remstof een constante remming van de

photosynthese waargenomen. Afhankelijk van de concentratie nam de photosynthese remming met CaD_2 in de tijd toe. Deze toename werd verklaard door een continue opname van CaD_2, welke alleen in het licht plaatst vindt (fig. 10). De resultaten van de glucose ademhaling, waarin ook na ongeveer 20 minuten een constante waarde van de remming werd bereikt, zijn hiermee in overeenstemming.

Zowel bij lichtlimitering als bij lichtverzadiging van de photosynthese werden overeenkomstige remmingspercentages waargenomen. Hoewel deze waarneming een aanwijzing is voor een narcotische werking van deze remstof, is aan te nemen, dat CaD_2 een specifiek enzym vergif is, dat behalve bepaalde donkerreacties ook de energieoverdracht bij de photosynthese aangrijpt, de voorlopige chlorophylfluorescentie en flitslicht proeven wijzen ook in deze richting. Bij de laatste werd geen vermindering van de remming waargenomen bij een langere donkerperiode, terwijl de fluorescentie zowel bij lichtverzadiging als bij lichtlimitering verlaagd werd.

Bij lagere temperaturen werd een sterke toename van de photosynthese remming met CaD_2 in *Chlorella* waargenomen. Dit effect van de temperatuur werd niet bij phenyl- en ethyl urethaan en bij hydroxyl amine geconstateerd en is vooralsnog niet geheel te verklaren.

Hoewel, zoals reeds eerder vermeld, de groei en de photosynthese reeds bij zeer lage concentraties geremd worden is een opbrengstvermindering in het veld bij kalibemesting met deze stof als verontreiniging voorshands niet te verwachten door enerzijds de hoge adsorptie van CaD_2 in de grond, speciaal aan humus, en anderzijds de geringe verontreiniging van het gehele veld.

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REFERENCES

1. ARNON, D. J., Microelements in culture-solution experiments with higher plants. Am. J. Bot. **25**, 322–325 (1938).
2. ASHBY, E. T. and A. OXLEY, The interaction of factors in the growth of *Lemna*. VI. An analysis of the influence of light intensity and temperature on the assimilation rate and the rate of frond multiplication. Ann. Bot. **49**, 309–337 (1935).
3. AUDUS, L. J., Studies on the pH relationships of root growth and its inhibition by 2,4-dichlorophenoxy acetic acid and coumarin. New Phytol. **48**, 97–114 (1949).
4. BEEVERS, H., 2,4-Dinitrophenol and Plant respiration. Am. J. Bot. **40**, 91–97 (1953).
5. BIERHUIZEN, J. F., Observations on potassium deficiency in *Lemna minor L.* Med. Landb. Hogesch. Wageningen/Ned. **54** (7), 311–319 (1954).
6. BIERHUIZEN, J. F. and H. J. WEZENBERG, Report to Mekog, IJmuiden.
7. BLACKMAN, G. E., Studies in the principles of phytotoxicity. I. The assessment of relative toxicity. J. Expt. Bot. **3**, 1–27 (1951).
8. BRADBURRY, D. and W. B. ENNIS, Jr., Stomatal closure in kidney bean plants treated with ammonium 2,4-dichlorophenoxyacetate. Am. J. Bot. **39**, 324–329 (1952).
9. BROWN, A. W. A., Insect control by chemicals. Edited John Wiley and Son, New York., 817 p. (1951).

10. BROWN, H. T. and F. ESCOMBE, Researches on some of the physiological processes of green leaves with special reference to the interchange of energy between the leaf and its surroundings. Proc. Roy. Soc. London series B, **76,** 29–112 (1905).
11. BROWN, J. W., Effect of 2,4-dichlorophenoxyacetic acid on the water relations, the accumulation and distribution of solid matter, and the respiration of bean plants. Bot. Gaz. **107,** 332–343 (1946).
12. BROWN, J. W. and R. L. WEINTRAUB, A leaf repression method for evaluation of formative activity of plant growth regulating chemicals. Bot. Gaz. **111,** 448–456 (1950).
13. EMERSON, R. and W. ARNOLD, A separation of the reactions in photosynthesis by means of intermittent light. J. Gen. Phys. **15,** 391–420 (1932).
14. EYMERS, J. G. and E. C. WASSINK, On the photochemical carbon dioxide assimilation in purple sulphur bacteria. Enzymol. **2,** 258–304 (1938).
15. FEIGL, F., Qualitative analysis by spot tests. Edited Elsevier Amsterdam, 462 p. (1939).
16. FRANCK, J., The Relation of the Fluorescence of Chlorophyll to Photosynthesis. In Photosynthesis in plants. 293–349 (1949).
17. FRANCK, J., C. S. FRENCH and T. T. PUCK, The fluorescence of chlorophyll and photosynthesis. J. Phys. Chem. **45,** 1268–1301 (1941).
18. FRANCK, J. and K. F. HERZFELD, Contribution to a theory of photosynthesis. J. Phys. Chem. **45,** 978–1025 (1941).
19. FREELAND, R. O., Effects of 2,4-D. and other growth substances on photosynthesis and respiration in *Anacharis*. Bot. Gaz. **111** (3) 311–324 (1950).
20. FREUNDLICH, H., Über die Adsorption in Lösungen. Zs. f. physikal. Chem. **57,** 385–471 (1907).
21. GAFFRON, H., Über auffallende Unterschiede in der Physiologie nahe verwandter Algenstämme, nebst Bemerkungen über Lichtatmung. Biol. Zentr. **59,** 302–314 (1939).
22. GAFFRON, H., Über die Kohlensäure-assimilation der roten Schwefelbakterien. Bioch. Zs. **269,** 447–453 (1934).
23. GAFFRON, H., The effect of specific poisons upon the photoreduction with hydrogen in green algae. J. Gen. Phys. **26,** 195–219 (1943).
24. GAVAUDAN, P., H. POUSSEL and M. DODÉ, Comparaison du pouvoir mito-inhibiteur des substances de la série aromatique en function de l'activité thermodynamique de leurs solutions. C.R. d.l. Soc. Biol. **138,** 267–268 (1944).
25. GENDEREN, H. VAN, Toxicity of crops grown on KD treated soil. I. Feeding experiments with rabbits and pigs. Scient. Report C 89, Mekog, IJmuiden (1952).
26. GORHAM, P. R., Growth factor studies with *Spirodella polyrrhiza (L) Schleid.* Am. J. Bot. **32,** 496–506 (1945).
27. GREENFIELD, S. S., Inhibitory effects of inorganic compounds on photosynthesis in *Chlorella*. Am. J. Bot. **29,** 121–131 (1942).
28. HEUBNER, W., Giftung aromatischer Nitroverbindungen. Arch. f. Expt. Path. u. Pharm. **205,** 310–311 (1947).
29. HILLE, J. C. VAN, The quantitative relation between rate of photosynthesis and chlorophyll content in *Chlorella pyrenoidosa*. Rec. Trav. bot. Neerl., **35,** 680–758 (1938).
30. HOLZER, H., Photosynthese und Atmungskettenphosphorylierung. Zs. Naturforsch. **6b,** 424–430 (1951).
31. JOHNSON, M. J., Manometric technics and related methods for the study of tissue metabolism by UMBREIT, W. W., R. H. BURRIS and J. F. STAUFFER. Minneapolis Burgess Publ. Co. 1951.
32. KAUTSKY, H. and A. HIRSCH, Chlorophyllfluoreszenz und Kohlensäure-assimilation. Das Fluoreszenzverhalten grüner Pflanzen. Bioch. Zs. **274,** 423–435 (1934).
33. KAUTSKY, H. and H. SPOHN, 1. Apparatur zur vergleichende Messung der Fluoreszenzänderungen lebender Blätter. 2. Der Einflusz der Temperatur auf die Fluoreszenzkurve. Bioch. Zs. **274,** 435–452 (1934).
34. KELLY, S. and G. S. AVERY, The effect of 2,4-dichlorophenoxyacetic acid and other physiologically active substances on respiration. Am. J. Bot. **36,** 421–426 (1949).
35. KOK, B., Some sensitive and recording volumeters. Bioch. Biophys. Acta, **16,** 35–44 (1955).
36. LINDEMAN, W., Over de betekenis van phosphaat in de photosynthese van *Lemna minor L.* Diss. A'dam, 1–83 (1952).
37. LIPSCHITZ, W. L., Giftung und Entgiftung aromatischer Nitroverbindungen. Arch. f. Expt. Path. u. Pharm. **205,** 305–311 (1948).
38. NAKAMURA, H., Über die Kohlensäure-assimilation bei niederen Algen in Anwesenheit des Schwefelwasserstoffs. Acta Phytochim. Japan, **10,** 271–282 (1938).

39. NAKAMURA, H., Über den Einflusz der Blausäure auf die Photosynthese von *Scenedesmus*. Acta Phytochim. Japan, **10**, 313–317 (1938).
40. NANCE, J. F., Inhibition of salt accumulation in excised wheat roots by 2,4-dichlorophenoxyacetic acid. Science, **109**, 174–176 (1949).
41. NEGELEIN, E., Über die Wirkung des Schwefelwasserstoffs auf chemische Vorgänge in Zellen. Bioch. Zs. **165**, 203–214 (1925).
42. NIEL, C. B. VAN, and E. M. MULLER, On the purple bacteria and their significance for the study of photosynthesis. Rec. Trav. Bot. **28**, 245–274 (1931).
43. ORANJE, P. J., Gasentladungslampen. Philips Technische Bibliotheek, 294 p. (1943).
44. ÖSTERLIND, S., Inorganic carbon sources of green algae. III. Measurements of photosynthesis in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. Phys. Plantarum **4** (2), 242–254 (1951).
45. PAAUW, F. VAN DER, The indirect action of external factors on photosynthesis. Rec. trav. bot. neerl. **29**, 497–620 (1932).
46. PIRSON, A., and F. SEIDEL, Zell und stoffwechselphysiologische Untersuchungen an der Wurzel von *Lemna minor L.* unter besonderer Berücksichtigung von Kalium und Kalziummangel. Planta **38** (4), 431–473 (1950).
47. PIRSON, A., C. TICHY and G. WILHELMI, Stoffwechsel und Mineral Salzernährung einzelliger Grünalgen. I. Vergleichende Untersuchungen an Mangelkulturen von *Ankistrodesmus*. Planta **40**, 199–253 (1952).
48. POTTER, C. and E. M. GILLHAM, Effects of atmospheric environment, before and after treatment on the toxicity to insects of contact poisons. Ann. Appl. Biol. **33**, 142–160 (1946).
49. PRATT, R., Studies on *Chlorella vulgaris*. VI. Retardation of photosynthesis by a growth inhibiting substance from *Chlorella vulgaris*. Am. J. Bot. **30**, 32–33 (1943).
50. PRATT, R., Studies on *Chlorella vulgaris*. VII. Influence of the age of the culture on the rates of photosynthesis and respiration. Am. J. Bot. **30**, 404–408 (1943).
51. PRATT, R., Studies on *Chlorella vulgaris*. VIII. Influence on photosynthesis of prolonged exposure to sodium bicarbonate and potassium bicarbonate. Am. J. Bot. **30**, 626–630 (1943).
52. RABINOWITCH, E. I., Photosynthesis and related processes. Vol. I. Interscience Publ. Inc. New York, 559 p. 1945.
53. RABINOWITCH, E. I., Photosynthesis and related processes. Vol. II. part 1, Interscience Publ. Inc. New York, p. 600–1208, 1951.
54. ROELOFSEN, P. A., On photosynthesis of the Thiorhodaceae. Diss. Utrecht, 1935.
55. SCHOL-SCHWARZ, M. B., Toxicity of dipicrylamine for soil organisms. Scient. Report C 87, Mekog, IJmuiden (1952).
56. SCHRIJFSMA, A. S. C., Toxicity of dipicrylamine for crops. Report 2, Toxic concentration of CaD_2 and poisoning symptoms for maize and tomato. Scient. Report C 53, Mekog, IJmuiden (1952).
57. SCHRIJFSMA, A. S. C., Toxicity of dipicrylamine for crops. Report 3, Anatomic abnormalities in poisoned maize roots. Scient. Report C 54, Mekog, IJmuiden (1952).
58. SEEL, H., Beiträge zur Toxologie einiger Schädlingsbekampfungsmittel aus der Gruppe aromatischer Nitroverbindungen. Arch. expt. Path. u. Pharmacol. **207**, 625–655 (1949).
59. SEIFERT, F., Bestimmung von Trinitrotoluol und Hexanitrodiphenylamin neben einander in Wasser und Abwasser. Wasser **17**, 89 (1949).
60. SHIBATA, K. and E. YAKUSHIJI, Der Reaktions Mechanismus der Photosynthese. Naturwissensch. **21**, 267–268 (1933).
61. SIMON, E. W. and H. BEEVERS, The effect of pH on the biological activities of weak acids and bases. 1. The most usual relationship between pH and activity. New Phytol. **51**, 163–190 (1952).
62. SIMON, E. W. and H. BEEVERS, The effect of pH on the biological activities of weak acids and bases. 2. Other relationships between pH and activity. New Phytol. **51**, 191–198 (1952).
63. SMITH, F. G., C. L. HAMNER and R. F. CARLSON, Changes in food reserves and respiratory capacity of bind weed tissues accompanying herbicidal action of 2,4-dichlorophenoxyacetic acid. Plant Physiol. **22**, 58–66 (1957).
64. SPIERINGS, F., in preparation. Report to Mekog, IJmuiden.
65. SPRUIT, C. J. P. and E. C. WASSINK, The simultaneous observation of oxydation-reduction potentials and chlorophyll fluorescence of *Chlorella* suspensions. Bioch. et Biophys. Acta **15**, 357–366 (1954).
66. TAMMES, P. M. L., Observations on the effect of temperature on the balance between entry of a poison and the process of detoxification. Scient. Report, Mekog, IJmuiden.

67. TAMMES, P. M. L., Report to Mekog, IJmuiden. Toxicity of waste water of the DK-process: I Effects of dipicrylamine on yeast. Scient. Report C 102 (1951).
68. TAMIYA, H., Analysis of photosynthetic mechanism by the method of intermittent illumination. Studies from the Tokugawa Inst. VI. 2, 1-132 (1949).
69. TRAUBE, J., Theorie der Narkose. Pflüg. Arch. f. Phys. **153**, 276–309 (1913).
70. VERHAGEN, H. J., Toxicity of dipicrylamine for crops. Report 6, Toxic concentration of CaD_3 and toxicity symptoms for barley. Scient. Report C 99, Mekog IJmuiden (1952).
71. WARBURG, O., Über die katalytischen Wirkungen der lebendigen Substanz. (1928).
72. WARBURG, O., Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. Bioch. Zs. **100**, 230–271 (1919) (I); Bioch. Zs. **103**, 188–218 (1920) (II).
73. WARBURG, O. and E. NEGELEIN, Über den Energieumsatz bei der Kohlensäure-assimilation. Zs. Phys. Chem. **102**, 235–267 (1922).
74. WASSINK, E. C., On the ratio between the uptake of carbon dioxide and of the hydrogen donor in purple sulphur bacteria. Enzymol. **10**, 257–268 (1942).
75. WASSINK, E. C. and E. KATZ, The initial changes of chlorophyll-fluorescence in *Chlorella*. Enzymol. **6**, 145–173 (1939).
76. WASSINK, E. C., E. KATZ and R. DORRESTEIN, On photosynthesis and fluorescence of bacteriochlorophyll in Thiorhodaceae. Enzymol. **10**, 285–354 (1942).
77. WASSINK, E. C. and J. A. H. KERSTEN, Observations sur la photosynthèse et la fluorescence chlorophyllienne des diatomées. Enzymol. **11**, 282–312 (1943/45).
78. WASSINK, E. C., D. VERMEULEN, G. H. REMAN and E. KATZ, On the relation between fluorescence and assimilation in photosynthezing cells. Enzymol. **5**, 100–110 (1938).
79. WASSINK, E. C. and H. J. WEZENBERG, On the behaviour of CaD_3 in the soil with some observations concerning the weed flora and some remarks on the microbiology of the soil. Scient. Report, Mekog, IJmuiden (1953).
80. WELLER, S. and J. FRANCK, Photosynthesis in flashing light. J. Phys. Chem. **45**, 1359–1374 (1941).
81. WEZENBERG, H. J., Toxicity of dipicrylamine for crops. Report 4, Field experiments with HD contaminated fertilizer. Scient. Report C 80, Mekog, IJmuiden (1952).
82. WHITE, H. L., The interaction of factors in the growth of *Lemna*. XIV. The interaction of potassium and light intensity in relation to growth and assimilation. Ann. Bot. N.S. **3**, 619–649 (1939).
83. WINTERMANS, J. F. G. M., Polyphosphate formation in *Chlorella* in relation to photosynthesis. Med. Landb. Hogesch. Wageningen/Ned. **55** (2), 69–126 (1955).

ERRATA

p. 27 Legend to figure 13, for: $NH_4OH.HCl$, read: $NH_2OH.HCl$

p. 46 In table XIII, last column, for: 70, read: 80

p. 54 14th line from below, for: $10^{-2}M\ CaD_2$, read: $10^{-3}M\ CaD_2$

p. 55 5e regel van onder, $10^{-2}M\ CaD_2$, moet zijn: $10^{-3}M\ CaD_2$

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