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ACTION OF SOME HERBICIDES IN PHOTOSYNTHESIS OF *SCENEDESMUS*, AS STUDIED BY THEIR EFFECTS ON OXYGEN EVOLUTION AND CYCLIC PHOTOPHOSPHORYLATION

J. J. S. VAN RENSEN

Laboratory of Plant Physiological Research,
Agricultural University, Wageningen, The Netherlands,
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I. GENERAL INTRODUCTION

I.1. Introductory remarks

Apart from their application in agricultural and horticultural practice, herbicides may claim a considerable interest of plant physiologists.

Besides the fact that the practical effects have their foundation in physiological reactions of the plant, this same property may be used in physiological research in attempts to further elucidate the mechanism of the underlying physiological processes. Conversely, it can be remarked that a further understanding of the physiological effects may have implications for the use of these substances, and their chemical relatives, in practice. The use of herbicides in physiological research is the more tempting since it has been known for a long time that several of these substances exert these effects in remarkably low concentrations, suggesting that these effects may be rather specific.

Among the various physiological processes that may be affected by poisons, photosynthesis deserves our attention in several respects. First of all, it is the main energy producing process, and secondly, general experience has taught that it shows an intermediate degree of sensitivity against many currently used poisons.

From this viewpoint, we have attempted to study the effects of some herbicides on the mechanism of photosynthesis, and on partial reactions of this process (e.g. photophosphorylation) in the hope that a contribution might be given to the understanding of the action mechanism of these herbicides on the modes of electron transport within the photosynthetic process.

I.2. Review of literature

I.2.1. *Some aspects of the primary reactions of photosynthesis*

In order to facilitate the understanding of the subsequent discussion of results obtained, it appears appropriate to present briefly current views about the mechanism of photosynthesis.

Photosynthesis can be defined as a light-mediated electron flow from water to carbon dioxide, ultimately leading to oxidation of water to oxygen, and reduction of carbon dioxide to carbohydrate. Light energy absorbed in chlorophyllous and related pigments, organized in lamellar systems in the plant cells is the primary driving force of this electron flow. Indications are available that this electron flow is accompanied by phosphorylations, leading to the formation of ATP. The energy of this ATP is partly reused in dark steps of the process e.g., in the carbon dioxide reduction chain.

Earlier and more recent research has revealed many detailed features of the electron flow which takes part stepwise, and has led to the characterization of several compounds involved.

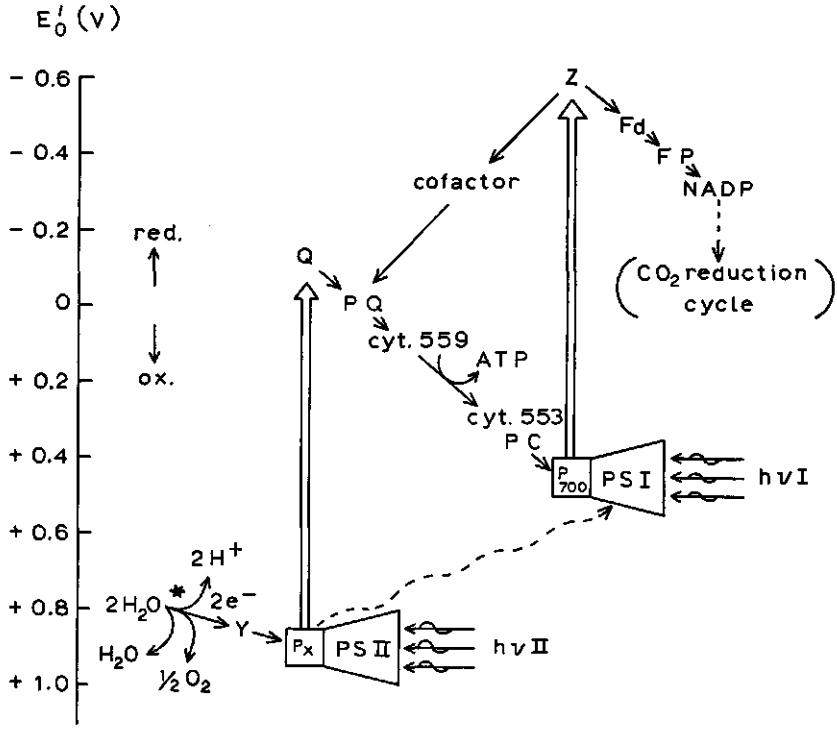


FIG. 1. Scheme for the primary reactions in photosynthesis.
* This represents a condensation of the following suggested reaction scheme for the photolysis of water:

$$2\,H_2O \rightleftharpoons 2\,OH^- + 2\,H^+ \quad (1)$$

$$2\,OH^- \xrightarrow{2h\nu} 2\,OH + 2e^- \quad (2)$$

$$2\,OH \longrightarrow H_2O + O \quad (3)$$

$$\text{sum: } 2\,H_2O \xrightarrow{2h\nu} 2\,H^+ + \tfrac{1}{2}O_2 + 2e^- + H_2O \quad (4)$$

One of the major discoveries in recent years has been that, most probably, at least two directly light driven steps are involved, owing to two quantum absorption in two different pigment systems.

Figure 1 shows a current scheme for electron (or hydrogen) transport in photosynthesis, demonstrating that two photochemical reaction systems act in series in photosynthesis. This concept was introduced by HILL and BENDALL (1960) and developed by DUYSENS *et al.* (1961), WITT *et al.* (1961), and KOK and HOCH (1961). This theory is apt to explain the second EMERSON effect (EMERSON, 1958) and a number of light-induced absorption changes of oxidation-reduction components in the electron transport chain (DUYSENS *et al.*, 1961).

The two photochemical reaction systems mentioned are connected with two different photosynthesis pigment systems, originally characterized by DUYSENS as System I and System II.

System I (PS I) contains most of the chlorophyll *a*, and transfers the absorbed light energy to the photochemical reaction centre I, currently denoted as P_{700} System II (PS II) contains a small portion of the chlorophyll *a*, and moreover, phycobilins, chlorophyll *b*, and carotenoids. It transfers the absorbed light energy to the photochemical reaction centre II, denoted as P_x.

These photochemical reaction centres P_{700} and P_x are assumed to become excited by the energy transferred to them and thus become able to transfer electrons to subsequent chemical steps of the reaction chain. Thus, P_x is supposed to reduce a compound Q, and, consequently, oxidize another compound Y which is supposed to play a role in a partial reaction system which ultimately serves the oxidation of water and is responsible for the photosynthetic oxygen production. Recent evidence suggests that Cl^- and Mn^{2+}-ions are active in this part of the reaction chain (IZAWA *et al.*, 1969, respectively CHENIAE and MARTIN, 1970).

Evidence suggests, moreover, that the reduced compound Q transfers electrons spontaneously along a chain in which several cytochromes and some other compounds are taken up. This transfer includes, e.g., production of ATP and further losses of potential energy along the chain. Consequently, the reduction capacity, or, probably, more correctly, the redox potential of the subsequent substances along the chain decreases, and reaches a level, insufficient for the reduction of carbon dioxide.

At this point, the action of P_{700} comes into play, and excitation of this compound by uptake of light energy from PS I, transfers electrons from the mentioned electron transfer chain to a compound Z, which thus acquires a redox potential, sufficient to reduce ferredoxin (Fd) and NADP, which latter substance then acts as the primary reductant for the CO_2-reduction cycle.

This position of Z enables the occurrence of another important electron transfer chain, viz., from Z probably via a 'cofactor' back to PQ or a neighbouring compound, and further along the already mentioned electron transfer chain to PC (with ATP formation included), and via renewed input of excitation energy from P_{700} back to Z. The ATP formation connected with this electron transfer has become known as 'cyclic photophosphorylation', (ARNON *et al.*, 1958); some of its properties will be discussed further in this paper.

Having thus outlined the main trends of electron transfer known to-day in the photosynthetic process, we will now deal further with a number of specific properties and discoveries regarding the various compounds involved. These properties will in part elucidate the evidence for the localization and mode of action of the various compounds at different places in the electron transfer chain.

P_{700}, the photochemical reaction centre I, is a chlorophyll *a* type with an absorption maximum at 700 nm, and (active at) a redox potential of + 0.43 V (KOK, 1961). The nature of the reaction centre of system II is so far unknown; DÖRING *et al.* (1967 and 1969) obtained indications that P_x might be a chlorophyll *a* type with an absorption maximum at 682 nm. For several reasons one has to assume that its E_o'-value is definity lower than that of P_{700}. Y is a cofactor, postulated by WITT *et al.* (1963) as an electron donor for P_x. Q is assumed to

be the first substance accepting energy from P_x, and thus acting as the quencher of fluorescence, postulated by DUYSENS (1963). According to MALKIN and KOK (1966) this factor still includes two components, which they call Q and P. Possibly, one of these is plastoquinone (PQ). The next substance along the energy transfer chain is assumed to be cytochrome 559 which has some of the properties of cytochrome b_3 and is supposed to transfer electrons to cytochrome 553 (LEVINE, 1969).

Cytochrome 553 is a *c*-type cytochrome, often called cytochrome *f*; its redox potential is + 0.37 V. Plastocyanin (PC) is a copper protein, first isolated by KATOH (1960); its redox potential is + 0.39 V. It was not clear, whether plastocyanin or cytochrome 553 is the electron donor for P_{700}; according to WESSELS (1966) and GORMAN and LEVINE (1966) plastocyanin is the one that serves as the electron donor for P_{700}. It may be mentioned in addition that the insoluble and tightly bound cytochrome 563 (= cytochrome b_6) is thought to play a role in cyclic electron transport (LEVINE, 1969 and HIND and OLSON, 1967). KOK *et al.* (1965), from studies on the photoreduction of viologen dyes having E_o' values between – 0.32 and – 0.74 V, concluded that the normal potential of the strong reductant Z, generated in the long-wave photoact of photosynthesis, is as low as or lower than – 0.7 V. WITT *et al.* (1963) already reported on an electron acceptor of light reaction I, also called Z, the redox potential of which should be below – 0.44 V. Reduction of NADP by ferredoxin is catalyzed by a flavoprotein enzym (FP), viz. ferredoxin-NADP reductase (E.C. 1.6.99.4).

The electron transport from water, via P_x, Q, the electron carriers, P_{700}, Z, Fd, to NADP is called *non-cyclic electron flow*. This electron flow ultimately produces oxygen, reduced NADP, and ATP. This ATP-production is called *non-cyclic photophosphorylation* (ARNON, 1959). Under certain conditions, to be specified later on, an electron flow occurs, called *cyclic electron flow*, accompanied by '*cyclic*' *photophosphorylation* (ARNON *et al.*, 1958) in which electrons move from P_{700} to Z, PQ (or possibly Q or cytochrome 559) and the other electron carriers, back to P_{700}. This electron flow produces no oxygen and no NADP-reduction. The only product one can measure is ATP. In chloroplasts, cyclic photophosphorylation only occurs after addition of a cofactor, e.g. PMS or vitamin K. It is possible to distinguish between cyclic and non-cyclic photophosphorylation, e.g. by irradiation with light of wavelengths beyond 700 nm. In this wavelength region only pigment system I absorbs light energy. Still another type of electron flow is the *pseudo-cyclic electron flow*, coupled to *pseudo-cyclic photophosphorylation* (ARNON *et al.*, 1961, 1964). This occurs when reduced ferredoxin does not reduce NADP, but is oxidized by oxygen. This pseudo-cyclic photophosphorylation looks like the cyclic one, because no oxygen is set free and no NADP is reduced. However, the electron flow corresponds more to the non-cyclic one, because the electrons are transported from water, via the electron carriers and ferredoxin, to oxygen.

The non-cyclic electron transport, which generates O_2, ATP, and reduced NADP, appears to be the main pathway of photosynthetic energy conversion, as long CO_2-assimilation proceeds. The fixation of each CO_2-molecule requires

2 reduced NADP and at least 3 ATP. The ATP needed for CO_2 reduction in excess of the amount supplied by non-cyclic photophosphorylation, can be provided by cyclic photophosphorylation (ARNON, 1965). ARNON also suggested that the regulatory mechanism in chloroplasts to switch from non-cyclic to cyclic electron transport is the availability of oxidized NADP which accepts electrons from ferredoxin. When all NADP is reduced, a cyclic electron flow, with coupled phosphorylation, will ensue. In general, the condition for this situation is shortage of CO_2. The electron transfer is of the pseudo-cyclic type when the electron carriers are strongly shifted to the reduced side and oxygen is present. The conditions for this mechanism occur especially at high light intensities.

Another important question is whether the possibility exists of energy transfer between the pigments from PS II to those of PS I. In 1963, MYERS and GRAHAM postulated two alternative models for energy distribution. In the first model, they assumed that the two pigment systems are so arranged, perhaps spatially, that transfer is exclusively within each pigment system to its specific reaction centre. They have called this possibility the *separate package model.* They have also imagined a second possibility, viz., the *spillover model.* In this they envisage that the energy of quanta absorbed by pigment system II is transferred preferentially to reaction centre II, but may also be transferred to reaction centre I, if the transfer capacity of centre II is constantly occupied. The results of their experiments did not allow them to discriminate between both models. MURATA *et al.* (1966) and KOK and RURAINSKY (1966) provided evidence for the correctness of the spillover model, based on results of fluorescence experiments. More recently, JOLIOT *et al.* (1968) demonstrated absence of photon transfer from PS II to PS I; WILLIAMS (1968) and WILLIAMS *et al.* (1969) also rejected the spillover model. However, MURATA (1969) found spillover of excitation energy from PS II to PS I which was suppressed by Mg^{2+}.

Further information about possible pathways of photosynthesis can be found in recent reviews by BOARDMAN (1968), FORK and AMESZ (1969) with a discussion of the spillover model, and LEVINE (1969).

I.2.2. *Polyphosphate formation in relation to photosynthesis*

Inorganic polyphosphates (poly P) occur in yeasts, green plant cells, fungi, and many bacteria (WIAME, 1947, and WINTERMANS, 1954; for reviews, see e.g., KUHL, 1960, and HUENNEKENS and WHITELEY, 1960). They have never been found in animals, except in a few insects (SCHMIDT, 1951; HUENNEKENS and WHITELEY, 1960). Metaphosphates and polyphosphates are distinguished in that metaphosphates are the low molecular weight phosphates, including cyclic tri- and tetrametaphosphate. Polyphosphates are the high molecular weight polymers, consisting of either linear or cyclic units, or are mixed polymers. Extraction with trichloroacetic acid yields two fractions: the soluble and the insoluble poly P fractions. The poly P of the insoluble fraction can be hydrolysed by heating to 100 °C in 1N HCl during 7 minutes, this fraction is called Δ 7 min. P. The chain of the insoluble poly P is longer than that of the soluble poly P, and correspondingly their molecular weight is higher.

Biologically, the insoluble poly P fraction is the most important one, because this fraction is in enzymatic equilibrium with ATP. With a purified enzyme (polyphosphate kinase; E.C. 2. 7. 4. 1.) from *Escherichia coli*, KORNBERG *et al.* (1956) found the formation of TCA-insoluble poly P, according to the equation:

$$x\ ATP + \underset{primer}{(PO_3^-)_n} \rightleftharpoons x\ ADP + (PO_3^-)_{n+x} \qquad (1)$$

A further study (KORNBERG, 1957) of the poly P synthesizing enzyme from *E. coli* showed that poly P, prepared by chemical or enzymatic synthesis, may be utilized in the quantitative phosphorylation of ADP to ATP, according to:

$$x\ ADP + (PO_3^-)_n \rightleftharpoons x\ ATP + (PO_3^-)_{n-x} \qquad (2)$$

Under unfavourable growth conditions, or at certain stages in the growth cycle, the accumulation of ATP might give rise to poly P formation via reaction (1). Upon resumption of growth, ATP utilization would yield ADP, which would then be recycled to ATP by reaction (2). The function of poly P would thus be analogous to that of the N-phosphate compounds, phosphocreatine and phosphoarginine, in animal tissues. It is of interest to note, that cells capable of accumulating large amounts of poly P, i.e. yeast, bacteria and algae, do not contain the N-phosphate compounds (HUENNEKENS and WHITELEY, 1960). For a more recent survey of poly P in biology, see review by HAROLD (1966).

Studies of the possible role of energy-rich phosphate compounds in photosynthesis started after initial work of VOGLER and UMBREIT (1942) and EMERSON *et al.* (1944) with the autotrophic sulfur bacterium *Thiobacillus thiooxidans* and with *Chlorella pyrenoidosa* respectively. In our laboratory, phosphate exchanges were studied in relation to photosynthesis in purple sulfur bacteria and in *Chlorella* (WASSINK *et al.*, 1949; WASSINK *et al.*, 1951 and WINTERMANS, 1955). Following the changes in phosphorus metabolism by extracting the cells with TCA, and measuring the shifts between the inorganic phosphate fraction and the TCA-insoluble phosphate fraction, the mentioned authors found that illuminated *Chlorella* cells can convert orthophosphate into high molecular weight polymers of phosphoric acid, particularly in the absence of CO_2. The formation of these polyphosphates in the light continues for several hours at a slowly decreasing rate. Poly P formation in the light does not require oxygen, an important distinction from phosphate fixation in the dark, the latter being oxygen dependent. WINTERMANS regarded polyphosphates as energy-rich phosphorus compounds formed via ATP at the expense of light energy in the primary reactions of photosynthesis. The energy-rich phosphate groups are supposed to be transferred from ATP and stored as poly P, particularly in the abesnce of CO_2, when the normal photosynthetic demands on energy-rich phosphate groups have been curtailed (see e.g. WASSINK, 1957). More recently, evidence for the role of energy reservoirs of poly P has been reported by MIYACHI *et al.* (1964) for *Chlorella ellipsoidea* and by SYKES and GIBBON (1967) for *Chlorobium*

thiosulphatophilum. Also in the laboratories of SIMONIS (KANAI and SIMONIS, 1968 and ULLRICH and SIMONIS, 1969) and KYLIN (KYLIN, 1966 and KYLIN and TILLBERG, 1967), poly P formation in relation to photosynthesis has been studied in *Ankistrodesmus braunii* and *Scenedesmus*, respectively.

VAN RENSEN (1969) demonstrated that, in analogy with WINTERMANS' data, in the absence of CO_2 and O_2, phosphate fixation by *Scenedesmus* cells represents polyphosphate formation by cyclic photophosphorylation *in vivo*.

I.2.3. *Effects of herbicides on photosynthesis*

Although the application of toxic chemicals for weed control has already been practiced for more than half a century (NORMAN *et al.*, 1950), the development of herbicides affecting photosynthesis started only about 15 years ago.
WESSELS and VAN DER VEEN were among the first to study the effect of herbicides on photosynthesis; in 1956 they investigated the action of some derivatives of phenylurethane and of 3- phenyl-1,1-dimethylurea on the HILL reaction. GAST *et al.* (1956) were the first to study the effect of simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine) on plants. The bipyridylium herbicides, such as diquat and paraquat, appeared still later in photosynthesis literature (MEES, 1960). Despite their short history, the effect of herbicides on photosynthesis has already been elaborately studied, mainly because they affect photosynthesis in extremely low concentrations, suggesting a large degree of specificity, and importance for elucidating detailed aspects of the mechanism of the process.

Among the substituted phenylurea derivatives, DCMU and CMU are most studied. The effect of both substances on photosynthesis is nearly the same, although DCMU is about ten times more effective. These herbicides inhibit the HILL reaction at very low concentrations, viz., 10^{-7} molar (WESSELS and VAN DER VEEN, 1956). Oxygen evolution in *Scenedesmus* was found inhibited for 50% at about 10^{-7}M (VAN STEEKELENBURG, 1959 and VAN RENSEN and VAN STEEKELENBURG, 1965).

GEOHEGAN (1957) reported that after glucose application, the concentration of three phenylurea compounds required to inhibit growth of *Chlorella vulgaris* is increased. GENTNER and HILTON (1960) fed sucrose to barley via the leaves, simultaneously with the application of fenuron, monuron (CMU), neburon or DCMU to the root medium in which the 10-day old barley plants were growing. The sucrose was found to decrease inhibition of new growth by the herbicides. The authors believed that the results proved that the toxic symptoms produced in barley by the phenylureas were primarily due to a herbicide-induced deficiency of photosynthate.

DUYSENS *et al.* (1961), studying the oxidation-reduction of a cytochrome, found that its reduction, mediated by photosynthetic system II, is inhibited by DCMU. ZWEIG *et al.* (1963) have shown that substituted phenylureas cause stimulation of fluorescence in *Chlorella*.

SWEETSER (1963) studied interactions between phenylurea herbicides and flavins, and found that FMN caused photochemical inactivation of CMU and

other substituted phenylureas; a compound of high molecular weight which was no longer antiphotosynthetic, was isolated from the CMU-FMN reaction mixtures. A close relationship was found between the ability of phenylurea compounds to react photochemically with FMN and their capacity to inhibit photosynthesis. Molecular models of FMN and DCMU show surprising similarity in structure. SWEETSER postulated that CMU exerts its inhibitory effect on photosynthesis through interaction with FMN or a flavoprotein in the electron transfer pathway.

IZAWA and GOOD (1965) and VAN RENSEN and VAN STEEKELENBURG (1965) showed that DCMU is accumulated by isolated chloroplasts and by *Scenedesmus* cells respectively. Yet, this compound can be removed by washing, as was demonstrated by WESSELS and VAN DER VEEN (1956), VAN STEEKELENBURG (1959), ZWEIG and GREENBERG (1964), and VAN RENSEN and VAN STEEKELENBURG (1965).

GINGRAS *et al.* (1963) and GINGRAS and LEMASSON (1965) showed that oxygen evolution of *Chlorella* is inhibited more strongly by CMU in the light-dependent part of the photosynthesis-light curve than in the light-saturated one. VAN RENSEN and VAN STEEKELENBURG (1965) demonstrated the same effects for DCMU in O_2-evolution of *Scenedesmus*.

DCMU has no effect on CO_2-fixation in algae, adapted to photoreduction with hydrogen (BISHOP, 1958). The phenylureas inhibit photophosphorylation coupled to oxygen evolution, but less so phosphorylation catalyzed by PMS (GOOD, 1961). Inhibition of non-cyclic photophosphorylation and NADP-reduction in chloroplasts by DCMU can be removed by the ascorbate-DCPIP couple as an electron donor (JAGENDORF, 1959 and VERNON and ZAUGG, 1960), although complete recovery is not always obtained (HOCH and MARTIN, 1963).

It thus appears that inhibition by phenylureas must be located close to PS II. Attempts to localize this inhibition site more precisely have not been successful until now, because of still elusive features in the mechanism of photosynthetic oxygen evolution. There are also some indications for another site of action. Results from experiments of ASAHI and JAGENDORF (1963) with disrupted and aged chloroplasts, of GINGRAS *et al.* (1963) with *Chlorella*, and from experiments on glucose-assimilation of TANNER *et al.* (1965) suggested that there is such a second site sensitive to high concentrations of CMU and DCMU, associated with PS I. However, according to VAN RENSEN (1969) it is as well possible to explain these effects by assuming one site of action only.

The group of substituted amino-triazines includes several compounds from which simazine and atrazine are best known. GAST (1958) showed that the accumulation of starch by *Coleus blumei* is inhibited after treatment with simazine. To determine whether simazine interferes with sugar formation or with starch accumulation, GAST kept starch-free *Coleus* leaves in the dark in a sucrose solution, found that they were able to form starch in the presence of simazine, and thus concluded that simazine must inhibit sugar formation. MORELAND *et al.* (1959) reported that glucose kept barley seedlings alive and growing in the

presence of otherwise lethal concentrations of simazine.

VAN STEEKELENBURG (1959) demonstrated that simazine inhibits oxygen evolution in *Scenedesmus* for 50% at a concentration of 10^{-7}M; for simetone the required concentration is 4×10^{-6}M (VAN RENSEN and VAN STEEKELENBURG, 1965).

ROTH (1958) observed that simazine inhibits photosynthesis in *Elodea*. EXER (1958) and MORELAND *et al.* (1959) have reported that simazine inhibits the HILL reaction in chloroplasts at concentrations of about 10^{-7}M. Most phytotoxic triazines seem to inhibit the HILL reaction in chloroplasts; however, the degree of inhibition is not always paralleled by a corresponding degree of herbicidal effectiveness (GYSIN and KNÜSLI, 1960). VAN OORSCHOT and BELKSMA (1961) monitoring by infrared analysis the CO_2-exchange of plants, maintained in a closed chamber, found that CO_2-uptake of several plant species is strongly inhibited by simazine.

IZAWA and GOOD (1965) showed that atrazine is accumulated by isolated chloroplasts. This is not so with simetone in *Scenedesmus* cells (VAN RENSEN and VAN STEEKELENBURG, 1965). Both simazine and simetone can be removed by washing, as was reported by VAN STEEKELENBURG (1959), BISHOP (1962), and VAN RENSEN and VAN STEEKELENBURG (1965). The last authors also demonstrated that simetone exerts a stronger effect on oxygen evolution of *Scenedesmus* in the light-limited part of the photosynthesis-light curve than in the light-saturated one.

Triazines inhibit chloroplast reactions when ferricyanide or FMN is the electron acceptor but fail to do so when PMS is the acceptor. This probably means that the mechanism inhibited is the mechanism normally responsible for the oxidation of water to molecular oxygen. Triazines with two imino-hydrogens are more strongly inhibitive than those with only one (GOOD, 1961). ZWEIG *et al.* (1963) showed that atrazine and simazine are able to stop oxygen evolution from illuminated *Chlorella*, and cause stimulation of chlorophyll fluorescence. BISHOP (1962) reported inhibition of photosynthesis in *Scenedesmus* by aminotriazines, however, no inhibition of photoreduction in hydrogen adapted algae. Concentrations which inhibit photosynthesis do not influence the respiration of the algae. In pea chloroplasts, the FMN-catalyzed photophosphorylation is much more sensitive to simazine than the vitamin K_3-catalyzed reaction. BISHOP (1962) reported that the various effects produced on photosynthesis, photoreduction, photophosphorylation, and the HILL reaction are the same for all triazines with two imino-hydrogens, as far as tested. It appears probable that also these triazines inhibit photosynthesis somewhere close to PS II.

The effect of bipyridylium compounds has been studied by various authors, see e.g. the review by MORELAND (1967). The two best known compounds are diquat and paraquat which both cause similar responses (HOMER *et al.*, 1960).

MEES (1960) showed that light increased the rate of killing of bean leaf discs by diquat. Furthermore, etiolated wheat seedlings treated with diquat in the light, showed a rate of killing comparable to that shown by light-grown seed-

lings treated with diquat in the dark, indicating that chlorophyll was required to obtain the maximum rate of killing. After pretreatment with CMU, or in the absence of oxygen, no stimulating effect of light on the rate of killing of chlorophyll containing samples by diquat was observed. Diquat sometimes caused initial stimulation of respiration, and inhibition later on. The stimulation was dependent on continuous supply of diquat to the tissues, while the inhibition was not. MEES considered free radical formation an essential step in the development of the toxic action of diquat.

BRIAN (1964) reported that the light induced reduction of NADP in chloroplasts is inhibited by diquat by competition for electrons. Reduction of diquat is preferent, owing to the redox properties of the two systems. In the dark, respiration supplies electrons for reduction of diquat. Both in light and in darkness, the situation leads to the production of free radicals. Secondarily, in the presence of oxygen, peroxides are formed which have been suggested to disrupt cell membranes, and ultimately cause the death of the cells.

LANG and SEAMAN (1964) found rapid chlorosis and death of *Lemna* and *Azolla* by low concentrations of diquat or paraquat. In both cases, chlorosis was directly related to concentration, light intensity, and duration of treatment. Electron microscope observations indicated major differences in the ultrastructure of chloroplasts between treated plants and controls; breakdown in the regular pattern of chloroplast lamellae and grana was frequently observed.

MERKLE *et al.* (1965) observed that anaerobiosis protected the pigment system of beans, exposed to light, from bleaching by paraquat. Changes in membrane permeability by paraquat in light occurred, also in the absence of oxygen, in mesquite, honeysuckle, and broadleaf bean. These changes in permeability were also temperature dependent.

VAN OORSCHOT (1964, 1966) showed that diquat strongly decreased CO_2-uptake of bean leaves in the light. The relative inhibition was almost equal at different light intensities. The reduction of CO_2-uptake was followed by a gradual development of chlorotic and necrotic spots on the leaves. Simultaneous application of diquat and simetone had more or less additive effects on CO_2-uptake, but the development of symptoms was suppressed.

Most results on the effects of bipyridylium herbicides on photosynthesis have been obtained in studies with higher plants or isolated chloroplasts. VAN RENSEN (1969*a*) and also TURNER *et al.* (1970) reported experiments on the effects of diquat on the gas exchange of unicellular algae, *Scenedesmus* and *Chlorella* respectively.

All the above results are consistent with the hypothesis that the reduction of diquat to a free radical is an essential step in the sequence of toxic reactions. In the light, diquat is reduced in the photosynthetic process; in the dark in respiration. Through interaction of the diquat free radical, water, and O_2, toxic peroxides are formed, and may be involved in the degradation of proteins and other large molecules in the protoplasm.

From experiments with diquat, VAN RENSEN (1969) concluded that there are, most probably, two phosphorylation sites in the cyclic electron transfer chain.

I.3. Scope of the present investigation

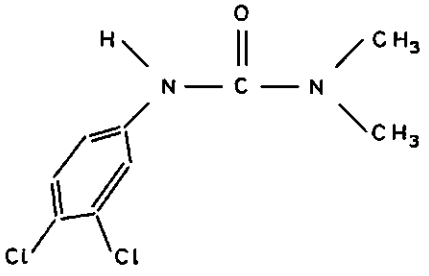
Against the background of the preceding discussion, an attempt has been made to contribute to the elucidation of the mechanism of action of three photosynthesis-inhibiting herbicides. Because these compounds act on photosynthesis already in very low concentrations, it might be expected, that their effects were rather specific, and, therefore, that they were a suitable tool in the study of the mechanism of photosynthesis.

Thus, we have investigated the effects of DCMU, simetone, and diquat on oxygen evolution in *Scenedesmus* with the aid of the Warburg technique, and moreover, collected observations on the effect of these herbicides on polyphosphate formation in relation to photosynthesis, in order to try to locate, if possible, their sites of action in the photosynthetic electron transfer chain.

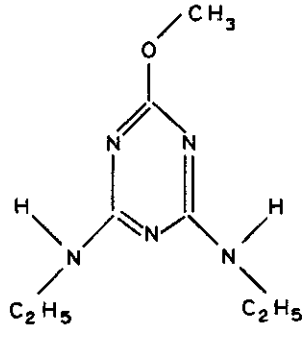
II. MATERIALS AND METHODS

II.1. Some notes on the herbicides used

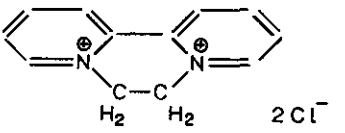
DCMU [N'-(3,4-dichlorophenyl)-NN-dimethylurea] is the best known representative of the substituted ureas. In agricultural research its name is diuron, but in photosynthesis research it is common to use the abbreviation, therefore the name DCMU is used in this paper. In higher plants, these ureas are taken up by the roots, transported by the xylem and accumulated in the leaves, where they inhibit photosynthesis at concentrations of 0.02 to 2 ppm. The solubility of DCMU in water is very low, viz., 42 ppm at 25 °C. The toxicity of the ureas to mammals is relatively low; the oral LD_{50} of DCMU for rats is about 3400 mg/kg (3400 ppm) (Würzer, 1969).



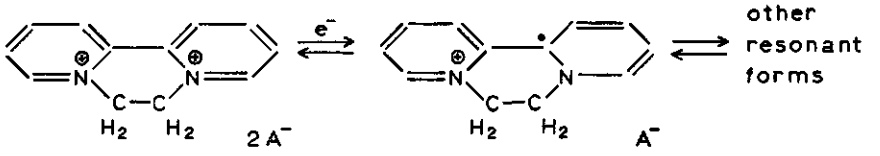
The chemical formula of simetone is 2-methoxy-4,6-bis (ethylamino)-1,3,5-triazine. The best known compound of the triazine group is simazine. The aqueous solubility of simazine at 26 °C and pH=7 is 5 ppm; that of simetone is 2340 ppm (Ward and Weber, 1968). Because of the very low solubility of simazine, simetone has been used in this investigation. Also the triazines in higher plants are taken up by the roots, transported via the xylem and accumulated in the leaves, where they inhibit photosynthesis at concentrations of 0.02 to 2 ppm. The toxicity of triazines to mammals is also low, the oral LD_{50} for rats varies from 3000–5000 mg/kg (3000–5000 ppm).



Diquat is used in the experiments as its dichloride monohydrate (1,1'-ethylene-2,2'-bipyridylium dichloride monohydrate), it is one of the bipyridylium herbicides. The compound forms a pale yellow monohydrate in water. The melting point is > 320 °C. The material is readily soluble; up to 70 g in 100 ml



water at 20 °C, and it is stable in acid and neutral solutions. Coloured complex products are formed in alkaline solution, which products appear to be due to to the opening of one of the pyridine rings. This process is associated with the uptake of one molecule of alkali (BRIAN *et al.*, 1958). Sprayed in the light, these herbicides cause rapid killing of leaves. Its distribution pattern is like that of a chemical transported through the xylem rather than through the phloem (BALDWIN, 1963). Upon contact with the soil, there is a rapid loss of activity. All quaternary bipyridylium salts with herbicidal activity are reducible to one-electron free radicals in a comparatively narrow range of redox potentials, viz., -349 to -548 mV (BOON, 1966). The redox potential of diquat is -349 ± 3mV. The reduction of diquat occurs according to the equation:



in which A^- may be chloride or bromide. The oral LD_{50} of diquat for rats is 400 mg ion/kg (400 ppm) (WÜRZER, 1969); photosynthesis is inhibited at concentrations of 2–20 ppm.

II.2. CULTIVATION OF THE ALGAE

The algae (*Scenedesmus* sp.) were kept as sterile stock cultures in tubes with 1.5% agar and a medium which per liter contains:

NH_4NO_3	0.33 g	$FeSO_4$	0.006 g
K_2HPO_4	0.2 g	Na-citrate	0.004 g
$MgSO_4$	0.2 g	EDTA	0.002 g

In addition, 2 ml of a combined A_4 and B_7 solution of trace elements, according to ARNON (1938), were supplied.

The cells for the experiments were cultured as follows: an inoculum taken from an agar slant, was suspended in about 5 ml sterilized culture medium.

With a sterilized pipette, 1 ml of this suspension was brought into a one liter erlenmeyer, containing 250 ml culture medium, which per liter contains:

a. KNO_3	2.5 g	d. $FeSO_4$	0.006 g
b. KH_2PO_4	0.135 g	Na-citrate	0.004 g
c. $MgSO_4$	0.50 g	EDTA	0.002 g

In addition, 2 ml of a combined A_4 and B_7 solution of trace elements according to ARNON (1938) were supplied. Concentrated stock solutions of the salts were sterilized separately (a, b, and c) or in mixtures as mentioned above (d, and the solutions of trace elements). The erlenmeyer flasks were placed on a rocking table and illuminated from below by fluorescent tubes. Room temperature was 22 °C. The suspension was flushed with a stream of air containing 5% CO_2, through a capillary tube closed by a cotton plug. After 4–6 days, the cells were used for an experiment. Cell density then was 3–5 mm^3 cells/ml.

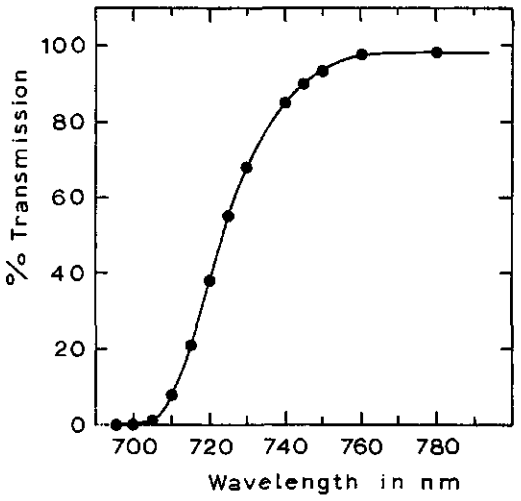
The algae were harvested by centrifugation, usually at 3000 rpm during 5 to 10 minutes and the density of the suspension determined with the aid of TROMMSDORFF tubes. Cells were then suspended in tap water, washed once, and resuspended in the medium used in the experiments, being adjusted to the desired density value. The final densities of these suspensions are given in mm^3 packed wet cells per ml.

II.3. MEASUREMENT OF GAS EXCHANGE

Measurements of gas exchange were made with a WARBURG apparatus. This apparatus had a thermostat bath of 100 × 30 cm, which could be kept at temperatures between 15 and 35 °C by an electric heater, controlled by a thermo relay with an accuracy of 0.05 °C, and a cooling system consisting of a continuous flow of tap water through a copper coil. The water in the thermostat bath was stirred by an electric pump. A rocking frame on which 6 manometers could be placed, was mounted in front of the bath. The reaction vessels, used in most of the experiments, had one side-arm and flat bottoms (diam. ca. 6 cm); their volume was about 28 ml.

The vessels were illuminated from below by incandescent lamps (Philips Attralux 24 V, 150 W, type 13378 E/06). They were cooled by two small (20 W) fans. The light intensity of the lamps was determined with a thermopile with and without a SCHOTT RG 8 filter. The range of transmission of this filter reaches from about 675 nm to about 3000 nm; its transmission is 70% at 700 nm and 90% at 750 nm. The light intensity values given in the text of this paper are corrected for the infrared radiation as transmitted by the SCHOTT RG 8 filter, and represent incident light intensities at the bottom of the vessels. Different light intensities were obtained by neutral metal screens, placed between the lamps and the vessels, below the water bath. Unless stated otherwise, the unweakened light intensity in the range up to about 690 nm was 4×10^5 ergs/cm^2. sec.

FIG. 2. Transmission of SCHOTT RG N9 filter.



In some experiments, SCHOTT RG N9 filters were used. They were placed at the bottom of the water bath, between the lamps and the vessels. The thickness of these filters was 3 mm and their transmission in the wavelength region of interest is shown in fig. 2. The transmission begins to decrease again at 800 nm; at 1000 nm it is 50%, and at 1500 nm about zero. Under the conditions of our experiments, the filters, at the level of the vessels, transmit about 50% of the total radiation of the tungsten lamps.

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, two of which will be discussed in some detail below.

a. *Measurements in which the CO_2-tension is kept constant*

It is readily understood that the change in manometric reading (h) is due solely to oxygen evolution or uptake if the partial pressure of CO_2 in the gas space of the reaction vessel is maintained constant. As is well-known, mixtures of carbonates and bicarbonates have been introduced by WARBURG (1919) to this purpose. In our experiments, mixture no 9 was used, consisting of 0.015 mol. carbonate and 0.085 mol. bicarbonate; pH was 9.37. This buffer is in equilibrium with a CO_2-concentration of 79×10^{-6} M at 25 °C, which corresponds to about 0.24 vol. % CO_2 in the gas phase. When properly used, this concentration is not a limiting factor for photosynthesis of *Chlorella*, as was already observed by WARBURG.

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

$$x = h.V_c \qquad \text{in which} \qquad V_c = \frac{V_g \times \frac{273}{T} + V_f.\alpha}{P_o}$$

In this formula, V_g represents the volume of the gas space in the vessel, the capillary tube and the manometer, V_f the volume of the liquid phase and α the absorption coefficient for oxygen at temperature T (the temperature applied in the experiment, in °K). P_o is the equivalent of 760 mm mercury expressed in mm of manometer liquid. By using BRODIE solution, a common manometer liquid, consisting of an aqeous solution of bile salts and NaCl in suitable concentrations, P_o = 10,000 mm. The bile salts serve as wetting agents in order to make the liquid move smoothly through the capillary measuring tube of the manometer. In most of the experiments, 10 ml algal suspension were introduced into the vessels. With a total volume of about 28 ml, V_c for oxygen was about 1.70 at 25 °C. One manometer vessel was provided with suspension liquid without algae and served as a thermobarometer, registering spurious manometer shifts for which the readings are to be corrected.

b.α. *Simultaneous measurements of CO_2 and O_2, using the indirect method of Warburg*

This method (WARBURG, 1926) is based upon the principle that changes in the volume of two gases of markedly different solubilities in the liquid used in the experiments can be measured by simultaneously following the manometric pressure changes occurring in the presence of identical reaction mixtures in two flasks of about equal total volume but with markedly different gas and liquid volumes. With respect to our experiments, identical samples of algae suspended either in 5 or 10 ml of the same suspension liquid under identical conditions of light intensity and temperature, may be assumed, under due precautions, to have the same gas exchange. This will cause unequal pressure changes in both manometers, owing to the different solubilities of CO_2 and O_2 in the suspension liquid. WARBURG has shown that the gas exchange in µl can be computed from the pressure changes of the two manometers by the formulae:

$$x_{O_2} = \frac{h\,k_{CO_2} - H\,K_{CO_2}}{\frac{k_{CO_2}}{k_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}} \qquad x_{CO_2} = \frac{h\,k_{O_2} - H\,K_{O_2}}{\frac{k_{O_2}}{k_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}}$$

in which

h is the manometric reading of the vessel with 5 ml suspension liquid in mm,

H is the manometric reading of the vessel with 10 ml suspension liquid in mm,

k_{O_2} and k_{CO_2} are the vessel constants for the vessel with 5 ml suspension liquid for O_2 and CO_2 respectively,

K_{O_2} and K_{CO_2} are the vessel constants for the vessel with 10 ml suspension liquid for O_2 and CO_2 respectively.

It should be observed that, of course, in this method, carbonate/bicarbonate mixtures can not be applied as suspension media, since the pressure change owing to CO_2-exchange should not be suppressed. The liquid used in our experiments was tap water or the medium used in the phosphate fixation experiments, as specified further on.

In order to provide sufficient amounts of CO_2 for photosynthesis, the algal suspension was saturated with a mixture of 95% air and 5% CO_2 by flushing during 15 minutes. The suspensions were then introduced into the WARBURG vessels, and the vessels were flushed with the same gas 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.

b.β. *Measurements with the one vessel method if the photosynthetic quotient is known*

This is an experimental simplification of the foregoing method (WASSINK, 1946). The principle is that it is possible to determine the rate of photosynthesis with one volume of liquid only if the photosynthetic quotient under the conditions of the experiments is known. If the quotient $-O_2/CO_2 = p$, the following relations hold:

$$x_{O_2} = \frac{p\, h\, k_{O_2}\, k_{CO_2}}{p\, k_{CO_2} - k_{O_2}} \quad \text{and} \quad x_{CO_2} = \frac{h\, k_{O_2}\, k_{CO_2}}{p\, k_{CO_2} - k_{O_2}}$$

These formulae are parallels of those derived for respiration by WARBURG (1924).

II.4. MEASUREMENT OF POLYPHOSPHATE FORMATION

WINTERMANS (1955) has shown that the major part of the inorganic phosphate which disappears during illumination of the algae in the absence of CO_2, can be recovered in the Δ 7 min. phosphate fraction. This is the fraction which contains the polyphosphates.

In our experiments, polyphosphate formation is measured as disappearance of inorganic phosphate during illumination of the algae. The method of WINTERMANS has been used with some modifications. For an experiment, the cells were centrifuged, washed once in a phosphate-free medium, containing K_2SO_4, and suspended in the medium used in the experiments. This medium contained 2.6 g K_2SO_4 and 200 mg KH_2PO_4 in 1000 ml distilled water, while pH was adjusted to 4.0 with dilute H_2SO_4. Suspensions containing 5 mm^3 cells per ml were introduced into 25 ml cylindrical vessels. The vessels were placed in the water bath of the WARBURG apparatus, kept at about 25 °C, and illuminated from below with the same lamps as used in the gas exchange experiments. Dur-

ing the 30 minutes dark pretreatment and during the incubation in the light, the vessels were continuously flushed with purified nitrogen, which contained less than 0.1 % argon, less than 0.001 % O_2, and less than 0.001 % of other contaminants.

For extraction and determination of total inorganic phosphate in the suspension, the procedure of LOWRY and LOPEZ (1946) was followed. This method is designed for the determination of orthophosphate in the presence of very labile organic phosphates (see also WASSINK, TJIA, and WINTERMANS, 1949). One and a half ml of the algal suspension were added to 0.5 ml cold 20% TCA. After 5 minutes, 8 ml 0.1 molar Na-acetate were added, and the mixture was centrifuged. Samples of 5 ml of the supernatant were pipetted and used for the determination of orthophosphate. To the sample, 4 ml of acetate buffer (pH = 4.0), 0.5 ml 1 % ammonium molybdate and 0.5 ml 1 % ascorbic acid were added. After standing for 10 minutes at room temperature, a blue colour, due to ammonium phosphomolybdate had developed, and the extinction of the samples at 700 nm was measured with a colorimeter. With the aid of the calibration curve, concentrations of orthophosphate were calculated in μg P/ml.

II.5. ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
Atrazine	2-chloro-6-ethylamino-4-isopropylamino-1,3,5-triazine
CMU	N′-(4-chlorophenyl)-NN-dimethylurea
DCMU	N′-(3,4-dichlorophenyl)-NN-dimethylurea
Diuron	see DCMU
Diquat	1,1′-ethylene-2,2′-bipyridylium ($2A^-$)
DCPIP	2,6-dichlorophenolindophenol
Fenuron	NN-dimethyl-N′-phenylurea
FMN	flavine mononucleotide
Monuron	see CMU
NADP	nicotinamide-adenine dinucleotide phosphate
Neburon	N-butyl-N′- (3,4-dichlorophenyl)-N-methylurea
Paraquat	1,1′-dimethyl-4,4′-bipyridylium ($2A^-$)
PMS	phenazine methosulphate
Poly P	inorganic polyphosphate
ppm	parts per million
PS I	photosynthesis pigment system I
PS II	photosynthesis pigment system II
rpm	revolutions per minute
Simazine	2-chloro-4,6-bis (ethylamino)-1,3,5-triazine
Simetone	2-methoxy-4,6-bis(ethylamino)-1,3,5-triazine
TCA	trichloroacetic acid

III. OXYGEN EVOLUTION AND POLYPHOSPHATE FORMATION IN *SCENEDESMUS*

III.1. INTRODUCTION

As already expounded in section I.2.2., WINTERMANS (1955) studied the poly P formation in *Chlorella* in relation to photosynthesis by following the changes in orthophosphate content of TCA-extracts of *Chlorella* suspensions. Figure 3 shows a typical picture from WINTERMANS' work. In the dark, the changes in phosphate content are small, both in the presence and absence of CO_2. In the light, the changes are much larger, while phosphate fixation is much larger in the absence of CO_2 than in its presence. The fixation in the light continues for hours at a slowly decreasing rate. WINTERMANS demonstrated that the major part of the phosphate that has disappeared, is recovered in TCA-insoluble polyphosphates. He explained his results by assuming the formation of energy-rich phosphates in the process of photosynthesis; these energy-rich phosphates are transferred to poly P when photosynthesis is curtailed by lack of CO_2.

Since that time, photosynthesis research has made great progress, and it lies at hand to assume that this process represents cyclic photophosphorylation *in vivo*. By illumination in the absence of CO_2, NADP and ferredoxin become fully reduced, consequently Z transfers electrons to the cyclic electron transport chain

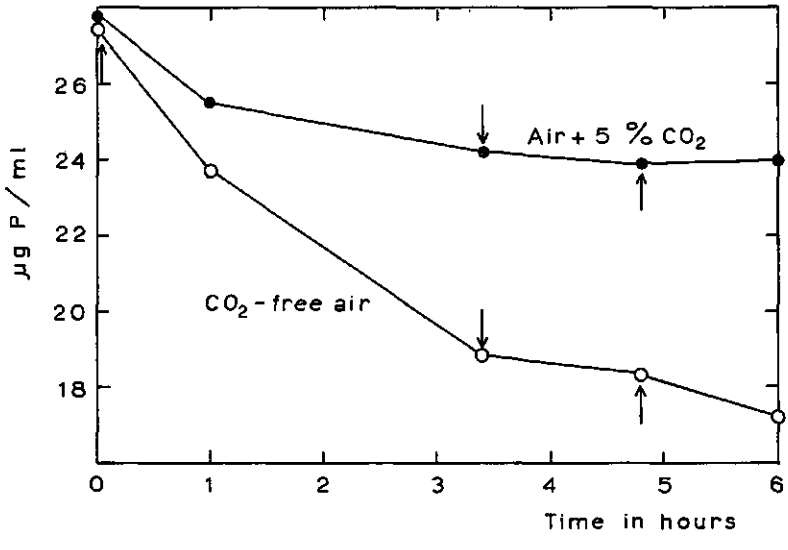


FIG. 3. Changes in orthophosphate in a suspension of *Chlorella*, in light and darkness, in the presence and absence of CO_2; pH $\pm$ 4.0, ca. 4 mm^3 cells/ml; ↑ shift to light, ↓ shift to darkness. From WINTERMANS (1955).

which results in cyclic photophosphorylation (see fig. 1). We decided to study this process in some more detail, also in its relation to oxygen evolution.

The experiments, described in this paper differ from those of WINTERMANS by the use of *Scenedesmus* instead of *Chlorella*. Moreover, the algal suspensions were flushed with highly purified nitrogen instead of CO_2-free air both during the 30 minutes pretreatment in the dark and the 90 minutes light incubation. In this way, pseudocyclic photophosphorylation and a contribution to the formation of energy-rich phosphates by oxidative phosphorylation were excluded. Recently, ULLRICH (1970) found ^{32}P-incorporation in poly P in *Ankistrodesmus braunii* increased in CO_2-free air as compared with a nitrogen atmosphere. This increase depended on light intensity and oxygen concentration. ULLRICH does not ascribe this increase to a contribution of oxidative phosphorylation, but thinks of the inhibition of photosynthesis by oxygen (WARBURG-effect), which leads to an increase in glycolate formation, and a decrease in pool sizes of CO_2-reduction cycle intermediates, even in the absence of CO_2; the excess ATP formation may be available for poly P synthesis.

III.2. OXYGEN EVOLUTION AND POLYPHOSPHATE FORMATION IN RELATION TO LIGHT INTENSITY

In order to study the relation between oxygen evolution and poly P formation, it was regarded necessary to suspend the algae in media of the same composition. Therefore, oxygen evolution was not measured in WARBURG buffer, but in the medium used in the phosphate fixation experiments. First, the photosynthetic quotient was determined under these conditions, according to the method as described in section II.3. b.α.; it was found to be 1.06. With the aid of this value, a light intensity series could be carried out according to the method, described

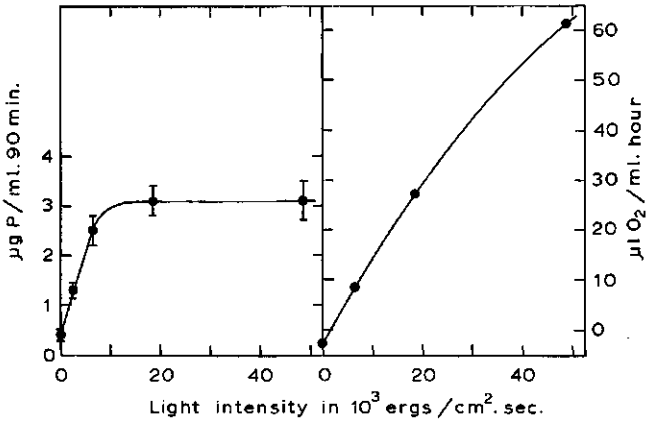


FIG. 4. Phosphate fixation under an N_2-atmosphere, and O_2-evolution, at a range of light intensities; pH ± 4.0, 5 mm^3 cells/ml.

in section II.3.b.β. From a harvest of algae, a sample was first taken to measure oxygen evolution, and afterwards another sample of the same harvest was used for determining phosphate fixation, in relation to light intensity. The results are shown in fig. 4.

It is striking that phosphate fixation is light saturated at about 13,000 ergs/cm^2.sec., while oxygen evolution continues to increase linearly in the entire light intensity range studied, confirming WINTERMANS (1955). In the dark, phosphate fixation reaches about 10% of the value observed in the light saturated part of the curve.

III.3. EFFECT OF AMMONIUM CHLORIDE ON POLYPHOSPHATE FORMATION

In view of results of HIND and JAGENDORF (1965) and others, showing that NH_4^+ inhibits photophosphorylation in chloroplasts, the effect of NH_4^+ on phosphate fixation was studied in intact *Scenedesmus* cells. The effect of 6.6×10^{-3}M NH_4Cl on phosphate fixation is shown in table 1. It is clear that, also in this case, NH_4Cl has an inhibitory effect on poly P formation.

TABLE 1. Effect of 6.6×10^{-3}M NH_4Cl on the fixation of phosphate in *Scenedesmus spec.* under an atmosphere of pure N_2

Exp. no.	Control	with NH_4Cl	% Inhibition
1	4.6	1.3	72
2	4.6	1.4	69
3	2.1	0.7	67
4	5.2	3.0	42
5	4.6	3.0	35
6	3.0	1.2	60
7	3.0	1.8	40
8	3.0	1.1	63
Average	3.8 ± 0.4	1.7 ± 0.3	56 ± 5

5 mm^3 cells/ml, pH ± 4.0; data in μg P/ml, fixed in 90 min.

III.4. OXYGEN EVOLUTION AND POLYPHOSPHATE FORMATION IN RELATION TO THE ACTIVITY OF SYSTEM I

It is well-known that the absorption spectrum of PS I reaches farther into the infra-red than the absorption spectrum of PS II. This makes it possible to select PS I dependent processes by illumination with long wavelength red light. Since poly P formation is observed especially under conditions promoting cyclic photophosphorylation, we have studied it in far-red light. To this purpose SCHOTT RG N9 filters (3 mm) were placed at the bottom of the water bath, between the lamps and the vessels. Under the conditions of our experiments,

TABLE 2. O_2-evolution in *Scenedesmus spec.*

	μl O_2/ml.hour	Same, corrected for dark uptake
Dark	−22	0
White light	300	322
With SCHOTT RG 8 filter	78	100
With SCHOTT RG N9 filter	−14	8

WARBURG buffer no 9, 10 mm^3 cells/ml, intensity of the white light 40 × 10^4 ergs/cm^2.sec.

these filters transmit 50% of the total irradiation of the tungsten lamps, at the level of the vessels. Table 2 shows the oxygen exchange in *Scenedesmus*. In the dark, there is O_2-uptake, in the light O_2-evolution. Both SCHOTT RG 8 and RG N9 filters decrease O_2-evolution as compared with the value in white light. After correcting for the dark O_2-uptake, oxygen evolution with SCHOTT RG 8 filter turned out to be 31%, however, with SCHOTT RG N9 filter only 2½% of the O_2-evolution in white light in the same experiment. This shows that in light transmitted by SCHOTT RG N9, there is very little activity of PS II. Nevertheless, poly P formation occurs in this light, as shown in fig. 5. The light intensity curve for this process, in RG N9- filtered light, shows a longer light limited range than it does in white light. A calculation from 17 comparable experiments revealed that phosphate fixation in white light was 4.1 ± 0.41 μg P/ml. 90 min., while in SCHOTT RG N9-filtered light the uptake was 3.9 ± 0.14 μg P/ml. 90 min. In these experiments, the intensity of the white light was 4 × 10^5 ergs/cm^2.sec. which is an over-saturating value for the white light experiments (fig. 3).

It thus appears that introducing the RG N9 filter causes a far greater depression in O_2-evolution than in poly P formation, as compared with white light.

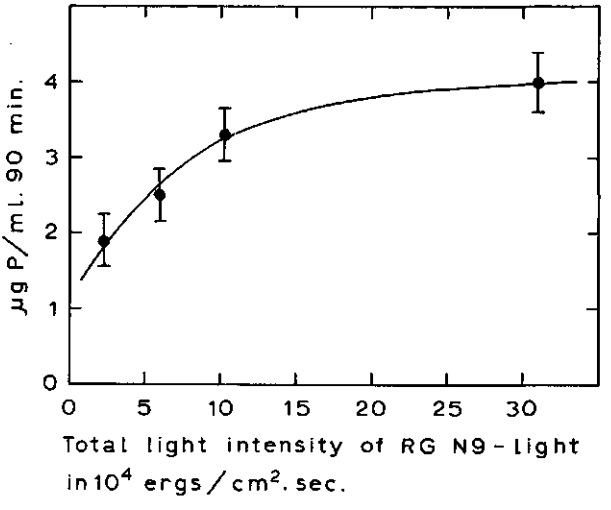


FIG. 5. Phosphate fixation under an N_2-atmosphere at a range of intensities of red light, obtained by neutral metal filters in combination with SCHOTT RG N9 filters; pH ± 4.0, 10 mm^3 cells/ml.

III.5. Discussion

Phosphate fixation in the dark is about 10% of that obtained under light saturation (fig. 4). Because the suspensions were flushed with nitrogen, dark fixation probably is due to glycolysis.

The light saturation of poly P formation at lower light intensities as compared with oxygen evolution was observed already by Wintermans (1955) for *Chlorella*, and was confirmed for *Scenedesmus* (fig. 4).

Table 2 demonstrates that in light, transmitted by Schott RG N9 filters, there is almost no oxygen evolution. This shows lack of activity of PS II in this long wavelength light. The fact that poly P formation continues in this light demonstrates that this is a PS I dependent process. Thus, it can be concluded that under the conditions of our experiments, poly P formation is the result of cyclic photophosphorylation, catalyzed by PS I.

In recent years, early light saturation has been observed for several other processes, probably connected with cyclic photophosphorylation, and several suggestions have been made regarding the discrepancy between light saturation of intact photosynthesis which is invariably found at substantially higher light intensities than that of the phenomena now under consideration.

Arnon (1969) reported that the rate of ferredoxin-catalyzed cyclic photophosphorylation reaches saturation at lower light intensities than the rate of ferredoxin-catalyzed non-cyclic photophosphorylation in spinach chloroplasts. A simple suggestion offered seems to be that the limiting factor for early light saturation of polyphosphate formation is the limited capacity of the poly P kinase. However, it is clear that this does not explain the early light saturation of several other phenomena (see e.g. Tanner and Kandler, 1969, Table 4). These authors concluded that for the best-studied PS I dependent process, viz., that of anaerobic photo-assimilation of glucose, the reaction limiting the rate observed at light saturation indeed is located in the process of cyclic photophosphorylation and not in other (dark) processes.

In a previous communication (Van Rensen, 1969), the evidence was stressed that in the case of O_2-evolution, the energy absorbed by PS II and PS I is used for the formation of ATP, O_2, and reduced NADP, while in the case of poly P formation there is a cyclic electron transport with ATP formation only. This situation may in principle explain the difference in light intensities for saturation. The causal mechanism possibly is the one suggested by Bonaventura and Myers (1969) (see also Murata, 1970), and Duysens (unpublished communication, 1970). Bonaventura and Myers proposed that there is a slow variation in the fraction (α) of total absorbed quanta delivered to reaction center 2, dependent on light intensity and wavelength of illumination. They suggested that conformational changes may control the distribution of quanta to the two Systems by altering the proximity of pigments to reaction centers. Duysens extended this suggestion by supposing that by illuminating preferently PS II, H^+-ions are produced at sites between PS II and PS I. Because of this acidification, the pigment proteins undergo conformational changes in a way that pigments belong-

ing to PS II, become connected with PS I. DUYSENS did this suggestion to explain the slow decrease (after a rapid increase) of fluorescence yield of chlorophyll a_2 in light preferently absorbed by PS II, after illumination with light preferently absorbed by PS I (DUYSENS and TALENS, 1969). These studies were carried out with the blue-green alga *Schizothrix calcicola.*

For measuring photo-assimilation of glucose or poly P formation, in our experiments algae are illuminated with white light in the absence of CO_2. Also under these conditions PS II should produce H^+-ions. According to DUYSENS' above mentioned picture, pigments 'bend' from PS II to PS I, and more energy becomes available to the PS I dependent process, which results in light saturation for poly P formation at lower light intensities. In this respect it is interesting to note that in light preferently absorbed by PS I, – then the only source of electrons for the reaction system – the light intensity curve for poly P formation less definitely shows light saturation than when both PS I and PS II are involved (fig. 5). Cyclic photophosphorylation *in vivo* is measured under conditions, not occurring in normal photosynthesis, since then non-cyclic and cyclic processes occur simultaneously. Therefore, it appears premature to discuss the lack of stoichiometric relationship between photosynthetic CO_2-fixation and cyclic photophosphorylation (TANNER *et al.*, 1969 and RAVEN, 1970) until more is known about the causal mechanism of light saturation of cyclic photophosphorylation *in vivo.*

TANNER *et al.* (1969) and RAVEN (1970) questioned the role of cyclic photophosphorylation in supplying the excess ATP, necessary for CO_2-assimilation. TANNER *et al.* (1969) stated that cyclic photophosphorylation generates ATP, not serving a specific function, but supporting quite a number of ATP-requiring reactions.

At the first sight, it looks surprising that NH_4^+, which is a normal constituent of many culture media, under conditions of poly P formation inhibits phosphate fixation (table 1). There are two possibilities to explain this observation. It is possible, that photosynthetic phosphorylation is uncoupled, probably via the mechanism proposed by CROFTS (1967). On the other hand, ammonium could induce an enhanced protein synthesis, which would compete for ATP with poly P formation. By utilization of ATP in protein synthesis, phosphate is set free, which results in decreased phosphate fixation, as is measured.

IV. EFFECTS OF THE PHENYLUREA DERIVATIVE DCMU

IV.1. Effects on oxygen evolution

IV.1.1. *Inhibition in relation to the concentration of the herbicide*

In this experiment, oxygen evolution was first measured in a series of vessels. Different concentrations of DCMU were then added to the separate vessels of the series; 30, 60, and 90 minutes after the addition, oxygen evolution was measured again. Figure 6 shows the effect, 30 minutes after the addition of the herbicide. After 60 and 90 minutes the effect of DCMU is the same. Oxygen evolution is inhibited for 50% at about 2×10^{-7}M DCMU.

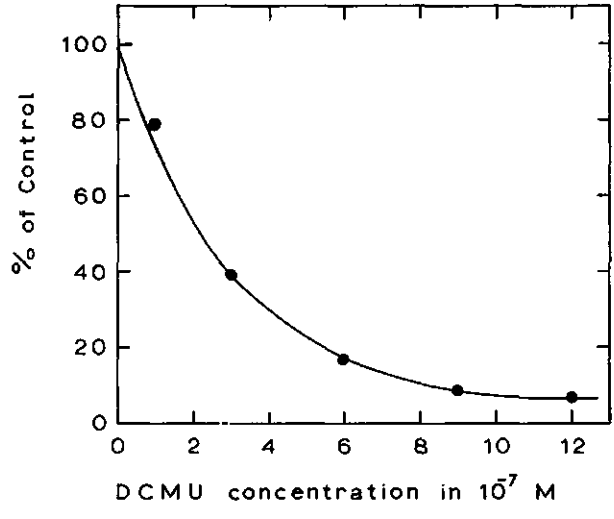


Fig. 6. Effect of different concentrations of DCMU on O_2-evolution, 30 min. after addition; 5 mm³ cells/ml.

IV.1.2. *Inhibition as affected by suspension density of the algae*

Figure 7 shows the inhibition of O_2-evolution by 2×10^{-7}M DCMU at different suspension densities. First, O_2-evolution was measured at 5 different suspension densities, and 30 minutes after addition of the herbicide, oxygen evolution was measured again. As follows from the linear course of the control curve, photosynthesis was light saturated, also at the highest suspension density. The degree of inhibition becomes lower at higher suspension densities. It seemed interesting to repeat this experiment with a lower DCMU concentration and higher suspension densities in order to check whether a DCMU concentration could be found, inhibiting at low suspension densities, but having no effect at high ones. The results are shown in fig. 8. With 2 mm³ cells/ml, 10^{-7}M DCMU inhibits O_2-evolution for 38%; with 6 mm³ cells/ml, inhibition is only 2%. It should be observed that there is 10% stimulation of oxygen evolution by 10^{-7}M DCMU at 8 mm³ cells/ml. This experiment strongly suggests that the

cells accumulate the poison, and that their number is decisive for how much each cell is going to store.

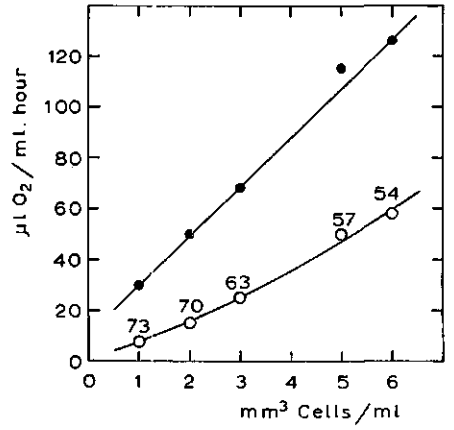


FIG. 7. Effect of 2×10^{-7}M DCMU on O_2-evolution at different suspension densities; ● = before addition of the herbicide, ○ = after addition; numbers along lower line: % inhibition.

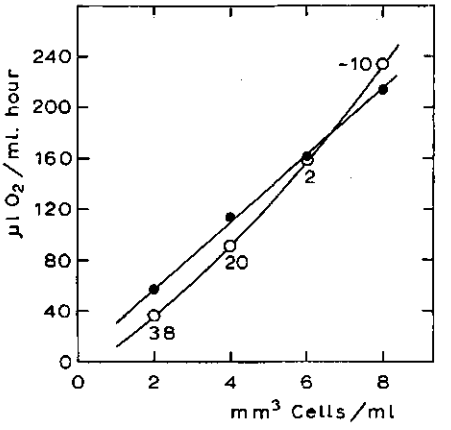


FIG. 8. Effect of 10^{-7}M DCMU on O_2-evolution at different suspension densities; ● = before addition of the herbicide, ○ = after addition; numbers along lower line: % inhibition.

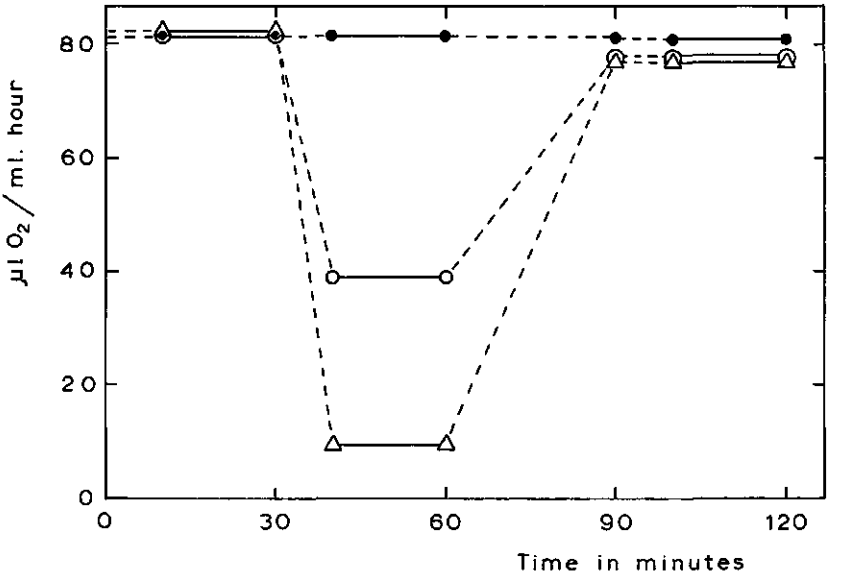


FIG. 9. Removal of DCMU-inhibition by washing; ● = control, ○ = 10^{-7}M, △ = 3×10^{-7}M; 4 mm³ cells/ml.

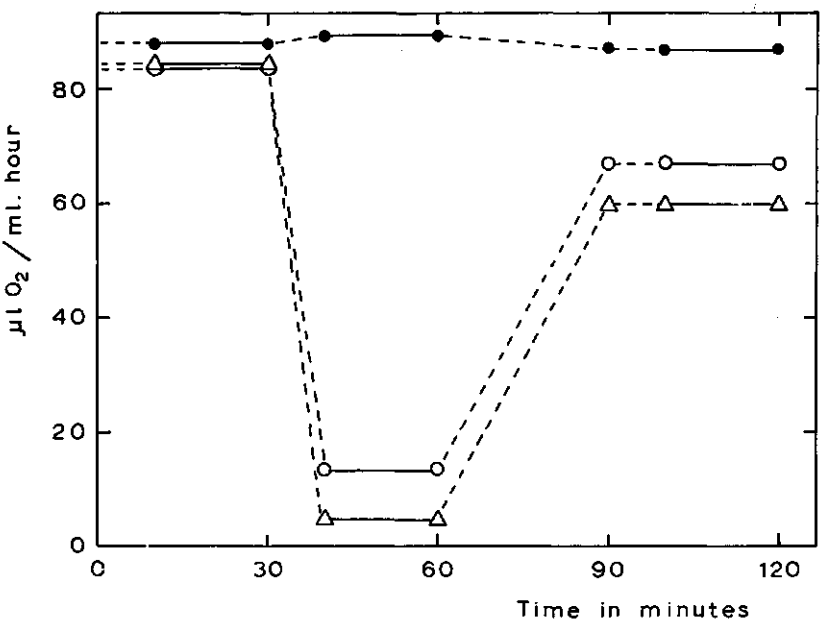


FIG. 10. Removal of DCMU-inhibition by washing; ● = control, ○ = 5×10^{-7}M, △ = 10^{-6}M; 4 mm³ cells/ml.

IV.1.3. *Washing experiments*

In view of the suggested accumulation of DCMU by the algal cells (section IV.1.2), it was of interest to investigate whether DCMU could be washed out. To this purpose, oxygen evolution was measured first, then two different concentrations of DCMU were added to separate vessels, and the effect of the herbicide determined. Now, the cells were centrifuged and washed twice with tap water. Hereafter, they were suspended in a fresh amount of buffer solution to the same suspension density as before, and the O_2-evolution was measured again. As found by VAN STEEKELENBURG (1959), and reported by VAN RENSEN and VAN STEEKELENBURG (1965), the inhibitions by 10^{-7}M and 3×10^{-7}M DCMU can be completely removed by washing (fig. 9). However, fig. 10 reveals that in cells which have been suspended in 5×10^{-7}M and in 10^{-6}M DCMU, O_2-evolution remains partly inhibited after washing.

IV.1.4. *Inhibition as affected by light intensity*

Figure 11 shows the effect of light intensity on the inhibition of O_2-evolution by 2×10^{-7}M DCMU. After the measurement of O_2-production at different light intensities, the herbicide was added to the suspensions, and oxygen evolution measured again. As can be seen from fig. 11, oxygen uptake in dark is not influenced by this concentration of DCMU. However, this does not affect the

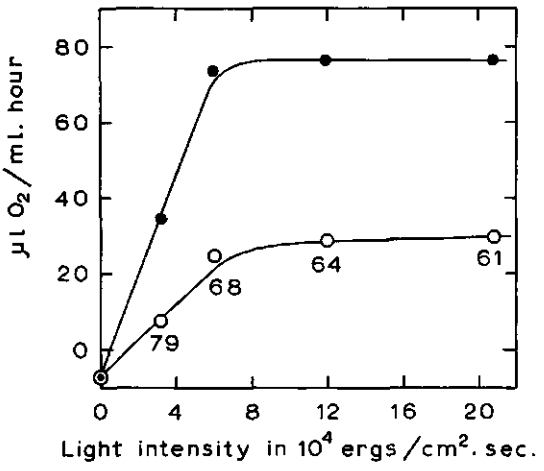


FIG. 11. Effect of 2×10^{-7}M DCMU on O_2-evolution at different light intensities; ● = control, i.e. before addition of the herbicide, ○ = after addition; numbers along lower curve: % inhibition; 3 mm³ cells/ml.

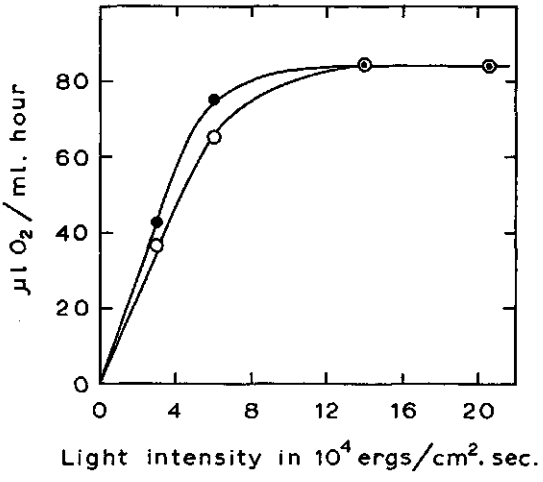


FIG. 12. Effect of 5×10^{-8}M DCMU on O_2-evolution at various light intensities; ● = control, i.e. before addition of the herbicide, ○ = after addition; 3 mm³ cells/ml; curves corrected for dark O_2-uptake.

conclusions, drawn in sections IV.1.1. and IV.1.2.. The inhibition in the light-limited part of the curve is stronger than that in the light-saturated part. In fig. 12 (corrected for respiration) it is demonstrated, that a DCMU concentration can be found, inhibiting in the light-limited part of the curve and without effect in the light-saturated part.

IV.1.5. *Inhibition as affected by temperature*

Light intensity series were carried out at two temperatures. Oxygen evolution was first measured at 30 °C, then at 20 °C. After the addition of 2×10^{-7}M DCMU to the vessels, the temperature was raised again to 30 °C, and the effect of DCMU determined. Then, another determination was carried out at 20 °C. Thus, four photosynthesis-light curves were obtained, shown in fig. 13. Table 3

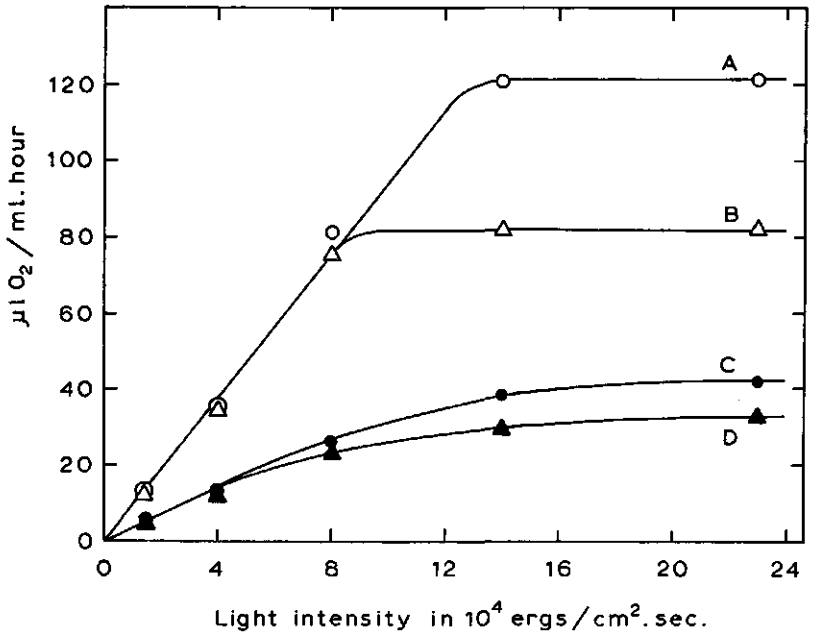


FIG. 13. Effect of 2×10^{-7}M DCMU on O_2-evolution at various light intensities and two temperatures; curves A and B: before addition of the herbicide, C and D: after addition; A and C: 30 °C, B and D: 20 °C; 4 mm³ cells/ml; curves corrected for dark O_2-uptake.

TABLE 3. Percentage of inhibition of O_2-evolution by 2×10^{-7}M DCMU at two light intensities and three temperatures

Light intensity in 10^4 ergs/cm². sec.	20 °C	25 °C	30 °C
4	71	74	72
23	60	61	65

contains data (calculated from figures 11 and 13) on the inhibition by the herbicide at three different temperatures and two light intensities, one of which was in the light limiting range, the other in the light saturated one. For all three temperatures the percentages of inhibition are higher at the limiting light intensity than at the saturating one. There is very little difference in inhibition between the three temperatures at equal light intensities.

IV.1.6. *Effect of DCMU on the photosynthetic quotient*

The photosynthetic quotient was determined according to the method, described in section II.3.b.α., in the absence and in the presence of 10^{-7}M DCMU. Table 4 demonstrates that in the non-buffered medium, 10^{-7}M DCMU has no effect on the photosynthetic quotient.

It should be observed that in this experiment, without DCMU, the rate of oxygen evolution per hour is 50 times the volume of the algae, while in experiments in WARBURG buffer, this value varied from 20 to 30. The lower rate of photosynthesis in alkaline media as compared with more neutral ones was reported e.g. by KOK (1951), WARBURG (1952), and MYERS (1960). This phenomenon is generally ascribed to the lower CO_2-concentration and the unphysiologically high pH of the most commonly used WARBURG buffers.

TABLE 4. Effect of DCMU on the photosynthetic quotient

	µl O_2-evolution	µl CO_2-uptake	Photosynthetic quotient
Control	1965	1822	1.08 ± 0.01
10^{-7}M DCMU	1635	1506	1.08 ± 0.02

40 mm³ cells/vessel, suspended in tap water; data in µl/vessel. hour, saturating light intensity, 25°C.

IV.2. EFFECTS ON POLYPHOSPHATE FORMATION

IV.2.1. *Inhibition in relation to the concentration of the herbicide*

Preliminary experiments had shown that polyphosphate formation is far less sensitive to DCMU than oxygen evolution.

The effects of different DCMU concentrations on phosphate fixation are demonstrated in fig. 14. The herbicide was added at the beginning of 30 minutes

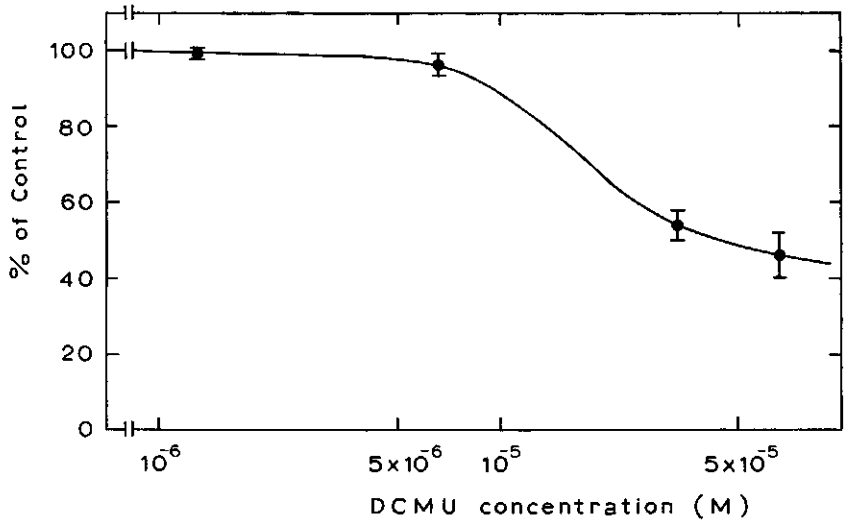


FIG. 14. Effect of various DCMU concentrations on phosphate fixation under an N_2-atmosphere; light intensity 40 × 10^4 ergs/cm².sec., pH ± 4.0, 5 mm³ cells/ml.

dark pretreatment. The two higher concentrations were obtained by preparing concentrated solutions of DCMU in methanol. These solutions were prepared in such a way that, after addition of equal volumes to the suspensions, the final concentration of methanol in the reaction media was 0.67%, and the required DCMU concentrations were obtained. For reasons of comparison, two control vessels were included, one without methanol, and one with the mentioned concentration of this substance. Even 1.3×10^{-6}M DCMU has not yet any effect on poly P formation, while oxygen evolution is almost completely inhibited at this concentration (fig. 6). Even at 6.6×10^{-5}M the inhibition of phosphate fixation is only 54%.

IV.2.2. *Observations in relation to light intensity*

In order to study the effect of light intensity on the inhibition of poly P formation by DCMU, two herbicide concentrations were chosen, one causing about 50% inhibition and the other almost without effect. The influence of these two concentrations was studied at three different light intensities. It can be concluded from table 5 that the inhibition at 33×10^{-6}M DCMU is the same at all three light intensities. However, at 6.6×10^{-6}M, the inhibition at 10×10^4 ergs/cm^2.sec. was 43%, while at the higher light intensities it was only 4%. It is important to notice that at 10×10^4 ergs/cm^2.sec. poly P formation is light saturated (fig. 4). It ensues that the inhibition by 6.6×10^{-6}M DCMU is light dependent; at low light intensities the inhibition is stronger than at higher ones. Also in the oxygen evolution experiments, inhibition was stronger at lower light intensities than at higher ones (fig. 11 and 12).

TABLE 5. Effect of two DCMU concentrations on phosphate fixation under a nitrogen atmosphere at three different light intensities

DCMU concentration	Light intensity in 10^4 ergs/cm^2.sec.	Phosphate Fixation		% Inhibition
		Control	with DCMU	
3.3×10^{-5}M	40	3.0 ± 0.4	1.6 ± 0.1	46 ± 4
3.3×10^{-5}M	20	2.8 ± 0.2	1.5 ± 0.2	46 ± 4
3.3×10^{-5}M	10	3.0 ± 0.2	1.4 ± 0.1	52 ± 5
6.6×10^{-6}M	40	2.6 ± 0.2	2.5 ± 0.1	4 ± 2
6.6×10^{-6}M	20	2.3 ± 0.1	2.2 ± 0.1	4 ± 2
6.6×10^{-6}M	10	3.4 ± 0.6	1.9 ± 0.3	43 ± 2

pH ± 4.0, 5 mm^3 cells/ml; data in μg P/ml fixed in 90 min.

IV.2.3. *Inhibition in light preferently absorbed by System I*

For reasons to be presented in more detail later on it appeared of interest to investigate whether DCMU-inhibition of poly P formation in light preferently absorbed by PS I, was different from the effect in white light. To this purpose the technique, mentioned before, using SCHOTT RG N9 filters, was applied; there is almost no activity of PS II in the light thus obtained. Figure 15 shows

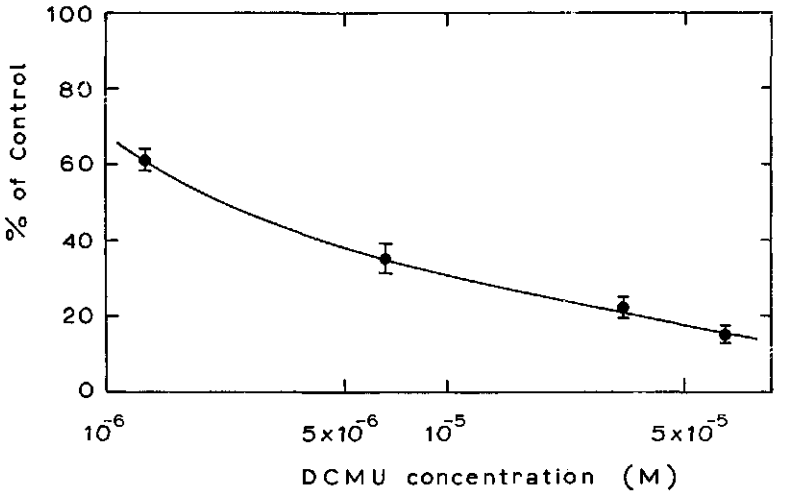


FIG. 15. Effect of various DCMU concentrations on phosphate fixation under an N_2-atmosphere and in light, filtered by SCHOTT RG N9 filters; intensity of white light 40×10^4 ergs/cm².sec., pH $\pm$ 4.0, 5 mm³ cells/ml.

the effect of DCMU on poly P formation in this wavelength region. It can be concluded that the DCMU effect is stronger than that on poly P formation in white light, but weaker than that on oxygen evolution. For instance, comparison of fig. 15 with figs. 6 and 14 shows that the percent inhibition of poly P formation by 1.3×10^{-6}M DCMU in the long wavelength red light is about 40%, while it is only 1% on poly P formation in white light (fig. 14) ; in the latter case oxygen evolution is almost completely inhibited (fig. 6). As mentioned before, oxygen evolution in the light, filtered by RG N9, is practically zero, also in the absence of any inibitor.

IV.3. DISCUSSION

a. *Oxygen evolution*

Oxygen evolution in *Scenedesmus*, at saturating light intensity and 5 mm³ cells/ml, is inhibited for 50% by 2×10^{-7}M DCMU (fig. 6). This value agrees with those reported in literature. WESSELS and VAN DER VEEN (1956) found 50% inhibition of the HILL reaction in chloroplasts at 2×10^{-7}M DCMU. ZWEIG *et al.* (1963) reported 50% inhibition of oxygen evolution in *Chlorella* at 1.7×10^{-7}M DCMU.

The fact, that the control curves in fig. 7 and 8 are linear, demonstrates that photosynthesis was light saturated, also at the highest suspension densities. The inhibition of oxygen evolution by DCMU decreases with increasing suspension density, which may be explained by accumulation of DCMU by the

algae. Fig. 8 shows that, at 10^{-7}M DCMU, the inhibition of O_2-evolution at lower suspension densities changes into a stimulation at 8 mm^3 cells/ml, crossing the control curve at about 7 mm^3 cells/ml. Also IZAWA and GOOD (1965) observed that with a denser chloroplast suspension higher amounts of DCMU were required to produce the same degree of inhibition. Moreover, they showed that the absorption of DCMU by isolated spinach chloroplasts involves at least three simultaneous processes: (*a*) an irreversible binding or destruction of DCMU in the proportion of one molecule of DCMU for about 1000 chlorophyll molecules; this is not associated with inhibition, (*b*) unequal partitioning between external and internal liquid phases, (*c*) an absorption of DCMU which corresponds closely to the degree of inhibition. Aside of accumulation of DCMU by the algal cells, also process (*a*) can contribute to the decrease of inhibition percentages with increasing suspension density. Process (*a*) can also explain the finding that 10^{-7}M DCMU does not inhibit oxygen evolution at a suspension density of about 7 mm^3 cells/ml. The stimulation of oxygen evolution by 10^{-7}M DCMU at 8 mm^3 cells/ml may be described as an example of the rather common phenomenon that agents, inhibitory for physiological processes at high concentrations, become stimulatory in regions of low concentrations. The effectivity of a very low concentration in the case of high suspension density may be due to the accumulation phenomenon. In our case, this stimulation could perhaps be explained by assuming that the low concentration of DCMU brings the components of the photosynthetic electron transport chain in a more favourable redox balance, probably via a mechanism which will be expounded in more detail in the general discussion. AVRON and NEUMANN (1968) described a stimulatory action of DCMU on cyclic photophosphorylation under some conditions, and inhibition under other conditions. They ascribed this to differences in the redox level during the reaction under different conditions. Also in the Botanical Laboratory of the University of Stockholm (TILLBERG, KYLIN, and SUNDBERG, personal communication), stimulation of oxygen evolution in *Scenedesmus* cells was observed at very low DCMU concentrations. The effect depended on the age and the phosphate state of the cells. For an explanation, the mentioned authors conceive the possibility of a change in redox level of electron transport chain intermediates, causing a decrease in pseudo-cyclic O_2-uptake (cf. HEBER and FRENCH, 1968), which results in an increased apparent oxygen evolution.

Fig. 9 shows that the inhibitory effect of 10^{-7} and 3×10^{-7}M DCMU can easily be removed by washing the cells twice with tap water. However, the effect of 5×10^{-7} and 10^{-6}M could not completely be removed by two washings (fig. 10). Also WESSELS and VAN DER VEEN (1956) reported, that the inhibitory action of CMU in isolated chloroplasts can be removed by simply washing away the CMU. SCHIFF *et al.* (1967) removed DCMU from *Euglena* by centrifugation. With *Chlorella*, ZWEIG and GREENBERG (1964) found equal in- and outflux diffusion rates of CMU, suggesting free in- and outflux. However, it was difficult to remove all the absorbed molecules from the cells. With respect to the remaining inhibition at the higher DCMU concentrations after washing (fig. 10),

probably complete removal of inhibition might be obtained after more than two washings. Although DCMU possibly is accumulated by the algal cells (fig. 7), it can be concluded from these washing experiments that the bond between DCMU and the site of action is a loose one.

Fig. 11 shows that a range of DCMU-concentrations exists which, while inhibiting photosynthesis, have no effect on respiration. The inhibition of oxygen evolution is higher under light-limited conditions than at light-saturation (fig. 11). According to fig. 12, it is possible to obtain inhibition of oxygen evolution in the light-limited part of the photosynthesis-light curve without an effect at the light saturation level. The same results were obtained by GINGRAS *et al.* (1963) and GINGRAS and LEMASSON (1965) for the effect of CMU on oxygen evolution in *Chlorella*. In the light-dependent part of a photosynthesis-light curve, the rate of photosynthetic oxygen evolution is determined by the amounts of ATP and reduced NADP formed. In the light-saturated part of the curve, the amounts of ATP and reduced NADP are in excess, and the rate of photosynthesis is determined either by dark enzyme activities (e.g. at low temperatures), or, incidentally, by limited CO_2-supply. The fact that it is possible, with DCMU, to inhibit oxygen evolution in the light-dependent range, leaving the light-saturated level unchanged (fig. 12), demonstrates that DCMU inhibits somewhere in the light dependent reactions leading to the formation of ATP and reduced NADP, and not in the dark reaction system belonging to the CO_2-reduction cycle. This situation explains why the inhibition of the oxygen evolution in the light limiting range – like the uninhibited process – is not temperature-sensitive (fig. 13 and table 3).

The apparent inhibition in the light saturation range by DCMU at high concentrations must probably be understood as a deficient supply of ATP and reduced NADP which, even in these cases, may be supposed to gradually disappear if light intensity was duely increased. Since this supply thus is mediated by light dependent reactions, it may explain why, also in the light saturating range, no effect of temperature on the degree of inhibition is observed (fig. 13, and table 3). GINGRAS and LEMASSON (1965) and GINGRAS (1966) also failed to find an effect of temperature on the inhibition of oxygen evolution in *Chlorella* by CMU either in the light-limited part of the intensity curve or in the saturated part.

The absence of an effect of DCMU on the photosynthetic quotient (table 4) demonstrates the strong coupling, *in vivo*, under stationary conditions, of O_2-evolution and CO_2-uptake.

b. *Photophosphorylation*

Fig. 14 shows that 1.3×10^{-6}M DCMU, which almost completely inhibits O_2-evolution (fig. 6), has no effect on poly P formation. However, at higher concentrations, poly P formation is also inhibited. Complete inhibition was not obtained even at the highest concentration applied. As discussed in Chapter III, poly P formation represents cyclic photophosphorylation *in vivo*. Other processes, dependent on cyclic photophosphorylation *in vivo* also are less sensitive to

DCMU than oxygen evolution. TANNER *et al.* (1965) showed that at DCMU concentrations, completely inhibiting O_2-production in *Chlorella*, anaerobic photo-assimilation of glucose still proceeds at a rate of 70 to 80% of that of the control. TANNER *et al.* (1966) also demonstrated that at higher DCMU concentions the inhibition of glucose-assimilation did not exceed 60%. SIMONIS (1967) reported that ^{14}C-fixation in *Ankistrodesmus braunii* is much more sensitive to DCMU than light-induced ^{32}P-incorporation into the organic phosphate fraction, under a nitrogen atmosphere. JESCHKE (1967) found that concentrations of DCMU that almost completely inhibit oxygen evolution in *Elodea* do not affect the light-induced Cl^--uptake in the absence of CO_2.

From the differences in sensitivity to DCMU between O_2-evolution and effects based on cyclic photophosphorylation *in vivo*, TANNER *et al.* (1965) concluded to two sites for DCMU inhibition: *a*) in the water splitting reaction, and *b*) somewhere in the system coupled to light reaction I at higher DCMU concentrations. In Chapter VII, part 2.1., a hypothesis on the mechanism of the action of DCMU will be discussed, in which only one site of inhibition is required to explain the mentioned difference in sensitivity.

From table 5, it is clear that the degree of inhibition by 6.6×10^{-6}M DCMU depends on light intensity; at the higher light intensities the inhibition is only 4%, while at the lowest one it is 43%. The degree of inhibition by 3.3×10^{-5}M DCMU is not influenced by light intensity. TANNER *et al.* (1965) found that also the degree of inhibition of glucose-assimilation in *Chlorella* by DCMU concentrations below 10^{-6}M was light intensity dependent. The results presented so far show that, in addition to the effect of light intensity on DCMU-inhibition of O_2-evolution, also the effect of light intensity on DCMU-inhibition of processes based on cyclic photophosphorylation strengthens the conclusion that the localization of the action of DCMU is in the light dependent reactions.

In far red light, preferently absorbed by PS I, the inhibition of poly P formation by DCMU is stronger than in white light (fig. 15 in comparison with fig. 14). SIMONIS (1967) found that the inhibition of light-induced ^{32}P-incorporation in *Ankistrodesmus* was stronger in white light than in far red. However, TANNER *et al.* (1966) showed that glucose-assimilation in *Chlorella* was more inhibited by DCMU in red light than in white light. Also in subchloroplast particles from isolated chloroplasts, DCMU abolished cyclic photophosphorylation catalyzed by PMS in red light, while there was much less effect in white light (ANDERSON and MCCARTY, 1969).

The already mentioned hypothesis (discussed in detail in chapter VII; p. 2.1.) suggested that in far red light inhibition of poly P formation by DCMU is stronger than that in white light, which was experimentally confirmed afterwards, as shown above. So much may be said here that the mentioned hypothesis does not contradict the conclusion, already drawn from the experiments in white light only, that the inhibition by DCMU is localized in light limited reaction sequences.

It should not be omitted that SIMONIS' cited result is at variance with this picture as well as with the other findings reported here.

V. EFFECTS OF THE *S*-TRIAZINE DERIVATIVE SIMETONE

V.1. EFFECTS ON OXYGEN EVOLUTION

V.1.1. *Inhibition in relation to the concentration of the herbicide*

To obtain an idea on the effectiveness of simetone as an inhibitor of photosynthesis in *Scenedesmus*, the influence of this compound on O_2-evolution was studied in a range of concentrations. Oxygen production was measured before, and 30, 60, and 90 minutes after addition of different concentrations of the herbicide to the vessels of a series of WARBURG manometers. Fig. 16 shows the effect of simetone on oxygen production, 30 minutes after addition; 60 and 90 minutes after addition the effect was the same. O_2-evolution is inhibited for 50% by about 4×10^{-6}M simetone.

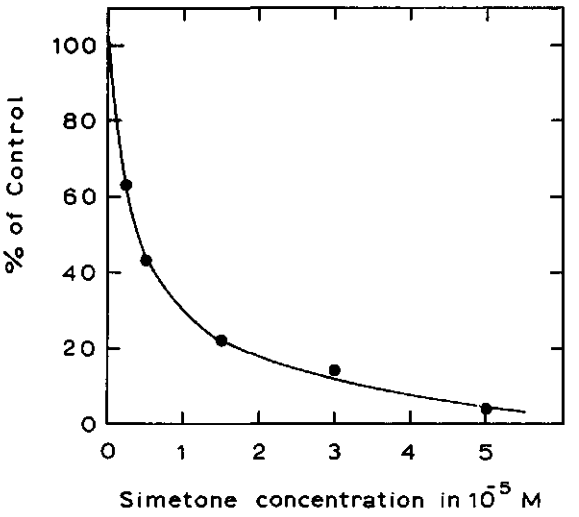


FIG. 16. Effect of various simetone concentrations on O_2-evolution, 30 minutes after addition; 4 mm³ cells/ml.

V.1.2. *Inhibition as affected by suspension density of the algae*

The percent inhibition of oxygen evolution by simetone, measured 30 minutes after addition, was more or less the same at different suspension densities (fig. 17). At a still higher density and a lower simetone concentration, viz. 2×10^{-6}M, the inhibition percentages again are nearly equal (fig. 18). This points to easy equilibration of concentrations in and outside the cells.

V.1.3. *Washing experiments*

In the experiment, illustrated in fig. 19, oxygen evolution was first measured in three pairs of vessels. Ten minutes after the addition of water to the control

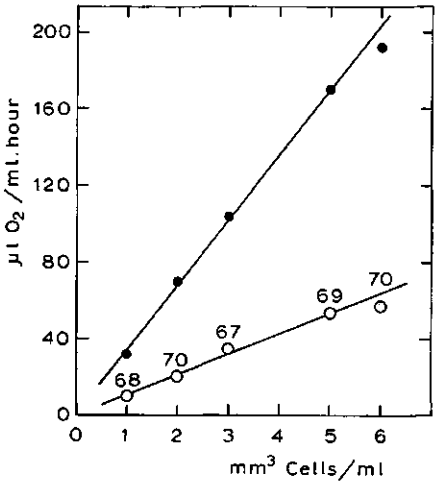


FIG. 17. Effect of 4×10^{-6}M simetone on O_2-evolution at different suspension densities; • = control, i.e. before addition of the herbicide, ○ = after addition; numbers along lower line: % inhibition.

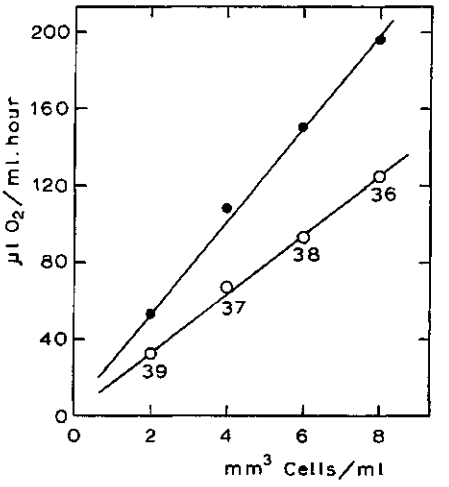


FIG. 18. Effect of 2×10^{-6} M simetone on O_2-evolution at different suspension densities; • = control, i.e. before addition of the herbicide, ○ = after addition; numbers along lower line: % inhibition.

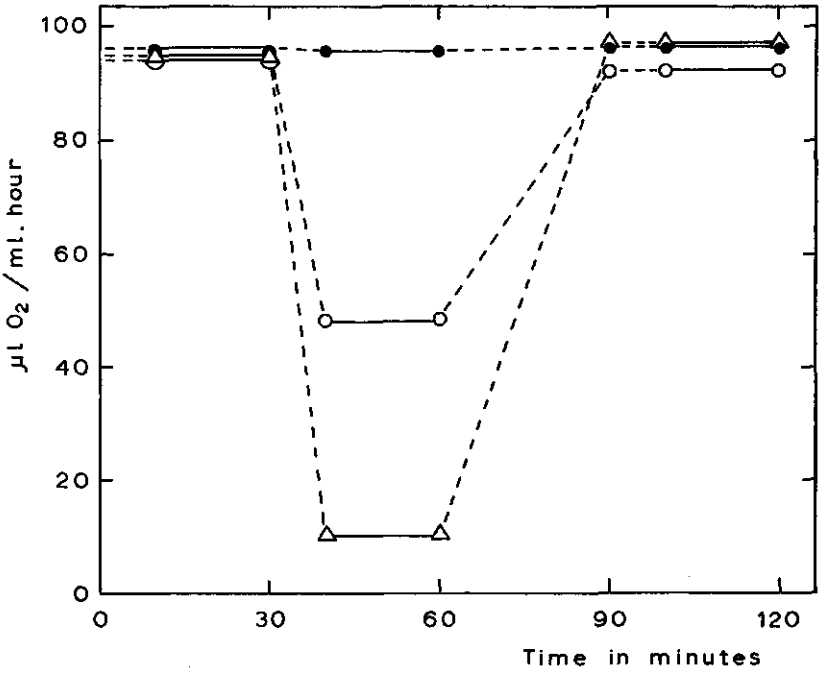


FIG. 19. Removal of simetone-inhibition by washing; • = control, ○ = 2×10^{-6}M, △ = 10^{-5}M; 4 mm³ cells/ml.

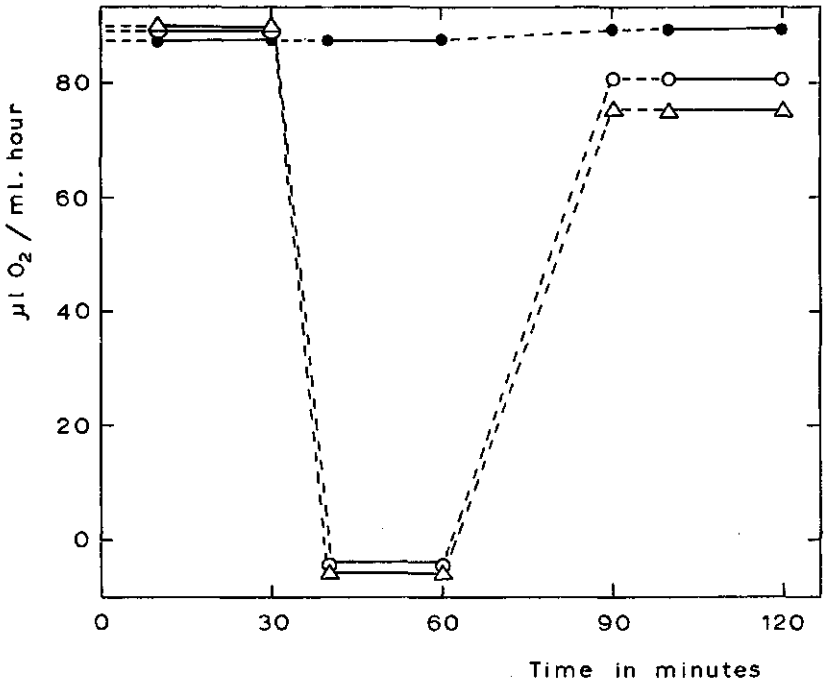


FIG. 20. Removal of simetone-inhibition by washing; ● = control, ○ = 5×10^{-5}M, △ = 10^{-4}M; 4 mm³ cells/ml.

pair, and 2×10^{-6}M and 10^{-5}M simetone respectively to the other pairs of vessels, the O_2-production was again determined. Now, aliquots of suspension of each pair of vessels were combined, centrifuged, and washed twice with tap water and resuspended in a fresh amount of buffer. The suspension density was made up to the same value as that at the beginning of the experiment. Oxygen evolution was then measured in three vessels, one for each treatment. The data show that the inhibiting effect could be removed by washing (fig. 19). With higher concentrations of simetone, resulting in nearly complete inhibition of photosynthesis, the inhibiting effect could not fully be removed after washing twice with tap water (fig. 20).

V.1.4. *Inhibition as affected by light intensity*

Fig. 21 shows the effect of 4×10^{-6}M simetone on oxygen evolution at different light intensities. The dark oxygen uptake is unaltered, so there is no effect on respiration at this concentration of simetone. The percent inhibition of oxygen production is higher in the light-limited part of the curve than in the light-saturated one. With 6×10^{-7}M simetone there is inhibition at limiting ight intensities and none at light saturation (fig. 22).

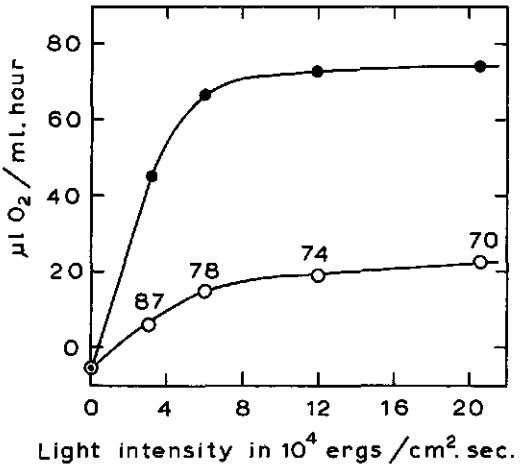


FIG. 21. Effect of 4×10^{-6}M simetone on O_2-evolution at various light intensities; • = before addition of the herbicide, ○ = after addition; numbers along lower curve: % inhibition; 3 mm^3 cells/ml.

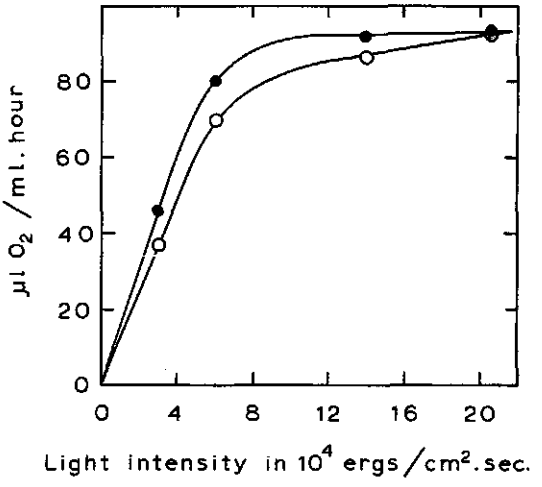


FIG. 22. Effect of 6×10^{-7}M simetone on O_2-evolution at various light intensities; • = before addition of the herbicide, ○ = after addition; 3mm^3 cells/ml; curves corrected for dark O_2-uptake.

V.1.5. *Inhibition as affected by temperature*

In order to study the effect of temperature on the inhibition by simetone, photosynthesis-light curves were determined at 20° and 30°C. After measuring oxygen evolution at 30°C at the various light intensities, the temperature of the water bath was lowered to 20°C, and O_2-production was again determined. Then, 4×10^{-6}M simetone was added, and the oxygen evolution measured at 20° and 30°C. It can be concluded from fig. 23 and table 6 that both at 20° and 30°C the percent inhibition is higher at light-limiting intensities than at light-saturated ones. At both light intensities, there is no effect of temperature on the percent inhibition (table 6).

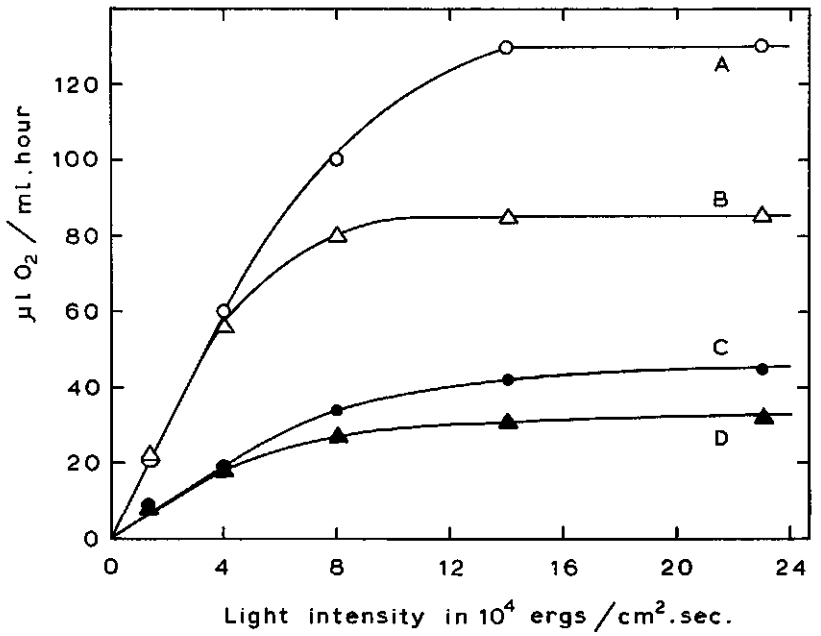


FIG. 23. Effect of 4×10^{-6}M simetone on O_2-evolution at various light intensities and two temperatures; curves A and B: before addition of the herbicide, C and D: after addition; A and C: 30°C, B and D: 20°C; 4 mm³ cells/ml; curves corrected for dark O_2-uptake.

TABLE 6. Percentage inhibition O_2-evolution by 4×10^{-6}M simetone at two light intensities and three temperatures

Light intensity in 10^4 ergs/cm².sec.	20°C	25°C	30°C
4	68	69	70
23	62	61	65

V.1.6. *Effect of simetone on the photosynthetic quotient*

According to the method, described in section II.3.b.α., the effect of simetone on the photosynthetic quotient was investigated. Table 7 shows, that the photosynthetic quotient is not influenced by 2×10^{-6}M simetone.

TABLE 7. Effect of simetone on the photosynthetic quotient

	µl O_2-evolution	µl CO_2-uptake	Photosynthetic quotient
Control	2183	2067	1.06 ± 0.02
2×10^{-6}M Simetone	1192	1138	1.06 ± 0.02

40 mm³ cells/vessel, suspended in tap water; data in µl/vessel. hour; saturating light intensity, 25°C.

V.2. Effects on polyphosphate formation

V.2.1. *Inhibition in relation to the concentration of the herbicide*

The influence of simetone on phosphate fixation was studied at a range of concentrations in order to compare the sensitivity of poly P formation with that of oxygen evolution. The compound was added at the beginning of the 30 minutes pretreatment. Fig. 24 shows that 3.3×10^{-5}M simetone which inhibits oxygen evolution for 90% (fig. 16), still has no effect on poly P formation. On the other hand, stronger inhibitions could be obtained than with DCMU (fig. 14); 6.6×10^{-4}M simetone inhibits poly P formation for 84%. This range of concentrations could not easily be applied in the case of DCMU, owing to its low solubility in water.

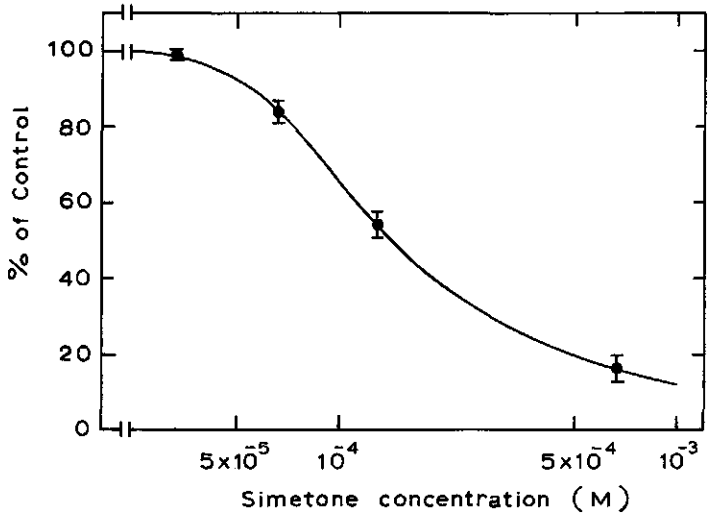


FIG. 24. Effect of various simetone concentrations on phosphate fixation under an N_2-atmosphere; light intensity 40×10^4 ergs/cm².sec., pH ± 4.0, 5 mm³ cells/ml.

V.2.2. *Observations in relation to light intensity*

Since we found that the inhibition of oxygen evolution by simetone was influenced by light intensity (fig. 21), it was of interest to study this effect in poly P formation (table 8). As in the case of DCMU (table 5), at a simetone concentration which inhibits poly P formation for about 50%, viz. 13×10^{-5}M, no effect of light intensity was observed. A simetone concentration of 3.3×10^{-5}M has no effect on poly P formation at high light intensity, but inhibition arises with decreasing light intensity. Lowering down to 2×10^4 ergs/cm².sec. was necessary to obtain a clear effect, which intensity, however, still is above light saturation for poly P formation (fig. 4).

TABLE 8. Effect of two simetone concentrations on phosphate fixation under a nitrogen atmosphere at various light intensities

Simetone concentration	Light intensity in 10^4 ergs/cm^2.sec.	Phosphate fixation		% Inhibition
		Control	with Simetone	
13×10^{-5}M	40	3.2 ± 0.8	1.7 ± 0.3	46 ± 4
13×10^{-5}M	20	2.3 ± 0.1	1.25 ± 0.1	46 ± 4
13×10^{-5}M	10	3.2 ± 0.8	1.8 ± 0.5	44 ± 5
3.3×10^{-5}M	40	5.8 ± 0.5	5.7 ± 0.5	1 ± 1.5
3.3×10^{-5}M	8	4.9 ± 0.1	4.8 ± 0.2	2 ± 3
3.3×10^{-5}M	4	4.55 ± 0.5	4.15 ± 0.6	9 ± 4
3.3×10^{-5}M	2	4.25 ± 0.5	3.35 ± 0.4	21 ± 3

pH ± 4.0, 5 mm^3 cells/ml; data in μg P/ml fixed in 90 min.

V.2.3. *Inhibition in light preferently absorbed by System I*

Fig. 25 shows the effect of a range of simetone concentrations on poly P formation in far red light. This light was again obtained by SCHOTT RG N9 filters on the bottom of the water bath, between the lamps and the vessels. The transmission of these filters has been shown in fig. 2, and table 2 demonstrates that there is almost no activity of PS II in the spectral region thus isolated. A comparison of fig. 25 with figures 16 and 24 shows that the inhibition of poly P formation in far red light by simetone is less strong than the inhibition of oxygen evolution, but stronger than the inhibition of poly P formation in white light.

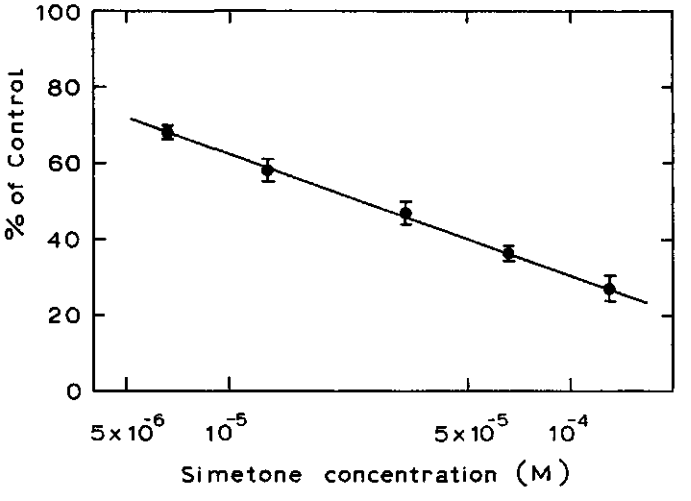


FIG. 25. Effect of various simetone concentrations on phosphate fixation under an N_2-atmosphere and in light, filtered by SCHOTT RG N9 filters; intensity of white light: 40×10^4 ergs/cm^2.sec., pH ± 4.0, 5 mm^3 cells/ml.

a. *Oxygen evolution*

A comparison of the results of this chapter with those of the previous one leads to the conclusion that the inhibitory effects of simetone show much the same features as those of DCMU. Only, simetone is clearly less effective than DCMU, shows no signs of accumulation by the algae, and hence, can be washed out more easily.

At saturating light intensities, oxygen evolution in *Scenedesmus* is inhibited for 50% by about 4×10^{-6}M simetone (fig. 16). There are only few reports in literature on the effect of simetone on photosynthesis. In our hands it was 20 times less effective than DCMU (VAN RENSEN and VAN STEEKELENBURG, 1965); GOOD (1961) reported that simetone inhibits the HILL reaction in isolated chloroplasts for 50% at 2×10^{-6}M, which was 5 times less effective than the closely related simazine.

There is no influence of suspension density on the rate of inhibition by simetone (fig. 17 and 18). This suggests an easy equilibration of concentrations in- and outside the cells. In this connection it is worth while to point to IZAWA and GOOD's conclusion (1965) that in the absorption of CMU, DCMU, and atrazine by isolated chloroplasts at least three simultaneous processes are involved (cf. section IV.3). Of these, process *b*) is concerned with the partitioning between external and internal liquid phases, and reflects the relative solubility of the inhibitor in the two phases. This process depends on the inhibitor used and it is here that one of the important differences between inhibitors is found. Although IZAWA and GOOD (1965) did not include simetone in their partitioning studies, their reasoning suggests that the difference with DCMU (fig. 7 and 8) probably is due to the much higher water-solubility of simetone.

Our finding that the inhibitory effect of simetone on oxygen evolution can easily be removed by washing the cells twice with tap water (fig. 19) confirms BISHOP'S (1962) report on simazine.

Recovery after washing two times with tap water was still within 10% with a concentration of simetone, about 10 times the concentration causing 50% inhibition of oxygen evolution. For DCMU, only the effect of concentrations, not exceeding 2 times that causing 50% inhibition of oxygen evolution could be removed by washing. This difference probably is due to the much higher solubility than DCMU of simetone in water. From these washing experiments it can be concluded that the bond between simetone and its site of action is a loose one.

Respiration is not influenced by 4×10^{-6}M simetone (fig. 21). The fact that low concentrations of simetone inhibit in the light dependent range of the photosynthesis-light curve, and leave the light-saturated level unchanged (fig. 22), suggests the localization of this inhibition in the light dependent reactions leading to the formation of ATP and reduced NADP. A similar conclusion was reached for DCMU in section IV.3. With reference to the discussions for DCMU, the situation for simetone may as well explain why, at a given light intensity, no effect of temperature on the inhibition of simetone was observed

neither in the light-limited nor in the saturated part of the intensity curve, even at relatively high concentrations of the inhibitor (fig. 23 and table 6).

The absence of an effect of simetone on the photosynthetic quotient (table 7) again shows the strong coupling of O_2-evolution and CO_2-uptake under stationary conditions.

b. *Photophosphorylation*

There are no reports in literature on the effect of simetone on cyclic photophosphorylation *in vivo*. We have demonstrated that 3.3×10^{-5}M simetone which inhibits oxygen evolution for 90%, has no effect on poly P formation (fig. 24). This is similar to what was found for DCMU at an appropriate concentration (fig. 14). In the case of DCMU, difficulties with the aqueous solubility prevented the use of concentrations causing more than 50% inhibition. The solubility of simetone being much better, 84% inhibition of poly P formation could be obtained (fig. 24).

Just as in the case of DCMU, no influence of light intensity was observed on the degree of inhibition of poly P formation by a high concentration of simetone, viz. 13×10^{-5}M. However, as with DCMU, inhibition by 3.3×10^{-5}M was light intensity dependent (table 8).

Again, like in the case of DCMU, the inhibition of poly P formation in far red light is higher than that in white light, but lower than the effect on oxygen evolution (see figures 16, 24, and 25).

The above discussion may have shown that also for poly P formation the results are similar to those obtained with DCMU. A discussion on the mode of action of DCMU and simetone in relation to current views on the course of photosynthesis will be attempted in the general discussion (section VII.2.1.).

VI. EFFECTS OF THE BIPYRIDYLIUM COMPOUND DIQUAT

VI.1. Effects on oxygen exchange[1]

VI.1.1. *Inhibition of oxygen evolution in relation to the concentration of the herbicide*

Literature (Mees, 1960, and Homer *et al.*, 1960) reports that darkness during treatment with diquat retards the increase of the effect of the herbicide with time. In order to test this, inhibition of oxygen evolution was determined in suspensions, kept in contact with various diquat concentrations for 30, 60, or 90 minutes in darkness (fig. 26). Besides, the effect of a range of concentrations on O_2-production was measured in suspensions that had been kept in contact with the herbicide for 30, 60, or 90 minutes in the light (fig. 27). Both figures show that the inhibition increases with the duration of the contact. Comparison of figures 26 and 27 shows that at diquat concentrations below 10^{-4}M, pretreatment in darkness yields a smaller effect on subsequent light-induced O_2-evolution than pretreatment in the light. In further experiments, the effect of 2×10^{-5}M diquat was mostly measured one hour after addition of the herbicide, while the algae were kept in the light during this time. This treatment yields an inhibition of about 50%.

VI.1.2. *Stimulation of oxygen uptake in relation to the concentration of the herbicide*

In view of results of Mees (1960), showing that oxygen consumption by bean

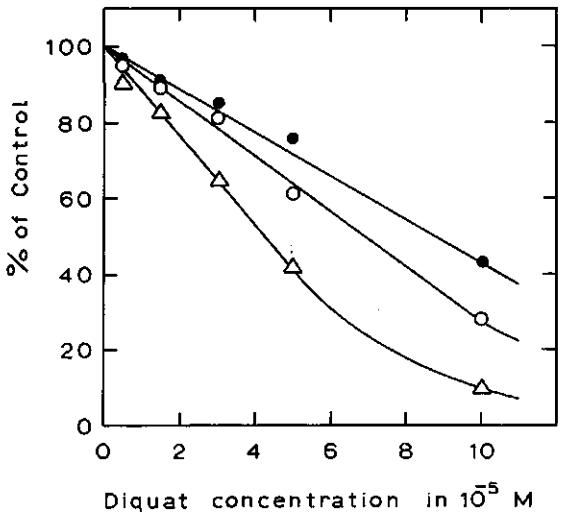


Fig. 26. Inhibition of O_2-evolution by different concentrations of diquat after 30 (●), 60 (○), and 90 (△) minutes dark incubation with the herbicide; 4 mm³ cells/ml.

[1] Some results from this section have been presented in: Van Rensen (1969*a*).

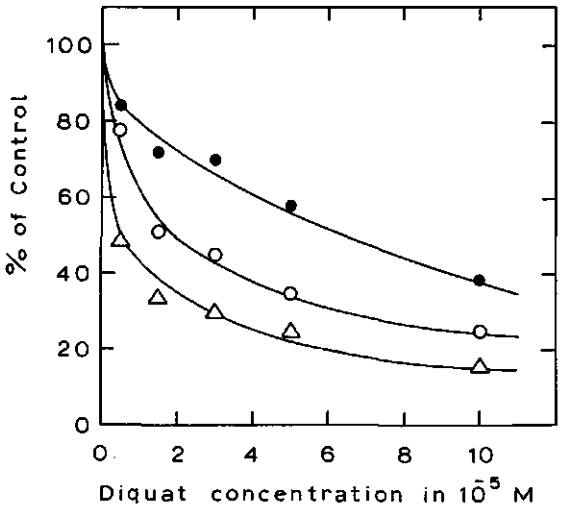


FIG. 27. Inhibition of O_2-evolution by different concentrations of diquat after 30 (●), 60 (○), and 90 (△) minutes incubation with the herbicide in light; 3 mm^3 cells/ml.

leaf discs sometimes was initially stimulated upon addition of diquat, the effect on O_2-uptake in *Scenedesmus* was studied. It follows from fig. 28 that oxygen consumption indeed is increased after addition of the herbicide. The stimulation increases with time; 30 minutes after addition of 2×10^{-5}M diquat O_2-uptake is 165% of the control, and 220% with 10^{-4}M. After 60 minutes, these values are 185% and 235%, for 2×10^{-5}M and 10^{-4}M respectively. It should be added that with still longer incubation times, e.g. 120 minutes after addition, 2×10^{-5} M diquat inhibits oxygen uptake for 20%, and 10^{-4}M diquat does so for 50%.

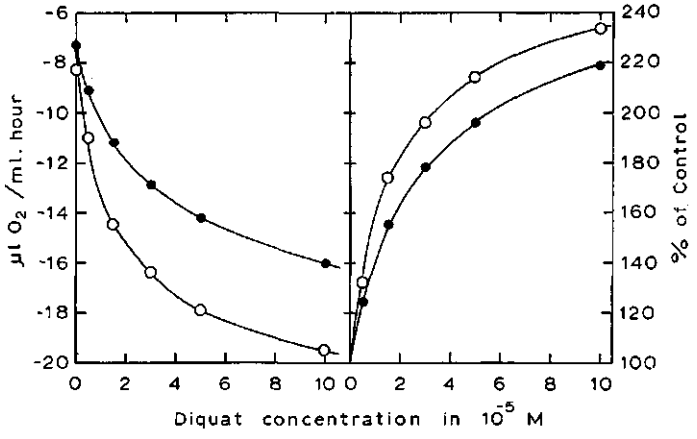


FIG. 28. Effect of various concentrations of diquat on O_2-uptake in the dark after 30 (●) and 60 (○) minutes dark incubation; 4 mm^3 cells/ml.

VI.1.3. *Inhibition as affected by suspension density of the algae*

Oxygen evolution was measured at five different suspension densities, then, 2×10^{-5}M diquat was added to the vessels, and one hour later the measurement of O_2-production was started again. Photosynthesis was measured under conditions of light-saturation, also at the highest suspension density. The inhibition percentage becomes lower at higher suspension densities (fig. 29), which points to some degree of accumulation of the herbicide by the cells.

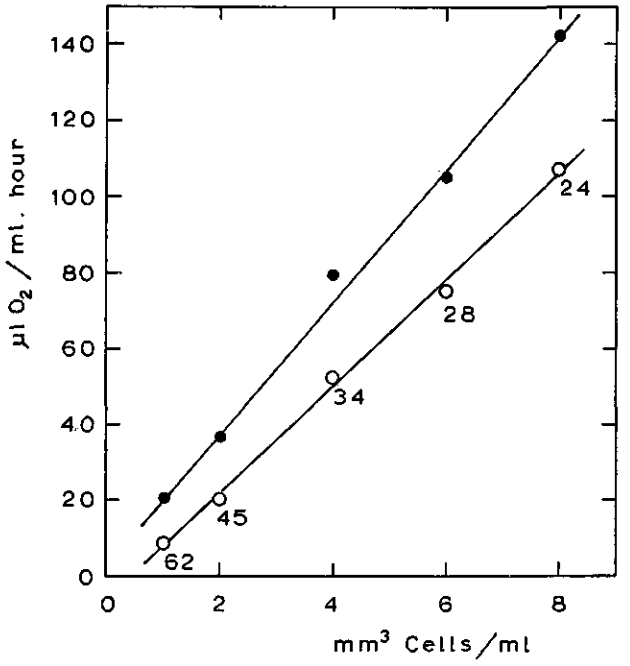


FIG. 29. Effect of 2×10^{-5}M diquat on O_2-evolution at different suspension densities; • = control, i.e. before incubation with the herbicide, ○ = after one hour incubation with the herbicide in the light; numbers along lower line: percent inhibition.

VI.1.4. *Washing experiments*

After the estimation of the influence of the herbicide upon oxygen evolution, the cells were centrifuged and washed twice with tap water. Hereafter, they were resuspended in a fresh amount of buffer solution to the same density as before, and the O_2-production was measured again. Fig. 30 shows that it is impossible to remove by washing the inhibition caused by diquat. Also with suspensions of cells, incubated with diquat for 60 minutes in the dark, the inhibition of photosynthesis after washing remains the same as before.

This result can be explained in two ways: either the herbicide cannot be washed out of the cells, or it has irreversibly damaged the photosynthetic apparatus. In order to distinguish between these two possibilities, we have tried to

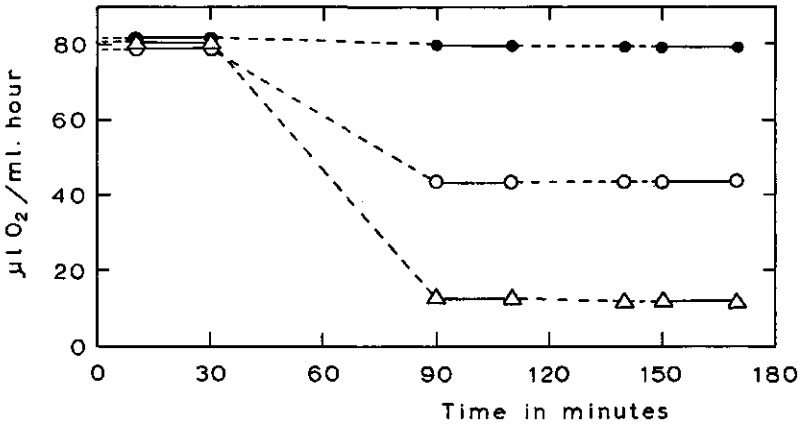


FIG. 30. Washing experiment; ● = control, ○ = 2×10^{-5}M diquat, △ = 10^{-4}M diquat, 4 mm³ cells/ml; 10–30 min.: measurement before addition; 90–110 min.: ditto in the presence of diquat; 150–170 min.: ditto after washing.

prevent damage to the photosynthetic apparatus by flushing the algal suspension with pure nitrogen. In fig. 31, at arrow 1, the lights were turned on. Between arrows 2 and 3, oxygen evolution was measured. At 3, the lights were turned off, and N_2-flushing was started. After 10 minutes, at arrow 4, the lights were turned on again, and 2×10^{-5}M diquat was added. Between arrows 5 and 6, oxygen evolution is measured again. At arrow 6, the algae were centrifuged, washed twice with tap water, and resuspended in fresh buffer at the same suspension density as before. At arrow 7, the lights were turned on, and at 8, oxygen evolution was measured for the third time. As is shown in fig. 31, under these conditions, the inhibition after washing is only about 1/3 of that in fig. 30.

Another, interesting way to protect the photosynthetic apparatus from damage is to prevent the reduction of diquat by blocking the electron transport chain at a site in front of the place of reduction of diquat. This can be done by adding the herbicide simetone which affects electron transport somewhere close to PS II. As has been shown (VAN RENSEN and VAN STEEKELENBURG, 1965), the inhibition caused by simetone concentrations up to 10^{-5}M can be completely removed by washing. In the experiment shown in fig. 32 and table 9, the lights were turned on at arrow 1, and the rate of oxygen evolution measured between arrows 2 and 3. At 3, two different concentrations of simetone were added to separate vessels; between arrows 4 and 5, the effect of simetone was measured. At 5, diquat was added (in the light in the presence of air), and the combined effect of both herbicides is shown between arrows 6 and 7. At 7, the algae were centrifuged, washed twice with tap water, and resuspended in fresh buffer at the same suspension density as before. At arrow 8, the lights were turned on and at 9, measurements of oxygen evolution were started again.

Fig. 32 and table 9 show that 2×10^{-6}M and 10^{-5}M simetone inhibit photosynthesis for 45 and 86 percent, respectively. The effects of simetone and diquat

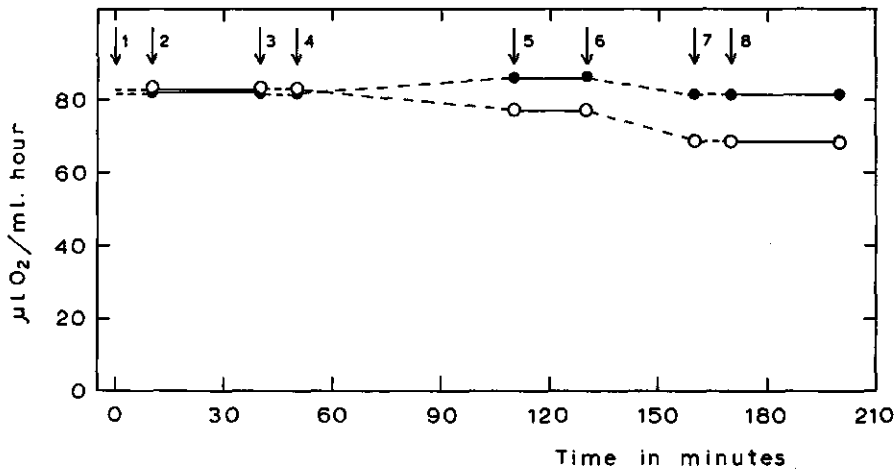


FIG. 31. Washing experiment; 2×10^{-5}M diquat added in the absence of oxygen; 4 mm³ cells/ml; 2–3: controls without diquat; at 4: diquat added to ○ only; 5–6: effect of diquat after one hour incubation in light, without oxygen; after 8: after washing; further explanation see text.

on photosynthesis are additive: 2×10^{-6}M simetone plus 2×10^{-5}M diquat inhibit for 84% and 10^{-5}M simetone plus 2×10^{-5}M diquat cause complete inhibition. After washing, it turns out that 2×10^{-6}M simetone has failed to prevent damaging of the photosynthetic apparatus caused by diquat, because

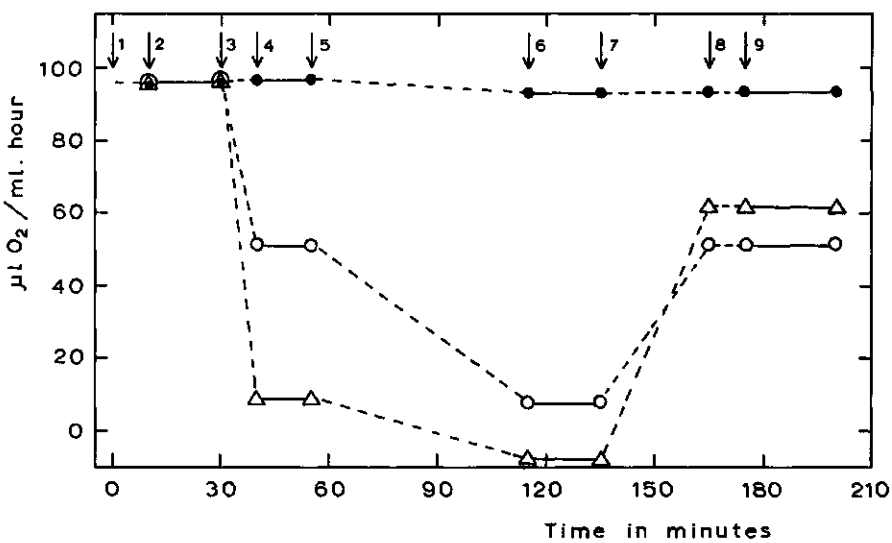


FIG. 32. Washing experiment; 2×10^{-5}M diquat added in the presence of simetone; ● = control, ○ = 2×10^{-6}M simetone, △ = 10^{-5}M simetone; 4 mm³ cells/ml; 2–3: controls; 4–5: effect of simetone; at 5: diquat added; 6–7: combined effects of simetone and diquat; after 9: after washing; further explanation see text.

TABLE 9. Washing experiment with diquat in the presence of simetone

	Before addition of herbicides	After addition of simetone	After addition of both herbicides	After washing
Control	102	103 (0)	99 (0)	99 (0)
2×10^{-6}M Simetone 2×10^{-5}M Diquat	102	56 (45)	16 (84)	58 (41)
10^{-5}M Simetone 2×10^{-5}M Diquat	102	14 (86)	0 (100)	69 (31)

Experimental procedure: the same as that for Figure 32; 4 mm³ cells/ml; data in µl O_2/ml. hour, corrected for respiration; numbers in parentheses: percentages of inhibition.

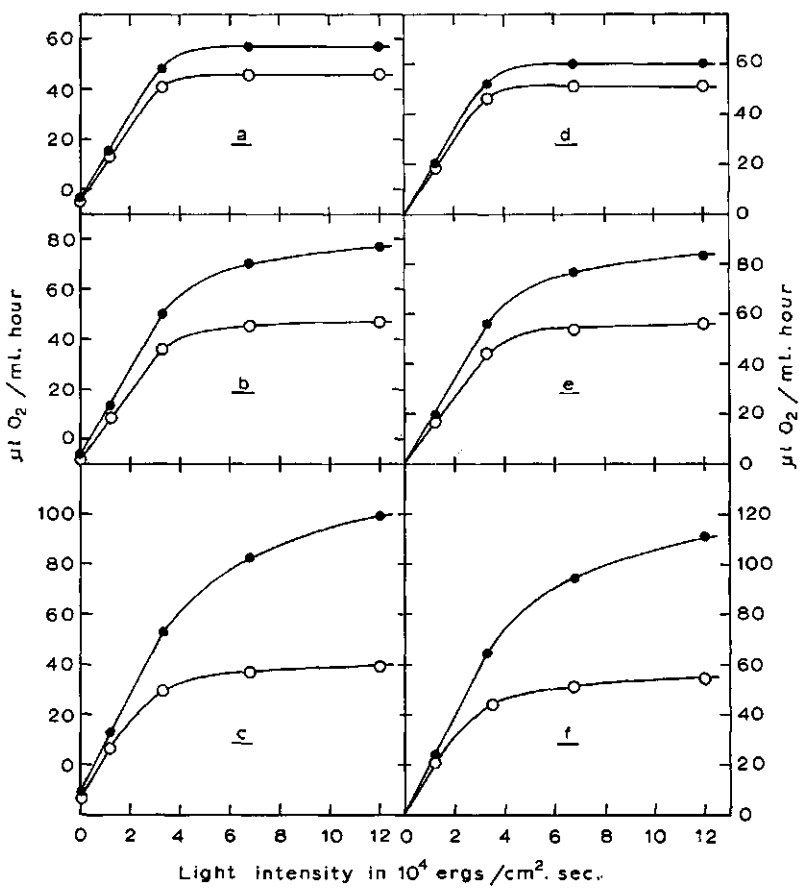


FIG. 33. Effect of 2×10^{-5}M diquat on O_2-evolution at different light intensities and different temperatures; ● = before addition, ○ = one hour after addition of the herbicide; a = 20°C, b = 25°C, c = 30°C; d, e, and f are the same experiments as a, b, and c respectively, data corrected for dark O_2-uptake; 4 mm³ cells/ml.

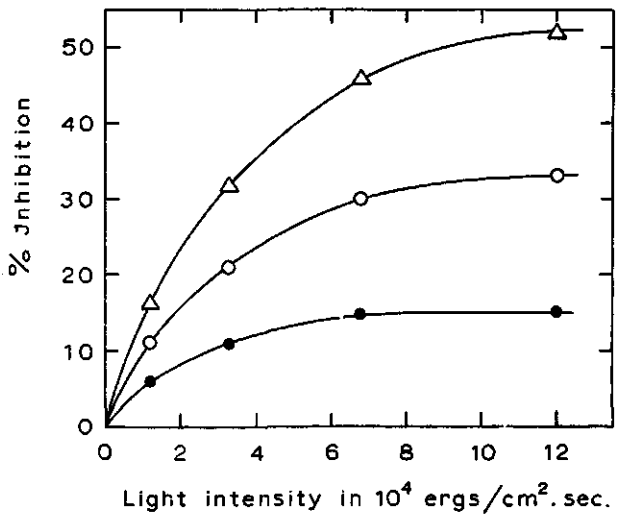


FIG. 34. Effect of temperature on inhibition percentages at different light intensities; calculated from the data of figure 33d, e, and f; ● = 20°C, ○ = 25°C, △ = 30°C.

the remaining inhibition is 41 %, which is about the same as in fig. 30. In the case of the higher simetone concentration, the inhibition left after washing is 31 % which is less than that found in fig. 30. Thus, 10^{-5}M simetone was found effective in protecting to some extent the photosynthetic apparatus from damage by diquat.

We had hoped to be able to apply a simetone concentration sufficiently high for complete inhibition of electron transport in photosynthesis. In that case, no diquat would be reduced and no inhibition of oxygen evolution would be left after washing. Unfortunately, the inhibition of photosynthesis, caused by still higher concentrations of simetone cannot completely be removed by washing (section V.1.3.), and therefore, a complete demonstration of the aim in view could not be achieved.

VI.1.5. *Inhibition as affected by light intensity and temperature*

Fig. 33 shows the effect of 2×10^{-5}M diquat on oxygen evolution at different light intensities and three different temperatures. Dark respiration is stimulated by diquat at all three temperatures (fig. 33a, b, and c). The percentage of inhibition of oxygen evolution is almost the same at all light intensities (fig. 33a, b, and c). However, when the curves are corrected for oxygen uptake in the dark, the percentages inhibition increase with temperature and with light intensity until light saturation is reached (fig. 33d, e, f, and fig. 34).

VI.1.6. *Effect of diquat on the photosynthetic quotient*

Additionally, the effect of diquat on the photosynthetic quotient was measured during a short period (45 mins) after the addition of the herbicide. Oxygen

TABLE 10. Effect of diquat on the photosynthetic quotient during the first 45 minutes after addition of the herbicide

	μl O_2-evolution	μl CO_2-uptake	Photosynthetic quotient
Control	1271	1207	1.06 ± 0.02
10^{-4}M Diquat	278	219	1.28 ± 0.05

40 mm^3 cells/vessel, suspended in tap water; data in μl/vessel. 45 min., saturating light intensity, 25°C.

evolution and CO_2-uptake were measured simultaneously by the method described in section II.3.b.α. Table 10 shows that CO_2-uptake is relatively more inhibited than O_2-evolution, leading to an increase of the photosynthetic quotient.

VI.2. EFFECTS ON POLYPHOSPHATE FORMATION

VI.2.1. *Inhibition in relation to the concentration of the herbicide*

Fig. 35a demonstrates the effect of various diquat concentrations on phosphate fixation (in contact with an N_2-atmosphere). The herbicide was added at the beginning of 30 minutes dark pretreatment. 3.3×10^{-5}M diquat has no effect on phosphate fixation, while 3.3×10^{-4}M inhibits it for about 50%. Difficulties with the phosphate determination at higher diquat concentrations prevented the use of concentrations higher than 3.3×10^{-4}M.

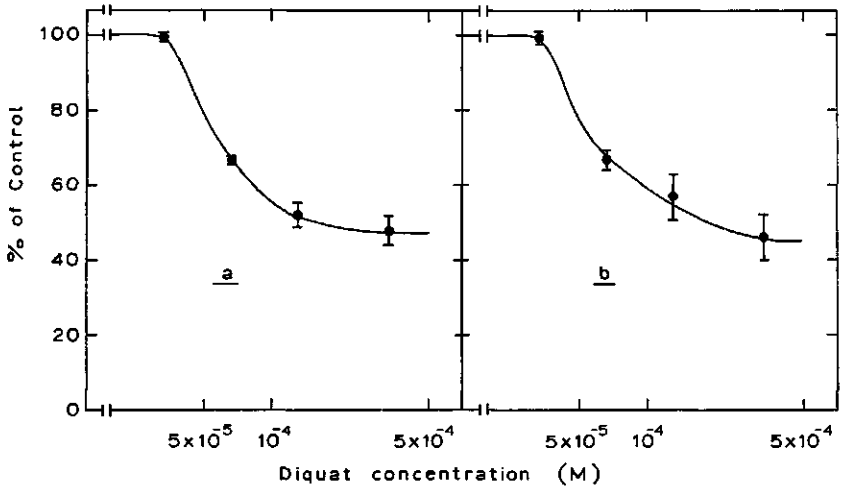


FIG. 35. Effect of different diquat concentrations on phosphate fixation under an N_2-atmosphere, in the absence (a) and in the presence (b) of 1.3×10^{-6}M DCMU; light intensity 40×10^4 ergs/cm^2.sec., pH ± 4.0, 5 mm^3 cells/ml.

To check whether the inhibition was due to damage of the photosynthetic apparatus by diquat in connection with oxygen produced, the experiment was repeated while 1.3×10^{-6}M DCMU was added which completely inhibits oxygen evolution, but still has no effect on phosphate fixation (fig. 36). In fig. 35b, DCMU was added first and, after 30 minutes of dark incubation under nitrogen flushing, diquat was added. After another 30 minutes dark period, the lights were turned on, and photophosphorylation measured 90 minutes later. It is clear that figures 35a and b are the same, thus it is unlikely that the inhibition is a result of damage to the photosynthetic apparatus in the light by produced oxygen.

VI.3. Discussion

a. *Oxygen evolution*

Surveying the results of this chapter, it can be concluded that the effects of diquat on photosynthesis are very different from those of DCMU and simetone.

In fig. 27, the effect of various diquat concentrations on oxygen evolution is shown after 30, 60, and 90 minutes incubation in the light. Incubation of the cells with 2×10^{-5}M diquat in the light during one hour results in about 50% inhibition of oxygen evolution; the inhibition increases with time. Incubation with diquat at concentrations lower than 10^{-4}M in darkness yields weaker inhibition of subsequent oxygen evolution in the light (fig. 26). The retarded inhibition after incubation in darkness is not due to slower uptake of diquat by the algae in the dark since uptake was the same as in the light (VAN RENSEN, unpublished data). A similar experience was reported by BRIAN (1967) who found that darkness even increased uptake of diquat and paraquat by tomato, sugar beet, and cocksfoot leaves.

Diquat reduction occurs both in relation to photosynthesis and in relation to respiration; in *Scenedesmus* cells, respiration has a far lower capacity than photosynthesis. Therefore as compared with the above explanation, it appears more attractive to explain the retardation by darkness of the inhibitory effect on subsequent oxygen evolution in the light by the fact that, in the dark, much less diquat is reduced than in the light. As a consequence, much smaller amounts of toxic substances, as e.g., hydrogen peroxide (DAVENPORT, 1963) are produced, resulting in less damage to the photosynthetic apparatus.

MEES (1960) and HOMER *et al.* (1960) also reported weaker herbicidal activity of diquat in darkness. DAVIES and SEAMAN (1968*a*) showed that, with diquat, darkness increased the incubation time needed to kill *Elodea*, as compared with light.

We have observed that diquat stimulates oxygen uptake in the dark 30 and 60 minutes after addition. Stimulation increases with diquat concentration and time (fig. 28). However, additional experiments have shown that, 120 minutes after addition, the stimulation of oxygen uptake changes into inhibition. MEES (1960) also found stimulation of oxygen consumption in bean leaf discs by

diquat, and inhibition later on. FUNDERBURK and LAWRENCE (1964) reported stimulation of O_2-uptake by *Lemna minor*, caused by diquat and paraquat. TURNER *et al.* (1970) observed a rapid drop in the rate of oxygen evolution upon addition of high concentrations of diquat to *Chlorella vulgaris*, followed by oxygen uptake which later on again decreased. This was explained by the assumption of an immediate, large increase in oxygen uptake, accompanied by irreversible inhibition of photosynthetic oxygen evolution, while later on, oxygen uptake again decreased.

According to fig. 29, the inhibition percentages decrease at higher suspension densities. This points to accumulation of diquat by the algal cells; because there are fewer sites of action at lower suspension densities so that the amount of diquat available per cell is higher. DAVIES and SEAMAN (1968) reported rapid initial uptake of diquat in *Elodea*, probably representing passive adsorption of the herbicide onto plant surfaces which was completed after the first 10 or 20 minutes, and was followed by a slower long-term uptake, probably due to metabolic accumulation.

The inhibiting effect of diquat on oxygen evolution cannot be removed by washing (fig. 30). Two possible explanations suggest themselves. First, it could be due to irreversible binding of diquat by the cells, secondly to irreversible damage of the photosynthetic apparatus by the supposed toxic peroxides. Flushing the algal suspension with nitrogen before and during the treatment with diquat was found to protect the photosynthetic apparatus from damage (fig. 31), the inhibition left after washing being only about 1/3 of that observed in a similar experiment in the presence of oxygen. The inhibition left after washing in the oxygen-free case could be due to some oxygen, left in the suspension or to some diquat left after washing. Anyhow, this experiment shows that diquat can be removed to a high extent by washing so that there is no irreversible binding. Similarly, BALDWIN *et al.* (1968) reported 90% loss in three washings of the other bipyridilium herbicide paraquat from an isolated chloroplast suspension. It is important to note that, one hour after the addition, there is more diquat in the algal cells under nitrogen flushing conditions than in the presence of air (VAN RENSEN, unpublished data).

TURNER *et al.* (1970) reported that the inhibitory effect of diquat on oxygen evolution in *Chlorella vulgaris* could be decreased by lowering the oxygen concentration in the WARBURG vessels. They also demonstrated lack of recovery of apparent photosynthesis in diquat-treated *Chlorella* cells after washing. This lack of recovery was not due to active diquat remaining in the washed cells, but to irreversible inhibition.

The effects of simetone and diquat on photosynthesis in our experiments were additive when simultaneously supplied (fig. 32 and tabel 9) as was also found by VAN OORSCHOT (1964). FUNDERBURK and LAWRENCE (1964) showed that the effects of a combination of CMU and diquat on O_2-production in *Lemna minor* were additive as well. On the other hand, 10^{-5}M simetone supplied separately beforehand, in our experiments was found to protect the photosynthetic apparatus with regard to the effect of diquat, supplied later (fig. 32 and table 9).

VAN OORSCHOT (1966) reported that a still higher simetone concentration prevented the development of symptoms, caused by simultaneously supplied diquat in bean leaves. This can be explained by the inhibitory effect of simetone on electron transport in the photosynthetic system (fig. 38) at a place in front of the site of diquat reduction.

In summary, our washing experiments have shown: 1) that diquat can be washed from the algal cells, 2) that simetone protects the cells against damage by diquat, and, 3) that reduction of diquat and the presence of oxygen are essential for the inhibiting effect on oxygen evolution.

A more close study of the effect of light intensity and temperature on the inhibition of O_2-evolution by diquat revealed that respiration is stimualted at the three temperatures applied, and that the percent inhibition of oxygen production is almost equal at different light intensities (fig. 33a, b, and c). However, after correction of the curves for dark O_2-uptake, the percentages of inhibition increase with light intensity, while, beyond a certain value, the procentual inhibition apparently shows light saturation (fig. 33d, e, f, and fig. 34). DAVIES and SEAMAN (1968*a*) found that, in the presence of diquat, the procentual dedrease in vitality of *Elodea* increased when the light intensity applied during the treatment with the herbicide was higher. The result obtained by VAN OORSCHOT (1966), who reported equal inhibition percentages at different light intensities, can be explained by the fact that he treated the leaves with diquat in the dark and measured the effect on subsequent CO_2-uptake in the light, whereas in our experiments the cells were treated with diquat in the light. As discussed above, darkness retards the inhibitory effect of diquat (fig. 26 and 27). VAN OORSCHOT, in the same paper, reported that, when plants were treated in the light, those at high light intensity developed earlier and more pronounced symptoms than those at low light intensity or in darkness.

Diquat was found to inhibit both in the light-limited and in the light-saturated part of the photosynthesis-light curves (fig. 33). This shows that diquat belongs to the group of inhibitors affecting light dependent reactions as well as dark reactions in photosynthesis. The observation that diquat itself can be washed out while its effect remains, leads to the conclusion that, in this case, the dual effect is due to secondary reactions leading to a general disturbance of the photosynthetic apparatus. Another indication for this conclusion is that the inhibition percentage is temperature dependent (fig. 34); an inhibition of light dependent reactions only, should be temperature independent. TURNER *et al.* (1970) also observed that, at a given light intensity, the degree of inhibition of photosynthesis in *Chlorella vulgaris* by diquat increased with increase in temperature.

It is clear that oxygen, light and increased temperature increase the percentage inhibition of photosynthesis by diquat. This is also in accordance with results reported by MEES (1960) and MERKLE *et al.* (1965)

The secondary effect of diquat and paraquat, the disturbance of cell structure by hydrogen peroxide or peroxide radicals, has been studied by various authors. MERKLE *et al.* (1965) showed rapid bleaching of the pigment system of broadleaf

bean by paraquat in the presence of light and O_2. Immersion of *Elodea* in diquat solutions caused increase in chlorophyll destruction in the course of time (DAVIES and SEAMAN, 1968*a*).

Breakdown of the regular pattern of chloroplast lamellae and grana was frequently observed. LANG and SEAMAN (1964) made electron microscope observations of *Lemna* and *Azolla* plants treated with diquat and paraquat, indicating major differences in the ultrastructure of chloroplasts in treated plants as compared with the controls. BAUR *et al.* (1969) studied the effect of paraquat on the ultrastructure of mesquite mesophyll cells by electron microscopic techniques. The first visible change induced by the herbicide was a rapid disintegration of the plasmalemma, followed by rupture of the chloroplast membranes and loss of chloroplast turgor. No changes were noted in mitochondria, GOLGI bodies. endoplasmic reticulum or nucleus, nor in the composition of the cytoplasm or cell walls of tissues treated with paraquat up to 5 hours. The observation that chloroplast structure is affected before an effect on other cell structures is found, is probably due to the fact that in photosynthetic tissue catalase is present only to a small extent in the chloroplast, as was reported by BALDWIN *et al.* (1968*a*) and GREGORY (1968). STOKES *et al.* (1970) demonstrated bleaching of chlorophyll in *Chlorella vulgaris* by diquat when the cells were illuminated in water or in phosphate or bicarbonate buffer. Studying the fine structure of the cells, they found considerable damage caused by diquat to cell membranes in less than 10 hours of illumination; however, inhibition of both photosynthesis and respiration of the plastids precedes any damage visible under the microscope.

ZWEIG *et al.* (1965) showed that in the photosynthetic electron transport chain, diquat reduction replaces reduction of NADP; this is to be considered as the primary effect of diquat on photosynthesis. Thus, during a short time after addition of diquat, it acts as a HILL-oxidant for O_2-evolution, while CO_2-uptake is decreased by lack of reduced NADP. This implies that the photosynthetic quotient should be increased during the first time span after addition of diquat, and it was indeed observed that, at adequate diquat concentrations, CO_2-uptake initially is somewhat more inhibited than O_2-evolution, resulting in an increased photosynthetic quotient during the first 45 minutes after addition of diquat (table 10). BAUR *et al.* (1969) state that paraquat causes waste of photosynthetic reducing potential by utilizing electrons produced in PS I for the reduction of the herbicide instead of ferredoxin. Ultimately, this leads to a decrease in starch deposit in chloroplasts, as observed in their experiments.

b. *Photophosphorylation*

These experiments were performed under flushing of the algal suspensions with nitrogen.

Diquat was found to inhibit phosphate fixation (fig. 35a). To be sure that the above effect was not due to oxygen production by the algae, the experiment was repeated in the complete absence of oxygen, which could be assured when diquat was added in the presence of 1.3×10^{-6}M DCMU. This completely inhibits oxygen evolution, but still has no effect on phosphate fixa-

tion (fig. 36). The curve in the presence of DCMU is the same as in its absence (fig. 35b). Inhibition of phosphate fixation by diquat in the absence of oxygen cannot be explained by the above expounded mode of action of diquat, and will be discussed in section VII.2.2.

VII. GENERAL DISCUSSION

VII.1. POLYPHOSPHATE FORMATION AS A TOOL FOR MEASURING CYCLIC PHOTOPHOSPHORYLATION *in vivo*

At the 1955 Gatlinburg Conference on photosynthesis, WASSINK proposed a scheme representing the relations between photosynthesis, polyphosphate formation, and the metabolism of the cell (WASSINK, 1957). This scheme suggested that the light dependent reactions of photosynthesis produce a pool of energy-rich phosphate. This pool can be emptied along three different pathways: (a) in the presence of CO_2, the major part is used in the CO_2-reduction cycle, whereby inorganic phosphate is regenerated, giving rise to a decreased overall phosphate fixation. In fact, this is illustrated in fig. 3 of the present paper; (b) in the presence of sugar, an additional pathway (denoted as 'oxidative assimilation') equally leads back to inorganic phosphate; (c) when both (a) and (b) are curtailed, the formation of polyphosphates is observed, obviously resulting from overfilling of the energy-rich phosphate pool. This also expresses itself in fixation of inorganic phosphate, as shown in our fig. 3. It appears that the principles of this scheme still form a useful basis to start a discussion of more recent results.

With respect to the energy-rich phosphate pool, KYLIN and TILLBERG (1967*a*) found an inhibition of ATP formation in *Scenedesmus* by phloridzin and high concentrations of inhibitor-β complex from potato, while poly P formation was not affected. The authors concluded herefrom to the existence of a common precursor $X \sim P$ in the formation of both ATP and poly P. The conversion of $X \sim P$ to ATP was supposed to be sensitive to phloridzin and high concentrations of inhibitor-β, while the conversion of $X \sim P$ to poly P was not. It is interesting to note that also WASSINK and ROMBACH (1954) obtained indications for the formation of a phosphate compound, accumulating more rapidly than ATP.

In the absence of CO_2, poly P formation as described in the present paper occurs under conditions of restricted ATP demands. With regard to poly P formation under these conditions, the question whether the energy-rich phosphate pool mainly contains ATP or other energy-rich intermediates or energy-rich states prior to the formation of ATP, appears irrelevant.

The demands for energy-rich phosphates induced by sugar supply has been thoroughly investigated in KANDLER's laboratory (see e.g. TANNER *et al.*, 1965, and KANDLER and TANNER, 1966). Addition of glucose to starved *Chlorella* cells results in assimilation of glucose to starch and oligosaccharides. The assimilation of one glucose was found to require two ATP. The ATP for this glucose-assimilation is generated by respiration (in the presence of oxygen) or by photosynthesis (in light). These authors demonstrated that, *in vivo*, under appropriate conditions (light, anaerobiosis, and absence of CO_2), glucose-assimilation is accompanied by cyclic photophosphorylation. These results are in accordance

with the finding of WASSINK *et al.* (1951*a*) that addition of glucose decreased the rate of phosphate fixation in *Chlorella* in the absence of CO_2. This can be understood as utilization of ATP in glucose-assimilation which tends to keep the inorganic phosphate in circulation.

AVRON and NEUMANN (1968) have considered several ways to determine the output of cyclic photophosphorylation *in vivo*.

In a direct approach, ATP levels or the rates of phosphate uptake were determined under conditions where the only ATP producing process was cyclic photophosphorylation. FORTI and PARISI (1963) showed a light-induced increase of ATP levels in leaves; this increase was observed also in the presence of CMU under strictly anaerobic conditions as well as in air. SIMONIS and co-workers measured the rate of phosphate uptake in *Ankistrodesmus braunii* under a variety of conditions (see e.g. SIMONIS, 1967).

In an indirect approach, reactions are measured which depend upon a steady supply of ATP. MARRÉ *et al.* (1963) reported stimulation by light of divalent ion uptake, also in the absence of CO_2. MACROBBIE (1965) demonstrated that potassium uptake in *Nitella* can be supported by cyclic photophosphorylation alone, because it could proceed in far-red light or in the presence of low concentrations of DCMU. Light-induced acetate assimilation in *Chlamydobotrys* has been shown by WIESSNER (1963) to depend on a photophosphorylation which is insensitive for DCMU concentrations, even for those completely inhibiting oxygen evolution. In leaves of *Elodea densa*, in the absence of CO_2, and also in far-red light, cyclic photophosphorylation provides the energy for active chloride uptake in the light (JESCHKE, 1967). Light-induced assimilation of glucose in *Chlorella* demonstrates the presence of cyclic photophosphorylation *in vivo* (TANNER *et al.*, 1965, and KANDLER and TANNER, 1966).

To AVRON and NEUMANN's enumeration we may now add the light-induced formation of polyphosphates in the absence of CO_2. That it demonstrates the presence of cyclic photophosphorylation *in vivo* is shown by its occurrence in far-red light (table 2 and figure 5) and its insensitivity to DCMU concentrations fully inhibitory for oxygen evolution (fig. 36). It is also similar to glucose-assimilation in its light saturation at low intensities as compared with light saturation of oxygen evolution (fig. 4).

With the method of cultivation of algae employed until now, in different harvests poly P formation is rather variable (see e.g. table 1). MARKAROVA and BASLAVSKAYA (1969) reported that the absolute amounts of poly P in cells are not the same in different experiments, owing to conditions of culturing, the supply of the cells with nutritional elements, the age of the culture, the relative proportions of cells at different phases of development, etc. It is our general experience that poly P formation is more pronounced in very young cultures than in older ones. This is related to the finding of CORRELL and TOLBERT (1962) that young cultures (< 5 mm^3 cells/ml) of *Anabaena* contained excessive amounts of poly P, while in older cultures the poly P content was very much lower. Possibly, the variability in phosphate fixation in different harvests could be decreased by

using continuous culturing devices, maintaining the suspension density at a low level. Also, synchronization and harvesting at a favourable time in the growth cycle (BAKER and SCHMIDT, 1964) perhaps could produce more constant results in different harvests.

VII.2. MECHANISMS OF ACTION OF THE HERBICIDES AND IMPLICATIONS OF THE OBSERVED EFFECTS FOR THE MECHANISM OF PHOTOSYNTHESIS

VII.2.1. *DCMU and simetone*

Owing to common use of DCMU as an inhibitor of PS II-dependent processes, numerous data on effects of DCMU in photosynthetic processes are available in literature. It is clear that not all these can be discussed. Three possible sites of action within the photosynthetic process have been reported and will be discussed in some detail.

WITT and coworkers (WITT *et al.*, 1961, and WITT *et al.*, 1966) assumed that DCMU interferes with the oxidizing side of PS II. Also KESSLER (1968) favoured the idea of an inhibition at this site. KESSLER proposed that the action at this site may account for the decreased steady-state fluorescence intensity under hydrogen-adaptation as compared with that under aerobic conditions in *Ankistrodesmus* cells in the presence of DCMU. WITT's group proposed this site-of-action of DCMU in order to explain its effect on the 515 nm absorbance change in *Chlorella* and in isolated chloroplasts. However, this conclusion has been questioned by FORK and DE KOUCHKOVSKY (1966); these authors assumed DCMU to act between the first reduced substrate (Q) of PS II and the pool of electron transport intermediates between PS II and PS I.

DUYSENS and SWEERS (1963) were the first to point out that DCMU might act at the reducing side rather than at the oxidizing side of PS II. This view now is accepted by most workers, using DCMU. Because DCMU prevented PS II from reducing cytochrome *f* in *Porphyridium cruentum*, DUYSENS *et al.* (1961) located the site of inhibition close to PS II.

At this point it seems appropriate to recall in mind that ORNSTEIN *et al.* (1938) suggested that the energy of the excited chlorophyll is transferred to a compound A_o, which then gives rise to the formation of a reducing substance. WASSINK and KATZ (1939) then demonstrated that high chlorophyll fluorescence is related with a reduced state of the photosynthetic apparatus. This group also showed that fluorescence of *Chlorella* is stimulated by concentrations of ethylurethane (related to DCMU), which partly inhibit photosynthesis (WASSINK *et al.*, 1938).

Additionally, WASSINK *et al.* (1942), with *Chromatium*, demonstrated in fluorescence experiments that the hydrogen donor system is much more closely related to the process of energy transfer than the CO_2-reducing system. This seems in a line with recent observations on the site of action of DCMU.

DUYSENS and SWEERS (1963) found that in the presence of DCMU chlorophyll a_2 fluorescence rapidly increased and remained high in the steady state, and

that light absorbed specifically by System I was not able to decrease this fluorescence. DUYSENS and SWEERS, therefore, concluded that DCMU prevents the reoxidation of quencher Q by PS I but not its reduction by PS II. Since low concentrations of DCMU inhibit the reduction of plastoquinone (DUYSENS, 1963*a*), the inhibition site could be more closely defined as between quencher Q and plastoquinone. JOLIOT (1966) compared the effects of CMU and NH_2OH on oxygen evolution and fluorescence in *Chlorella pyrenoidosa* and found that hydroxylamine, unlike CMU, does not increase the fluorescence intensity of the cells (cf. the analogous observations in WASSINK *et al.*, 1942, pp. 330–334) even when oxygen evolution is completely inhibited, and that addition of CMU to cells inhibited by hydroxylamine increases the fluorescence to the same level as obtainable with CMU alone. From these and other results Mrs. JOLIOT concluded that NH_2OH interacts with the component Y (fig. 40) and that CMU prevents the reoxidation of Q (fig. 40). Also IZAWA *et al.* (1969) and ELSTNER *et al.* (1970), studying the effects of NH_2OH and DCMU on electron transport in isolated chloroplasts, concluded that hydroxylamine inhibits between PS II and the 'watersplitting system', and that DCMU acts on the reducing side of PS II. Among others, also MURATA *et al.* (1966) from a study on the kinetics of fluorescence, and YAMASHITA and BUTLER (1968), from photoreduction studies with tris-washed chloroplasts, proposed the same site of action of DCMU. A full discussion of the implications of these several findings is beyond the scope of our present paper. We may state, however, that our hypothesis about the mechanism of action of DCMU (see below) is also compatible with the views expressed.

An inhibition site close to PS I was proposed by ASAHI and JAGENDORF (1963). They found inhibition of pyocyanine-mediated cyclic photophosphorylation in broken, aged chloroplasts by rather high (10^{-4}M) concentrations of CMU. Therefore, they postulated that the electron transport on the reducing side of pyocyanine (i.e. PS I) involves another CMU-sensitive site. A factor, capable of reversing this inhibition was purified and seemed to be associated with diaphorase activity. From studies of the effects of DCMU on oxygen evolution and photo-assimilation of glucose in *Chlorella*, also TANNER *et al.* (1965) concluded that DCMU, besides inhibiting PS II, at high concentrations also inhibits cyclic photophosphorylation which depends only on PS I. GOOD (1961) reported partial inhibition of PMS-catalyzed (cyclic) photophosphorylation in isolated chloroplasts by CMU, and complete inhibition of FMN-catalyzed (non-cyclic) photophosphorylation already at lower concentrations. Several authors report that processes dependent on non-cyclic electron transport (mediated by PS I) are not affected by DCMU. BISHOP (1958) demonstrated that DCMU has no effect on photoreductive CO_2-fixation by hydrogen-adapted algae. Photoreduction of NADP or low-potential acceptors with reduced DCPIP as electron donor was not inhibited by DCMU (VERNON and ZAUGG, 1960). According to DUYSENS and AMESZ (1962) DCMU has no effect on the oxidation of cytochrome *f* in *Porphyridium cruentum*. HEALY (1970), and STUART and KALTWASSER (1970), studying the mechanism of photoproduction of hydrogen-gas in *Chlamy-*

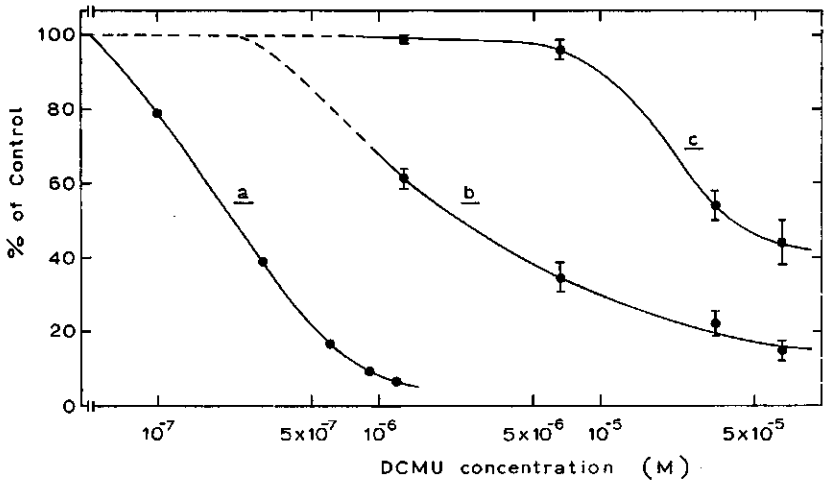


FIG. 36. Effect of DCMU on O_2-evolution in white light (curve a), phosphate fixation in white light (curve c), and phosphate fixation in red light (curve b); light intensity of white light 40 × 10^4 ergs/cm².sec., 5 mm³ cells/ml.

domonas and *Scenedesmus* respectively, did not find DCMU-inhibition of this process.

GINGRAS and LEMASSON (1965) and GINGRAS (1966) have proposed a model for the mode of action of CMU to explain their experimental results. They suggested that CMU removes a certain number of molecules of the oxidized state of the primary substrate (or electron acceptor) E of PS II. So there should be a competition for E^+ between the light reaction at PS II and CMU. It should be noticed that, at saturating light intensity, this substrate is not the limiting factor for photosynthesis. Although DCMU is about ten times more effective than CMU, its mode of action probably is the same. We (VAN RENSEN, 1969) extended the model of GINGRAS and LEMASSON in assuming that DCMU affects the oxidized state of a substance X, which is very close to, or might even be identical with the primary substrate of PS II and that X, moreover, takes part in the cyclic electron transport chain[1]. So there is a competition for X^+ between DCMU and the electron carriers Q and 'cofactor'. This hypothesis explains the following observations:

1. Both in oxygen evolution and in cyclic photophosphorylation, at higher light intensities, the redox balance of X will be shifted to the reduced side, resulting in a lower concentration of X^+. Consequently, inhibition by DCMU will be smaller at higher light intensities, as was demonstrated in figures 11 and 12, and table 5.
2. Inhibition of cyclic photophosphorylation (fig. 36, curve c) is possible, since X is supposed to take part also in the cyclic electron transport chain.

[1] The reader is requested, at this point, to consult fig. 40.

3. In the case of oxygen evolution, according to the above propositions, there is only a single electron donor for X^+, i.e. Q. In the case of cyclic photophosphorylation in white light there are two electron donors, i.e., Q and the 'cofactor' (or electron carrier *in vivo*). For arriving at equal inhibition, a higher DCMU-concentration is therefore needed in the case of phosphate fixation (fig. 36, compare curve a and c).
4. In red light, preferently absorbed by PS I, electron pressure from Q is strongly decreased. So, the inhibition by DCMU of cyclic photophosphorylation in red light should be higher than that in white light, as actually found (fig. 36, compare curve b with curve c).

This hypothesis also explains why processes dependent on cyclic photophosphorylation require higher DCMU concentrations than O_2-evolution for inhibition, as reported e.g. by SIMONIS (1967) for ^{32}P-incorporation in the organic phosphate fraction of *Ankistrodesmus braunii* under nitrogen atmosphere, by TANNER *et al.* (1965) for photo-assimilation of glucose in *Chlorella*, and by JESCHKE (1967) for the light-induced Cl^--uptake in *Elodea* in the absence of CO_2.

AVRON (1967) stressed the necessity of the maintenance of certain catalysts of cyclic electron flow in their proper redox state to obtain maximum rates of photophosphorylation. According to our model, DCMU competes with Q and the 'cofactor' for X^+ which takes part also in the cyclic electron transport. In this way, DCMU can influence the redox balance of the electron transport components. Under some conditions, DCMU can prevent 'over-reduction' of the electron carriers (BOSE and GEST, 1963, and WHATLEY, 1963), resulting in stimulation of ferredoxin-dependent photophosphorylation in argon atmosphere (TAGAWA *et al.*, 1963), while in air, and also with 714 nm light in argon, this reaction was inhibited by CMU (ARNON *et al.*, 1964) Also KYLIN and TILLBERG (1967), in the presence of DCMU, found stimulation of ATP formation in *Scenedesmus* cells in nitrogen atmosphere. On the other hand, TREBST and ECK (1961) found that in the case of photophosphorylation, catalyzed by vitamin K in an atmosphere of nitrogen, DCMU-inhibition can be prevented by bringing the cofactor in the reduced state at the beginning of the reaction.

Photophosphorylation in subchloroplast particles mediated by pyocyanine was much less sensitive to DCMU when pyocyanine was partly reduced by dithiothreitol (ANDERSON and MCCARTY, 1969). HAUSKA *et al.* (1970) confirmed the previous finding of JAGENDORF and MARGULIES (1960) that in chloroplasts, DCMU strongly inhibits phosphorylation in the presence of pyocyanine, whereas with PMS the rate was much less affected. This was explained by the finding of JAGENDORF and MARGULIES (1960) that white light causes a fast non-enzymatic reduction of PMS, however not of pyocyanine. Reduction of pyocyanine however, could be brought about by borohydride or dithiothreitol, and then also eliminated the inhibition by DCMU.

So, several sorts of evidence tend to the conclusion that DCMU exerts its effect by blocking the oxidized form of a component X, which is located in the electron transport pathway close after PS II. In this way, it can inhibit both

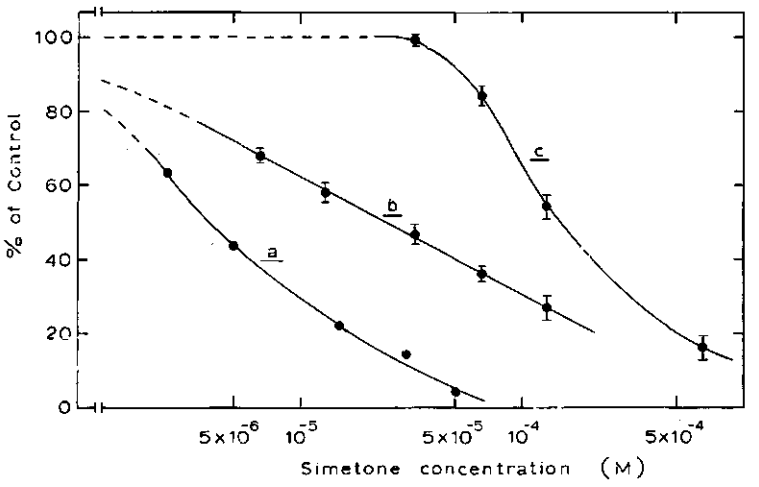


FIG. 37. Effect of simetone on O_2-evolution in white light (curve a), phosphate fixation in white light (curve c), and phosphate fixation in red light (curve b); light intensity of white light 40 $\times$ 10^4 ergs/cm^2.sec.; curve a: 4 mm^3 cells/ml, curves b and c: 5 mm^3 cells/ml.

non-cyclic and cyclic electron transport and thus only one site of inhibition by DCMU needs to be assumed.

With respect to simetone, it may be remarked that symmetrical triazines behave like the phenylureas, as was reported by BISHOP (1962), IZAWA and GOOD (1965), MURATA *et al.* (1966), and GABBOT (1969). Also comparison of the results of Chapter IV and V of this paper shows that the effects of simetone resemble those of DCMU. Simetone is only somewhat less effective than DCMU and it differs from DCMU in being not accumulated by the algal cells and being washed out more easily. These differences, however, can be accounted for by different partition characteristics of the two compounds (DCMU has a higher liposolubility than simetone). Because also simetone has the four above-mentioned DCMU-characteristics (cf. p. 62–63, and illustrations for simetone in figures 21, 22, table 8, and fig. 37), we believe the mode of action of simetone to be the same as for DCMU. As suggested by WESSELS and VAN DER VEEN (1956), GOOD (1961), VAN OVERBEEK (1964), and others, this common mode of action probably depends on the capacity to form hydrogen bonds with a component indispensable for photosynthesis. The observation that both compounds can be removed from the cells by washing, favours the assumption of weak bonds, e.g., hydrogen bonds. The common structure, suited to form hydrogen bonds is R–NH–CX–R', where X = O for DCMU, and X = N for simetone (fig. 38.)

In our opinion, plastoquinone is a possible candidate for being substance X in fig. 40. AMESZ (1964) found that 6 $\times$ 10^{-6}M DCMU in *Anacystis nidulans* inhibits reduction of plastoquinone by light preferently absorbed by PS II, but not its oxidation by light mainly absorbed by PS I. The demonstration of

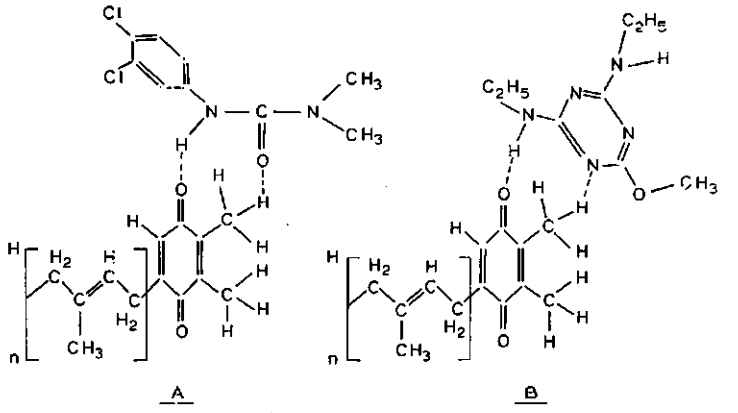


FIG. 38. Proposed formation of hydrogen bonds between the oxidized state of plastoquinone and DCMU (a); the bonds with simetone are presented in (b).

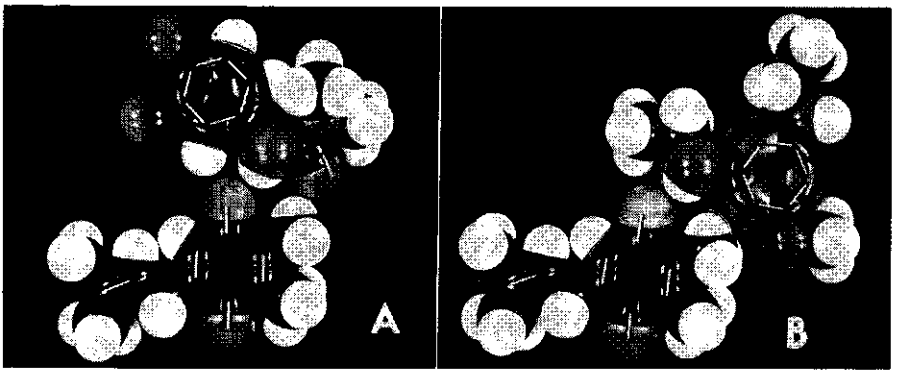


FIG. 39. Molecular models of the complex of the oxidized state of plastoquinone with DCMU (a); (b) same with simetone.

KROGMANN (1961) and KROGMANN and OLIVERO (1962) that addition of plastoquinone to heptane-extracted chloroplasts partially restores PMS-catalysed photophosphorylation, indicates that plastoquinone acts as a cofactor in cyclic photophosphorylation. From a theoretical viewpoint, hydrogen bonds between the oxidized state of plastoquinone and DCMU or simetone appear quite possible, whereas these bonds are much less probable when plastoquinone is in the reduced state (cf. figs. 38 and 39).

VII.2.2. *Diquat*

MEES (1960), BALDWIN (1969), and others have put forward a hypothesis on the mode of action of diquat and paraquat according to which these herbicides are reduced to their free radicals during photosynthesis and, more slowly, by

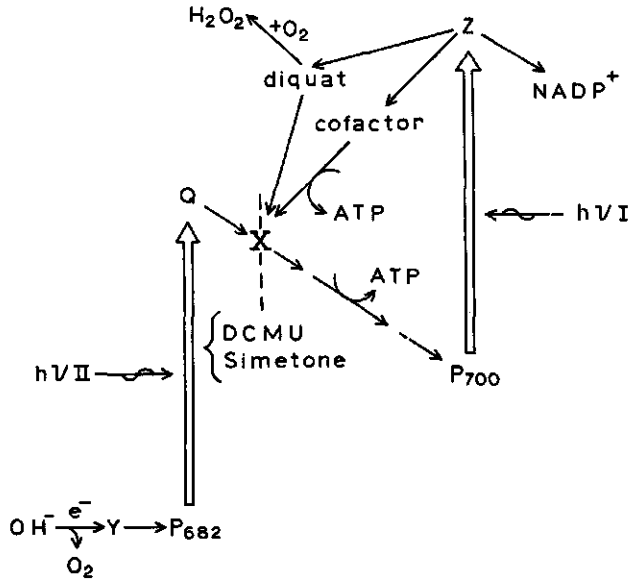


FIG. 40. Proposed scheme for the interactions of the herbicides DCMU, simetone, and diquat with the electron flow in photosynthesis; explanation see text.

respiration in the dark or in the absence of chlorophyll, and that these radicals are reoxidized by oxygen. It is postulated further that after this reoxidation, accompanied by the conversion of oxygen to hydrogen peroxide or possibly to a hydroxyl radical, oxidative damage occurs in the membranes of the chloroplast, disturbing the photosynthetic apparatus. This damage then spreads further to disrupt cell membranes as reported by MERKLE *et al.* (1965) upon treatment with paraquat.

Most results on the effects of bipyridylium herbicides on photosynthesis are obtained from studies with higher plants or isolated chloroplasts. VAN RENSEN, (1969*a*) and TURNER *et al.* (1970) studied the effects of diquat on gas exchange of unicellular algae, *Scenedesmus* and *Chlorella vulgaris*, respectively. The results obtained by both studies support the above mentioned hypothesis (see also fig. 40).

This hypothesis, however, cannot explain the inhibition of phosphate fixation in the complete absence of oxygen (fig. 35). ZWEIG *et al.* (1965) have demonstrated that under strictly anaerobic conditions, diquat can act as a cofactor for cyclic photophosphorylation in isolated chloroplasts. Cyclic photophosphorylation in isolated chloroplasts is only possible after addition of cofactors, such as PMS or vitamin K. *In vivo*, however, cyclic photophosphorylation occurs without the addition of such cofactors, and probably a physiological electron carrier is present which enables cyclic electron transport and, therefore, renders superfluous the addition of cofactors (fig. 40). Fig. 35 shows that, under *in vivo* conditions, diquat inhibits cyclic photophosphorylation in the absence of oxygen.

This may indicate the presence of a second phosphorylation site in the cyclic electron transport chain (fig. 40) and, moreover, that diquat acts as a bypass for the normal electron transport including this second phosphorylation site. Thus, all the electrons, transported via diquat, are lost for this second phosphorylation site. Although sufficiently high concentrations of diquat could not be applied, the shape of the curve indicates that probably not more than 50% inhibition can be reached, suggesting that still one of two phosphorylation sites remains in operation. Evidence for a second phosphorylation site in the cyclic electron transport chain was also presented by SIMONIS (1967), KYLIN and TILLBERG (1967), AVRON and NEUMANN (1968), HAUSKA *et al.* (1970), and others for different reasons.

VII.2.3. *Implications of the observed effects for the mechanism of photosynthesis*

The results presented in this paper allow some conclusions with respect to the mechanism of photosynthesis. From Chapter III it is clear that cyclic photophosphorylation occurs also *in vivo*, as it occurs also in light in which only PS I is operative.

The experiments with DCMU and simetone have made it very probable that the cyclic electron transport chain contacts the non-cyclic one at the level of plastoquinone. In brief recapitulation, the evidence is that these herbicides seem to have the correct properties to block plastoquinone, the inhibition is to be located in the neighbourhood of plastoquinone in the electron transport chain, and evidence discussed above indicates that, in heptane-extracted chloroplasts, plastoquinone may act as a cofactor for cyclic photophosphorylation. With respect to the place of connection between the cyclic and non-cyclic electron transport chain it is worth noting that FAN and CRAMER (1970) recently reported the redox potential of cytochrome 563 to be −0.18 V. It is assumed that this cytochrome takes part in the cyclic electron transport pathway, thus it may act as the physiological 'cofactor' or electron carrier in figures 1 and 40.

From the effects observed with diquat, one may conclude that there are two phosphorylation sites in the cyclic electron transport chain. The evidence herefor is that cyclic photophosphorylation is inhibited also in the absence of oxygen, diquat probably causing a bypass of one phosphorylation site while the observation that inhibition probably never exceeds 50% indicates the presence of a second, non-bypassed phosphorylation site. In connection with relevant literature, it seems most reasonable to locate this non-bypassed phosphorylation site between cytochrome 559 and cytochrome 553 and the other one in the cyclic electron transport chain via PS I, between Z and plastoquinone (fig. 40).

SUMMARY

The mode of action of some herbicides, viz., DCMU, simetone, and diquat, was investigated by studying their effects upon oxygen evolution and cyclic photophosphorylation in the unicellular green alga, *Scenedesmus* spec.

Oxygen evolution was measured with the aid of the WARBURG technique, the suspension medium mostly was WARBURG buffer no 9, the gas phase was air, in general, the temperature 25 °C.

Cyclic photophosphorylation was determined by measuring inorganic phosphate uptake by the algae during a 90 minutes illumination period under a nitrogen atmosphere.

In Chapter III, it is shown that phosphate uptake under the conditions described is saturated at much lower light intensity than oxygen evolution (fig. 4). In far-red light, preferentially absorbed by PS I, there is almost no oxygen evolution (table 2), while the fixation of phosphate proceeds equally well as in white light (fig. 5). This demonstrates that the latter process represents cyclic photophosphorylation *in vivo*.

In Chapter IV, it is shown that DCMU, at light saturation and at 5 mm^3 cells/ml, inhibits oxygen evolution for 50% at a concentration of 2×10^{-7}M (fig. 6). This concentration has no effect on dark O_2-uptake (fig. 11). The degree of inhibition of oxygen evolution by DCMU decreases with increasing suspension density (section IV.1.2.). Moreover, the degree of inhibition depends on light intensity (section IV.1.4.), but not on temperature (section IV.1.5.). By washing the cells, the inhibiting effect of DCMU can be removed (section IV.1.3.). DCMU has no effect on the photosynthetic quotient (table 4).

Phosphate fixation in white light is much less sensitive to DCMU than oxygen evolution; in far-red light phosphate fixation is more strongly inhibited than in white light, but less than oxygen evolution (fig. 36).

In Chapter V, the effect of simetone is studied. Simetone has effects, qualitatively similar to those of DCMU on oxygen evolution and phosphate fixation. It differs from DCMU only in being less effective, not accumulated by the algae, and washed out more easily.

In Chapter VI, experiments on the influence of diquat on oxygen evolution and phosphate fixation are presented. The inhibition of O_2-evolution by diquat increases with time; treatment in light for one hour with 2×10^{-5}M gives an inhibition of about 50% (fig. 27). The degree of inhibition decreases with increasing suspension density (fig. 29) which points to an accumulation of diquat by the cells.

The inhibiting effect cannot be washed out, but when diquat is added in the absence of oxygen or in the presence of 10^{-5}M simetone, the inhibition after washing is decreased (section VI.1.4.). It is concluded that diquat can be removed to a large extent by washing, and that oxygen is required to bring about the inhibiting effect.

Diquat initially stimulates dark O_2-uptake which subsequently changes into inhibition (section VI.1.2.). The degree of inhibition of O_2-evolution increases with light intensity. Inhibition was observed both in the light-limited and in the light-saturated part of the photosynthesis-light curve. The degree of inhibition increases with temperature (section VI.1.5.).

During the first 45 minutes after addition of diquat (with air as a gas phase), the photosynthetic quotient is increased (table 10). This shows that during this time diquat acts as a HILL-oxidant for O_2-evolution, while CO_2-uptake is decreased by lack of reduced NADP.

Diquat was found to inhibit phosphate fixation to about 50% (in the complete absence of oxygen) (fig. 35).

The results are consistent with the hypothesis that diquat is reduced to a free radical in the photosynthetic process and also, to a smaller extent, in respiration. The reaction of this free radical with water and oxygen leads to the formation of toxic peroxide radicals or hydrogen peroxide (fig. 40). These peroxides are assumed to disrupt cellular organization, structure and function.

In Chapter VII, the mode of action of the various herbicides studied, and the implications of the results for the mechanism of photosynthesis are discussed. The results obtained give rise to the following interpretation of the mode of action of DCMU and simetone: both herbicides affect the oxidized state of a substance X, which may represent, or is very close to, the primary electron acceptor of PS II. Moreover, X takes part in the cyclic electron transport chain (fig. 40). X might be plastoquinone; the binding with the herbicides probably occurs via hydrogen bonds (figs. 38, 39).

With respect to the mechanism of photosynthesis it is concluded that cyclic photophosphorylation occurs also *in vivo*. The cyclic electron transport chain probably contacts the non-cyclic one at the level of plastoquinone. Evidence suggests that there are two phosphorylation sites in the cyclic electron transport chain: one between cytochrome 559 and cytochrome 553, and another one in the chain of PS I between Z and plastoquinone. For more details regarding these considerations, cf. section VII.2.3.

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SAMENVATTING

In dit proefschrift wordt een onderzoek naar het werkingsmechanisme van enkele herbiciden beschreven. Daartoe werd hun invloed op zuurstofontwikkeling en cyclische fotofosforylering bestudeerd aan het ééncellige groenwier *Scenedesmus* spec.

Zuurstofontwikkeling werd gemeten met behulp van een WARBURG apparaat; de algen werden meestal gesuspendeerd in WARBURG buffer no 9, de gasfase was lucht en de temperatuur 25 °C.

Cyclische fotofosforylering werd gemeten door het anorganisch fosfaatgehalte in een algensuspensie te bepalen aan het begin en aan het eind van een belichtingsperiode van 90 minuten. Gedurende deze periode werd zuivere stikstof door de suspensies geleid.

In hoofdstuk III is aangetoond, dat fosfaatopname onder deze condities bij veel lagere intensiteit licht-verzadiging vertoont dan zuurstofontwikkeling (fig. 4). In donker-rood licht, voornamelijk geabsorbeerd door PS I, is er bijna geen zuurstof-ontwikkeling (tabel 2). Het feit, dat in dit donker-rode licht toch fosfaatopname optreedt (fig. 5), toont aan, dat dit proces de cyclische fotofosforylering *in vivo* weergeeft.

Hoofdstuk IV laat zien, dat DCMU, bij lichtverzadiging en een suspensiedichtheid van 5 mm^3 cellen/ml, de zuurstofontwikkeling remt voor 50% bij een concentratie van 2×10^{-7}M (fig. 6). Door deze concentratie wordt de ademhaling niet beïnvloed (fig. 11). De remming van de O_2-ontwikkeling door DCMU neemt toe met de suspensiedichtheid (sectie IV.1.2.). Lichtintensiteit (sectie IV.1.4.) heeft invloed op de remming van de zuurstofontwikkeling door DCMU; de temperatuur echter niet (sectie IV.1.5.). Het is mogelijk, de remming door DCMU op te heffen, door de algen met leidingwater te wassen (sectie IV.1.3.). DCMU heeft geen effect op het fotosynthetisch quotient (tabel 4).

De zuurstofontwikkeling is veel gevoeliger voor DCMU dan de fosfaatopname in wit licht; in donkerrood licht is de fosfaatfixatie sterker geremd dan in wit licht, echter minder sterk dan de zuurstofontwikkeling (fig. 36).

In hoofdstuk V is het effect van simeton besproken. De invloed van simeton op de zuurstofontwikkeling en fosfaatfixatie is kwalitatief gelijk aan die van DCMU. Simeton is alleen iets minder actief, wordt niet door de algen geaccumuleerd en is gemakkelijker uit te wassen dan DCMU.

Hoofdstuk VI vermeldt de invloed van diquat. De remming van de zuurstofontwikkeling neemt toe met de tijd; één uur nadat dit herbicide (in het licht) aan de algensuspensies is toegevoegd, is de remming door 2×10^{-5}M ongeveer 50% (fig. 27). De remmingspercentages zijn bij hoge suspensiedichtheden kleiner dan bij lagere dichtheden (fig. 29); dit wijst op accumulatie van diquat door de algen.

De remming kan niet opgeheven worden door de cellen uit te wassen, maar wanneer diquat wordt toegevoegd in afwezigheid van zuurstof of in aanwezigheid van 10^{-5}M simeton, is de remming na het uitwassen minder (sectie VI.1.4.).

Het blijkt, dat diquat voor een groot deel verwijderd kan worden door de cellen uit te wassen en dat zuurstof nodig is om de remming tot stand te brengen.

De zuurstofopname in het donker wordt door diquat in eerste instantie gestimuleerd, later geremd (sectie VI.1.2.). De remming van de zuurstofontwikkeling neemt toe met de lichtintensiteit. Zowel het licht-gelimiteerde als het verzadigde deel van de fotosynthese-lichtcurve worden geremd. De remming neemt toe met de temperatuur (sectie VI.1.5.).

Het fotosynthetisch quotient wordt door diquat verhoogd gedurende een korte periode (45 min.) na het toevoegen (tabel 10). Dit herbicide werkt in eerste instantie als een HILL-oxidans voor de zuurstofontwikkeling, terwijl de CO_2-opname geremd wordt door gebrek aan gereduceerd NADP.

Bij volledige afwezigheid van zuurstof kan diquat de fosfaatfixatie tot ongeveer 50% remmen (fig. 35).

De verkregen resultaten zijn in overeenstemming met de hypothese, dat diquat in het fotosyntheseproces en in mindere mate tijdens de ademhaling, wordt gereduceerd tot een vrij radicaal. In aanwezigheid van zuurstof worden dan toxische peroxiden gevormd (fig. 40), waardoor de structuren in de cel worden verstoord.

In hoofdstuk VII worden de werkingsmechanismen van de herbiciden en de consequenties van de resultaten voor het mechanisme van de fotosynthese besproken. De verkregen resultaten hebben geleid tot de volgende hypothese over het werkingsmechanisme van DCMU en simeton: Beide herbiciden blokkeren de geoxideerde vorm van een component X; deze X is, of is zeer dicht gelocaliseerd bij, het primaire substraat van lichtreactie II. Bovendien is X een component van de cyclische electrontransportenketen (fig. 40). Het is mogelijk, dat X plastochinon is; de binding met de herbiciden vindt waarschijnlijk plaats door waterstofbruggen (figuren 38 en 39).

Met betrekking tot het mechanisme van het fotosyntheseproces wordt geconcludeerd dat cyclische fotofosforylering ook *in vivo* optreedt. De cyclische electronentransportketen ontmoet de niet-cyclische waarschijnlijk op het niveau van plastochinon. Behalve de fosforyleringsplaats in de niet-cyclische electronentransportketen is er ook nog een extra plaats in de cyclische en wel in de keten van PS I, tussen Z en plastochinon. De details van deze beschouwingen zijn vermeld in sectie VII.2.3.

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