THE EFFECT OF OZONE ON PHOTOSYNTHESIS AND RESPIRATION OF *SCENEDESMUS OBTUSIUSCULUS* CHOD., WITH A GENERAL DISCUSSION OF EFFECTS OF AIR POLLUTANTS IN PLANTS

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(met een samenvatting in het Nederlands)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
PROF. DR. IR. H. A. LENIGER,
HOOGLERAAR IN DE TECHNOLOGIE,
IN HET OPENBAAR TE VERDEDIGEN OP
WOENSDAG 18 DECEMBER 1974 DES NAMIDDAGS
TE VIER UUR IN DE AULA VAN DE
LANDBOUWHOGESCHOOL TE WAGENINGEN

STELLINGEN

I

De werking van ozon is niet bijzonder specifiek, wat blijkt uit de overeenkomstige invloed op fotosynthese en ademhaling van *Scenedesmus obtusiusculus*.

Dit proefschrift

II

De werking van ozon op de fotosynthese van *Scenedesmus obtusiusculus* berust zowel op donker-oxydatieve als op photo-oxydatieve reacties.

Dit proefschrift

III

De primaire reactie van ozon in een cel is waarschijnlijk membraanbeschadiging en/of inactivering van enzymen.

Dit proefschrift

IV

De opvatting van BALDRY *et al.* en SELWYN dat een interpretatie van het kinetisch gedrag van de fotosynthese op basis van 'feedback' mechanismen de voorkeur verdient boven die op basis van limiterende factoren is niet gebaseerd op een adequate experimentele analyse.

BALDRY, C. W., C. BUCKE and D. A. WALKER: Biochim. Biophys. Acta 126, 207–213 (1966)
SELWYN, M. J.: Biochim. Biophys. Acta 126, 214–224 (1966)
WASSINK, E. C.: Meded. L.H. 74–22 (1974)

V

De aanname in de hypothese van LEVITT, betreffende de regeling van de huidmondjesbeweging in bladeren onder invloed van een protonentransport mechanisme, dat mesophylcellen geen en huidmondjescellen wel PEP-carboxylase zouden bezitten, is onjuist.

J. LEVITT: Protoplasma 82, 1–17 (1974)

VI

Tegen de opvatting van DUGGER en TING dat de beschadigde werking van peroxyacetylnitraat bij planten in het licht groter is dan in het donker door de photoreductieve werking van het licht, zijn bezwaren aan te voeren.

DUGGER Jr., W. M. en TING, I. P.: Phytopath. 58, 1102–1107 (1968)

VII

Het optreden van een kristalstructuur in het stroma van verschillende plastiden in bladeren onder invloed van fysiologische 'stress' factoren behoeft niet per se een beschadiging van enzymen of membranen in te houden. Een faseverandering van het eiwit, met behoud van activiteit, kan ook de oorzaak zijn.

De Greef, J. A. and J. P. Verbelen: Ann. Bot. 37, 593–596 (1973)
Thomas, W. W., W. M. Dugger Jr. and R. L. Palmer: Can. J. Bot. 44, 1677–1682 (1966)

VIII

De geconstateerde parallelliteit tussen de werking van ozon en röntgenstralen, betreffende vrije radicaal productie, opent de mogelijkheid van selectie op ozon-gevoeligheid van biologisch materiaal door middel van röntgenbestralingsonderzoek.

Brinkman, R. and H. B. Lamberts: Nature 181, 1202–1203 (1958)

IX

Verschillende luchtverontreinigende gassen kunnen de groei van planten verminderen zonder dat er van zichtbare beschadiging sprake is.

Adedipe, N. O., R. E. Barrett and D. P. Ormrod: J. Amer. Soc. Hort. Sci. 97, 341–345 (1972)
Spierings, F. H. F. G.: Neth. J. Pl. Path. 77, 184–200 (1971)

X

Bepaalde cultuurmaatregelen (temperatuur, bemesting en licht) bij gewassen kunnen de tolerantie voor luchtverontreinigende gassen verhogen.

Heck, W. W., J. A. Dunning and I. J. Hindawi: J. Air Pollut. Contr. Assoc. 15, 511–515 (1965)
Knabe, W., Sonderheft Landwirtsch. Forsch. 26 (1), 41–54 (1971)

XI

'Evergreens' zijn het meest geschikt bij de bestrijding van geluidshinder langs auto-snelwegen, doch zijn het gevoeligst voor luchtverontreiniging.

Cook, D. I. and D. F. van Haverbeke: Research Bull. 246, Forest Service, U.S. Dept. of Agriculture, Nebraska U.S.A. (1971)

XII

Het toepassen van kunstgrasmatten voor bermen en taluds, als mogelijkheid geponeerd in wegenbouwkringen, moet vanwege esthetische en biologische overwegingen worden ontraden.

Sticht. Studie Centr. Wegenbouw, Meded. 29 (1972)

XIII

In verband met de steeds verder gaande specialisatie van leerlingen van middelbare scholen en van studenten in het tertiair onderwijs is een goede begeleiding in verband met maatschappelijke implicaties een gerechtvaardigde eis.

XIV

Het lijkt zinvol om belangrijke onderzoekresultaten aan de Universiteiten en Hogescholen periodiek samen te vatten en in de openbaarheid te brengen.

XV

De jazzmuziek heeft zich na John Coltrane in experimentele vormen verstrikt.

VOORWOORD

Het verschijnen van dit proefschrift is een vreugdevolle afsluiting van een promotie-assistentschap aan de Landbouwhogeschool.

Hoewel ik me nu alweer enige tijd bezighoud met het biologieonderwijs aan middelbare scholieren, heb ik de beste herinneringen aan de voorbije onderzoekperiode.

Mijn belangstelling voor natuur en landbouw was reeds vroeg gewekt door het kontakt op het ouderlijk tuinbouwbedrijf. Mijn vader, die helaas deze gebeurtenis niet meer mocht meemaken, heeft mij in deze zeer gestimuleerd.

Gedurende de ingenieursstudie in Wageningen ging mijn interesse in steeds sterkere mate uit naar de meer fundamentele problemen van de natuurwetenschap.

Ik was de Landbouwhogeschool en in het bijzonder mijn hooggeachte promotor, PROF. Dr. E. C. WASSINK, dan ook zeer erkentelijk om mij in de gelegenheid te stellen fundamenteel onderzoek te doen omtrent het werkingsmechanisme van ozon in algen. Ozon, een luchtverontreinigende stof, begon zich toen (1967) juist sterker in Nederland te manifesteren. Ik prijs me dan ook gelukkig in die periode in kontakt te zijn gekomen met PROF. Dr. J. G. TEN HOUTEN, die mij waardevolle adviezen heeft gegeven bij de keuze van het onderwerp.

Hooggeleerde WASSINK, hooggeachte promotor, ik heb bij U op het laboratorium een zeer leerzame en inspirerende periode gehad en denk met dankbaarheid terug aan Uw veelzijdige suggesties in de experimentele fase en tijdens de besprekingen van de resultaten.

Zeer geachte MEVROUW WASSINK, ik ben U zeer erkentelijk voor de prettige gesprekken.

Alle medewerkers van het laboratorium ben ik dankbaar voor hun belangstelling en hulp.

Zeer geachte heer KEETMAN, beste ARIE, jouw belangstelling en medewerking worden zeer gewaardeerd.

WIL, het is niet mogelijk, op deze plaats, jouw hulp onder woorden te brengen.

CONTENTS

1. GENERAL INTRODUCTION

1.1. GENERAL ASPECTS OF AIR POLLUTION EFFECTS ON PLANTS

1.1.1. *Introduction*

Air must be considered as an important natural resource, vital to plants, animals and human beings. The quality of the air may be considered to depend on the chemical nature of its minor constituents, and varies as a result of contaminants emitted by man's activities, including industry, traffic, and house-heating. The amounts of the major constituents of the air, nitrogen and oxygen, are fairly constant and account for 99 % of the atmosphere near the earth's surface. In clean air, the remaining one percent is made up of carbon dioxide, water vapor and a variety of other compounds. The minor gaseous constituents of the air resulting from man's activities may have profound effects on growth and development of plants by interfering with metabolic processes, occasionally resulting in visible injury.

1.1.2. *Classification of air pollutants*

The pollutants considered in this paper are: HF (hydrogen fluoride), SO_2 (sulphur dioxide), NO_2 (nitrogen dioxide), PAN (peroxyacetyl nitrate) and O_3 (ozone). They are gaseous and usually the most important air contaminants responsible for plant injury, but other gaseous pollutants (such as ethylene, chlorine, hydrogen chloride, ammonia) and particulate materials (dust and sulphuric acid droplets) can be just as harmful in local situations.

HF and SO_2 are primary pollutants, produced directly by combustion processes. PAN and O_3, on the other hand, are secondary pollutants resulting from sunlight-initiated (photochemical) reactions between certain hydrocarbons (olefins) and oxides of nitrogen. NO_2 is partly produced primarily by combustion, but most of it is formed secondarily from NO (nitric oxide) by a photochemical reaction. While SO_2 is the most important constituent of the chemically reducing type of air pollution, O_3, PAN and NO_2 (often grouped together as 'photochemical oxidant') are the most important compounds of the chemically oxidizing type, called photochemical smog (PITTS, 1969 [1]).

1.1.3. *Injury considerations*

The severity of injury varies with the nature of the pollutant, its concentration, duration of exposure, and the plant species. In severe cases, visible injury occurs in two types:
1. Acute injury in which markings on the leaves of susceptible plants are quite characteristic and may result in death of cells or tissues. Such injury occurs when the rate of absorption of gas exceeds the capacity of the tissues to remove the pollutant by oxidation, reduction, respiration or translocation;
2. Chronic injury which usually is not characteristic; there may be a chlorosis

or yellowing of leaf tissue, but usually no death of cells. Chlorosis or chronic injury occurs when the rate of absorption is slower, and the plant is able to accommodate the toxicant with less severe effects.

In cases of less severe injury, a physiologically important effect of the pollutants is a suppression of growth in the absence of visible symptoms. This occurs when the plant is exposed to a subnecrotic or sublethal concentration. Growth suppression in the absence of visible symptoms may occur by alterations in photosynthesis and respiration, interference with enzyme activity, and changes in cell wall permeability.

The effects of the pollutants on plants are usually noted on the leaves, because the leaves are the site of gas exchange and of the photosynthetic process.

Typical acute injury symptoms (after DARLEY, 1969 [2] and JACOBSON and HILL, 1970 [3]) are the following:

HF: Necrosis of the margin of the leaves of dicotyledonous or broad-leaved plants and of the tips of grass-like, parallel-veined leaves of monocotyledonous plants. The affected areas may be light tanned to brown or dark red in colour; the injury is bifacial. The symptoms appear when the accumulation of fluoride reaches the toxic level for the species in question.

SO_2: A white to tan bleaching of tissues at the leaf margin, the tip or the intercostal areas; also brown, red or black colours may predominate in the injured area, causing bifacial injury. The injury is due to sulfite production from SO_2. At lower concentrations of SO_2, the sulfite ion is oxidized to the less toxic sulfate ion.

NO_2: Necrotic lesions between the veins; they may be located anywhere on the leaf surface, but are most prominent at the apex and along the margins. The symptoms first appear on the upper leaf surface, followed by bifacial injury. The necrotic areas are usually white to tan or brown and closely resemble SO_2-induced symptoms. Concentrations of 8–50 ppm are required to mark plants, and these concentrations far exceed those found in the atmosphere, so that there are no field observations as yet.

PAN: A silvering, glazing or bronzing sheen on the lower surface of affected leaves, with no injury to the upper leaf surface. Later on bifacial necrotic lesions can appear, which can be white to light brown to black. Very characteristic for PAN-injury is a banding type symptom associated with cell age and cell susceptibility.

O_3: Stippling, mottling or bleaching of the upper leaf surface of affected leaves with no injury to the lower leaf surface. Palisade cells, and, when the injury is more severe, upper-epidermis cells collapse and become bleached. The colour of the marking varies from light tan to red and to almost black, depending upon the species affected. When all of the tissue through the leaf is killed, relatively large bifacial necrotic areas develop. Markings often consist of a band of injured tissue across the leaves of monocotyledons, in which only tissue of a certain age is affected. Also in dicotyledons zonal markings are associated with cell age and cell susceptibility.

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1.1.4. *The effects of air pollutants on metabolic processes in plants in relation to photosynthesis and respiration*

The effect of the air pollutants on photosynthesis and respiration can be divided into two phases. The first phase is that in which the dose (concentration × exposure time) is not high enough to cause visible injury. The second phase is that of visible injury.

HF: Phase I

Reduction of photosynthesis without visible injury was observed, e.g. in gladiolus (THOMAS, 1958 [4], 1961 [5]) and strawberry (HILL, 1969 [6]). This proceeds at relatively high HF-concentrations and short fumigation times. In these instances, after the fluoride treatment was discontinued, photosynthesis slowly recovered. For any sensitive plant there is a threshold value for observable reduction of photosynthesis. Because the threshold concentration for reduction of photosynthesis in the most sensitive gladiolus is about 7 ppb, and outdoor concentrations rarely exceed 1–5 ppb, this impairment is improbable in the field. Under conditions of high HF-concentrations, there can be a decrease of photosynthesis above that caused by the leaf necrosis then occurring (phase II).

Stimulation of respiration before any visible injury appears, has been observed in many plant species, e.g. in bush bean (*Phaseolus vulgaris*) leaves (McNULTY and NEWMAN, 1957 [7]; APPLEGATE and ADAMS, 1960 [8]) and tomato and bean leaves (WEINSTEIN, 1961 [9]). However, under certain conditions respiration might also be inhibited at this phase (APPLEGATE and ADAMS, 1960 [8]; McNULTY and NEWMAN, 1956 [10].

Phase II

THOMAS (1958 [4]) and HILL (1969 [6]) showed that, for many plant species, there was no reduction in photosynthesis until necrosis appeared; the reduction in the photosynthetic rate then was proportional to the amount of leaf necrosis.

Increase in respiration is associated with the presence of leaf necrosis (McNULTY and NEWMAN, 1957 [7]; ROSS *et al.*, 1962 [11]). Necrotic tissue itself, whether due to fluoride toxicity or mechanical or thermal injury, produces increased O_2-uptake of adjacent tissue (HILL *et al.*, 1959 [12]), and it is difficult to ascertain which changes in metabolism are causes or consequences of necrosis.

Mode of action of HF

Once fluoride has penetrated into the leaf through the stomata, it is rapidly transported in the transpiration stream (JACOBSON *et al.*, 1966 [13]), and accumulates preferably in the leaf tips and margins. The chloroplasts are the major site of fluoride accumulation (CHANG and THOMPSON, 1966 [14]) and they become disrupted early in the sequence of events in necrosis (SOLBERG and ADAMS, 1956 [15]).

The way in which atmospheric fluorides affect the metabolism of the plant is not well understood. An effect of fluoride on a physiological process may re-

flect the affection of one or more enzyme systems by fluoride. Enzymes, coenzymes or activators may be the targets of the pollutant (McCune and Weinstein, 1971 [16]). Fluoride is known as an enzyme inhibitor; such enzymes as enolase (Warburg and Christian, 1942 [17]), phosphoglucomutase (Chung and Nickerson, 1954 [18]) and succinic dehydrogenase (Slater and Bonner, 1952 [19]) are inhibited by fluoride *in vitro*. The enolase system is also inhibited *in vivo* by fluoride, e.g. in *Chlorella pyrenoidosa* (Sargent and Taylor, 1972 [20]). However, in plants treated with HF, also enhancement of enzyme activities is found, associated with changes in the pool sizes of metabolites (McCune et al., 1964 [21]). The concentration and distribution of fluoride within the cell may determine, by affecting specific enzymes, not only the degree to which metabolism is affected, but also the type of effect. Since, in the sequence of events induced by fluoride, it appears that respiration is affected earlier than photosynthesis, the pathways of respiratory activity are perhaps the primary sites of fluoride toxicity. Changes in respiration and photosynthesis that are not associated with chlorosis or necrosis are manifest above threshold values of each crop for HF, which depend upon duration of exposure and HF-concentration. Translocation or inactivation of fluoride and the nonrate-limiting character of a fluoride-sensitive step or pathway might explain the existence of a threshold. The apparent recovery after some time might well be explained by the removal of fluoride from a sensitive site.

In general, plants have been found to be more susceptible to HF than to other pollutants, e.g., about 8 ppb HF v/v caused visible injury of sensitive plants while for the other pollutants much higher concentrations are needed, so for SO_2-injury about 0.2 ppm (Jacobson et al., 1966 [13]). This greater susceptibility might be explained partly by the accumulation of fluoride in leaf tissues, otherwise from the discovery of Miller and coworkers (Cheng et al., 1968 [22]; Lovelace et al., 1968 [23]) that HF, in some plants at least, can be metabolized to fluororganic acids: fluoroacetate and fluorocitrate. Fluorocitrate is a very toxic compound and might cause inhibition of the enzyme aconitase in the Krebs cycle. Moreover, by this block in the Krebs cycle, citric acid accumulates and migh inhibit in its turn the enzyme phosphofructokinase in the glycolytic pathway (Lowry and Passonneau, 1964 [24]). This could also be the explanation for the decrease of respiration by fluoride.

In addition to a relatively specific effect on enzymes, fluoride may exert a general effect on metabolism by the formation of metal fluoride complexes (e.g. CaF_2). Thus, the altered nutrient status of the cell could result in a decreased activity of its metal-requiring enzymes or a weakening of its structural integrity in case calcium is involved. Moreover, interference with the water household is possible (Van Raay and Spierings, 1969 [25]; LeBlanc et al., 1971 [26]). The decrease of photosynthesis may be associated with the breakdown of chlorophylls (LeBlanc et al., 1971 [26]) and carotenoids (Arndt, 1971 [27]). In the case of the chlorophylls, the breakdown might take place by precipitation of MgF_2. The chlorophylls are more sensitive in lichens (LeBlanc et al., 1971 [26]), while the carotenoids are so in higher plants (Arndt, 1971 [27]).

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The HILL reaction of bean chloroplasts is inhibited by KF in a pH range of 4.8–5.7, so that HF may be the active agent. Sodium monofluoroacetate was found rather ineffective in inhibiting the HILL reaction (BALLANTYNE, 1972 [28]).

SO₂: Phase I

Reduction of photosynthesis without the appearance of visible markings is known, e.g. in alfalfa (THOMAS and HILL, 1937 [29]), spruce and fir (VOGL et al. 1964 [30]), and tobacco and broad bean (LÜTTGE and FISCHER, 1971 [31]). This effect is most significant at relatively high SO_2-concentrations and short exposure times. Generally, partial or total recovery occurs after fumigation is stopped. For reduction of photosynthesis there seems to be a threshold value for sensitive plants, however, the threshold concentration mostly is high in relation to concentrations present under field circumstances. Thus, VOGL and BÖRTITZ (1969 [32]) did not find any depression of photosynthesis in sensitive firs in the field with maximum concentrations of 1.15 mg SO_2/m^3/day (equivalent with 43.9 pphm), while 10–20 ppm SO_2 in laboratory experiments caused depression of photosynthesis in sensitive firs before visible injury is produced.

THOMAS and HILL (1937 [29]), in their experiments with alfalfa, which is very sensitive to SO_2, showed that, for a 4 hr. exposure, the threshold concentration is about 0.4–0.6 ppm. In prolonged exposures, interference occurred at still lower concentrations, although a 45-day fumigation at 0.14 ppm failed to show any significant effect on photosynthesis. Field concentrations of SO_2 normally range from the country air level of 10–20 $\mu g/m^3$ to that of 100–150 $\mu g/m^3$ (equal to 3.8–5.7 pphm) typical for the region of industrial districts (SPEDDING, 1969 [33]), although peak values can reach around 1 ppm (MAJERNIK and MANSFIELD, 1970 [34]); probably these concentration levels are too low to interfere with photosynthesis.

Before visible damage occurs, respiration in higher plants generally is not affected, although in spruce (BÖRTITZ, 1964 [35]) and larch (VOGL and BÖRTITZ, 1965 [36]) stimulated respiration was found. In experiments on *Euglena gracilis*, an exposure of the algae to 1 hr. air bubbling with 5 ppm SO_2 caused an increase of respiration (14%), while photosynthesis is reduced by about the same amount (12%) (DE KONING and JEGIER, 1968 [37]). In experiments with bryophytes, respiration is stimulated by 5 ppm SO_2, and generally increased with extended exposure time (SYRATT and WANSTALL, 1969 [38]).

Phase II

With alfalfa (THOMAS and HILL, 1937 [29]), spruce and fir (VOGL et al., 1964 [30]) it is shown that, when necrosis occurred, photosynthesis sometimes is reduced somewhat stronger than could be accounted for by the amount of leaf necrosis. There thus seems to be an extra impairment of photosynthesis above the loss of photosynthetically active area.

Concerning respiration at this stage, both a stimulation (in larch: VOGL and BÖRTITZ, 1965 [36]) and an inhibition (in lichens: KLEE, 1970 [39]) have been observed.

Mode of action of SO$_2$

SO$_2$ enters the leaf through the stomata and can dissolve in the tissue by forming HSO$_3^-$ and SO$_3^{2-}$:

$$SO_2 + H_2O \overset{k_1}{\rightleftharpoons} H^+ + HSO_3^- \overset{k_2}{\rightleftharpoons} 2H^+ + SO_3^{2-}$$

Bisulphite will predominate over sulphite because k_2 is much smaller than k_1 (TERRAGLIO and MANGANELLI, 1967 [40]). (Bi)sulphite may be oxidized to sulphate (WEIGL and ZIEGLER, 1962 [41]). Above a certain dosis value (the product of fumigation time and SO$_2$-concentration), the (bi)sulphite concentration may become toxic. For SO$_2$-treated rice plants it has been shown that glyoxylate bisulphite occurs (TANAKA et al., 1972 [42]).

Bisulphite and glyoxylate bisulphite may inhibit photosynthesis by interference with enzyme systems, photosynthetic redox systems and membrane systems (LÜTTGE and FISCHER, 1971 [31]). Reduction of (bi)sulphite in the light may also produce toxic levels of hydrogen sulfide (H$_2$S); H$_2$S production in the light in SO$_2$-treated tomato plants has been recorded (DE CORMIS, 1969 [43]).

There is a significant effect of SO$_2$ on stomatal movement. WEIGL and ZIEGLER (1962 [41]) found accumulation of sulfur in the guard cells of the stomata of spinach plants after exposure to ^{35}SO$_2$. More recently, it has been found that SO$_2$ may cause either closure (FISCHER, 1971 [44]; MANSFIELD and MAJERNIK 1970 [45]) or opening (MANSFIELD and MAJERNIK, 1970 [45]) of stomata, dependent on environmental conditions. FISCHER (1971 [44]) found that the closing reaction in *Nicotiana tabacum* was a quenched vibration of the stomata, which was concluded from an analogical oscillation of CO$_2$-fixation, SO$_2$-uptake and transpiration. This was found in the light and in the dark. FISCHER suggested that such a mechanism may more or less protect plants against SO$_2$. In his experiments, it was not or hardly found in broad beans; photosynthesis at a certain concentration of SO$_2$ is more inhibited in broad beans than in tobacco. MANSFIELD and MAJERNIK (1970 [45]) found a different reaction in the stomata of broad bean leaves, dependent on the relative humidity. It appeared that a closing reaction is induced by SO$_2$ in relatively dry air (less than 40% R.H. at 18 °C), and an opening reaction in relatively moist air (more than 40% R.H. at 18 °C). The consequences of a stimulation of stomatal opening might be twofold: a considerable increase in the access of gas to the mesophyll so that the damage to the mesophyll will be greater, and moreover, an increase in transpiration (WEIGL and ZIEGLER, 1962 [41]), which for plants growing with a limited water supply might lead to damage or even to lethal water stress.

Photosynthesis seems to be very sensitive to SO$_2$. The chloroplasts are known to disintegrate before total tissue collapse occurs; no microscopic injury is detected with the light microscope before macroscopic injury is developed (in pinto bean and tomato leaves, SOLBERG and ADAMS, 1956 [15]). More recently, WELLBURN et al., (1972 [46]), using an electron microscope, and exposing broad bean plants to 0.25–1 ppm SO$_2$ for 1–2 hrs., observed thylakoid swelling in the chloroplasts of the mesophyll and palisade parenchyma cells. The first symptom is a swelling of the stroma thylakoids, but at higher pollutant concentrations or

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at prolonged exposures also swelling of the granum thylakoids appears; the swelling could be reversed by a treatment with pure air. At more extended exposure times, a severe disruption of the chloroplast structure was observed. Also a shrinkage of the starch granules, due to a sudden mobilisation of starch, is observed. At this stage, no alteration is observed in the extra-chloroplastic cytoplasm or in the cellulose wall. FISCHER (1967 [47]) also found the early stages of damage of broad bean to occur in the chloroplasts. By extensive damage, an increase in the number of osmophilic globules is detected, produced by deterioration of membranes (LÜTTGE and FISCHER, 1971 [31]).

The thylakoid swelling may result in reduced rates of net CO_2-assimilation by the impairment of enzyme activities (WELLBURN et al., 1972 [46]). ZIEGLER (1972 [48]) showed that sulphite in an in vitro system inhibits the activity of ribulose-1,5-diphosphate carboxylase, isolated from spinach chloroplasts. In the leaf tissue of broad bean and tobacco plants, just next to necrotic spots, FISCHER (1971 [44]) found a decrease in activity of the CALVIN cycle enzyme glyceraldehyde-3-phosphate dehydrogenase.

Besides possible interference of SO_2 with enzyme activity, redox systems, membrane systems, structural configuration, and water balance, also the breakdown of chlorophyll and carotenoid pigments may be of importance for photosynthesis, although probably only in the case of visible injury. Destruction of chlorophylls, when visible damage occurred, is reported, e.g. for alfalfa (THOMAS and HILL, 1937 [29]), bryophytes (SYRATT and WANSTALL, 1969 [38]) and lichens (KLEE, 1970 [39]; RAO and LEBLANC, 1966 [49]). It is clearly pointed out that the degradation of chlorophyll is accompanied by phaeophytin a and Mg^{2+}-production. Chlorophyll a is more sensitive than chlorophyll b, thus causing a decrease of the chl a/chl b ratio (KLEE, 1970 [39]). KLEE, in the same paper, also reported a loss of carotenoid pigments in lichens. With ARNDT (1971 [27]) it can be concluded that in higher plants, as oat, barley, and rye, chlorophyll loss is not observed in the early phases of visible damage, but that β-carotene is more sensitive.

In literature, no studies were found on the mechanism of SO_2-toxicity on respiration.

NO_2: Phases I and II

A photochemical reaction is essential for the production of NO_2 from NO, and under similar conditions NO_2 is consumed with the production of ozone and PAN. Since no clear observations of visible damage in the field seem to be available, a distinction of phases I and II is not so much indicated in this case, essentially all observations are related to phase I.

The NO_2-concentration in the atmosphere rarely exceeds 1 ppm, but concentrations of 0.2–0.5 ppm are not uncommon in polluted areas (TEBBENS, 1968 [50]). Within this concentration range, significant visible damage probably would not yet occur, because it is known from artificial fumigations that visible symptoms on sensitive plants, such as tobacco, bean and tomato, are produced only at concentrations of about 2.5–10 ppm (THOMAS, 1969 [51]). Significant

growth suppression is found by prolonged exposures to NO_2-concentrations of 0.25–0.5 ppm in tomato (TAYLOR and EATON, 1966 [52]; SPIERINGS, 1971 [53]) and pinto bean (TAYLOR and EATON, 1966 [52]).

HILL and BENNETT (1970 [54]) reported inhibition of photosynthesis at sublethal concentrations for alfalfa and oats, without the production of visible injury. Both NO_2 and NO, and also NO_2 + NO, the latter causing an additional effect, inhibit photosynthesis above a threshold concentration of about 0.5–0.7 ppm for 45–90 min. It seems that NO_2 is more toxic for photosynthesis than NO. Complete recovery of non-visibly injured plants was consistently noted. Growth suppression of plants owing to the presence of NO_2 in the ambient air could probably be partly due to periodic reduction of the rate of photosynthesis during periods of increased concentrations of NO_x (nitrogen oxides).

Mode of action of NO_2

NO_2, which enters the leaf through the stomata, may dissolve in the tissue and lead to the formation of NO_3^- and NO_2^-. The concentrations then will increase with the duration of the exposure and with the NO_2-concentration. Above a certain level, nitrite will become toxic.

HILL and BENNETT (1970 [54]) suppose two possible reaction mechanisms to play a role in the inhibition of photosynthesis. NO_2^- can be reduced to ammonia in the presence of chloroplasts, ferredoxin and light (PANEQUE et al., 1964 [55]). Once ferredoxin is reduced in the light, electrons can be transferred via the appropriate reductase enzymes to the NO_2^--system or to the NADP-system (to be reduced to NADPH). This indicates the possibility of a competitive effect, resulting in a decrease in the rate of CO_2-reduction. From the experiments of HILL and BENNETT (1970 [54]) it appeared that the suppression of photosynthesis can not wholly be explained by such a competitive inhibition. Another possible mechanism is that photosynthesis can be depressed by the formation of transitory iron-NO complexes with compounds of the electron transport system. In biological systems nitrite can be converted to NO by reducing agents, such as ascorbic acid, pyridine nucleotides and ferrous iron (EVANS and McAULIFFE, 1956 [56]). Both NO and NO_2^- may serve as precursors for iron-NO complexes. Iron-containing redox compounds with a function in energy transfer in photosynthesis (e.g. ferredoxin, cytochromes) may be complexed by NO and thus interfere with the normal electron transport.

The effects on photosynthesis are in agreement with the observation of WELLBURN et al. (1972 [46]) in broad bean leaves, that the first ultrastructural change is a thylakoid swelling in the chloroplasts, if treated with 1–3 ppm NO_2 for 1 hr. This swelling disappears quickly after a treatment with unpolluted air.

PAN: Phase I

Already DUGGER et al. (1963 [57]) have reported that inhibition of CO_2-absorption by bean leaves, treated with 1 ppm PAN for 30 min., did not occur until visible symptoms developed. In experiments with sorghum, oats, petunia and tomato, treated with 10–30 pphm for 2 hrs., TAYLOR (1969 [58]) has also

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shown that the inhibition of CO_2-absorption did not occur unless visible symptoms (water-soaked areas) became apparent. Therefore, it may be suggested that no direct impairment of photosynthesis is caused by PAN before visible injury is produced.

Phase II

Because ambient concentrations in, e.g., California range from 1-3 pphm on average days and up to about 5 pphm on 'smoggy' days (TAYLOR, 1969 [58]) acute injury on sensitive plants, such as petunia, bean and tomato, in the field can be expected and has been reported. Photosynthesis, as already noted (DUGGER *et al.*, 1963 [57] and TAYLOR, 1969 [58]), is decreased when visible markings appear. In experiments with PAN on *Chlamydomonas reinhardtii* it is shown that photosynthesis is more affected than respiration (GROSS and DUGGER, 1969 [59]).

Mode of action of PAN

After PAN has penetrated the leaf through the stomata, it may be decomposed spontaneously in the tissue. PAN has a short half-life in solution. One of the decomposition products may be nitrite (MUDD and DUGGER, 1963 [60]). On the other hand, PAN may be directly toxic by its peroxide nature.

Because of its short half-life it is most likely to have its effects on susceptible chemical targets close to the port of entry into the leaf, the stomata. This is just what appears as primary markings of PAN on susceptible leaves; the typical injury symptom is a silvering or glazing on the lower surface, where PAN is entering the leaf by the stomata.

It was observed that protoplasts of mesophyll cells in the region of the stomata collapsed, and large air pockets took their place (GLATER *et al.*, 1962 [61]; THOMSON *et al.*, 1965 [62]; TAYLOR and MACLEAN, 1970 [63]). STEPHENS *et al.* (1961 [64]), and DUGGER *et al.* (1966 [65]), labeling PAN with ^{14}C, showed that much of the ^{14}C in treated plants appeared in the chloroplasts and here the first injury might be expected. This was indeed found by THOMSON *et al.* (1965 [62]) in the mesophyll cells of bean plants. The primary ultrastructural change is in the stroma of the chloroplasts: a granulation, followed by the formation of ordered rods and plates in the stroma. Later on, the cellular membranes break down, and the chloroplasts and cell contents clump together. It has been suggested that these changes in the chloroplast stroma play a dominant role in inhibiting primary CO_2-fixation, which is located in the stroma.

The exact targets for PAN, important in inhibition of photosynthesis, are unknown. However, enzyme systems are potentially susceptible. MUDD (1963 [66]) in studies in *in vitro* systems, suggested that the inactivation of enzymes, such as isocitric dehydrogenase, glucose-6-phosphate dehydrogenase and malic dehydrogenase, active in respiration, might be due to the oxidation of sulfhydryl (SH) groups, since sulfhydryl oxidizers caused similar effects. SH-proteins, such as ovalbumin, haemoglobin and papain, are also oxidized by PAN *in vitro*, but the protein structure can protect the susceptible sites (SH-groups) against PAN (MUDD *et al.*, 1966 [67]). ORDIN *et al.* (1971 [68]), in tobacco plants treated

with PAN (1 ppm, 1 hr.), found that the activity of the enzymes cellulose- and alkali-soluble glucan synthetases has already decreased before necrosis occurred. These enzymes play an important role in cell wall biosynthesis. Growth suppression without detectable leaf injury in tomato, bean and citrus (TAYLOR and MACLEAN, 1970 [63]; DARLEY, 1971 [2]) may be explained partly by an impairment of cel wall metabolism.

Not only enzymes, but also coenzymes, such as the cofactors NADH and NADPH, are sensitive to PAN. MUDD and DUGGER (1963 [60]) showed that these cofactors are reversibly oxidized in vitro, so that the oxidation does not alter their biological activity.

TAYLOR et al. (1961 [69]) showed that for plant damage from PAN there is an absolute requirement for light prior to, during and after fumigation of sensitive plants, such as pinto bean and petunia. DUGGER et al. (1963 [70]) found in action spectrum studies maximum response at 419, 480 and 641 nm, while there was another maximum at a wavelength shorter than 370 nm. The much weaker response at 641 nm than at 419 nm and 480 nm suggests, that the chlorophyll pigments were not initially involved in the PAN – light interaction which leads to damage. The observed response indicated a possible photoreaction between carotenoid pigments and the oxidant.

GROSS and DUGGER (1969 [59]) observed that the levels of carotenoids and chlorophyll a pigments in Chlamydomonas had decreased shortly after exposure of the algae to PAN. DUGGER and TING (1968 [71]) suggested a relation between the activity of PS I and PS II and plant susceptibility to PAN. Susceptible bean plants exposed to red light (660 nm), activating system II, were damaged during PAN treatment. At equal energies of far-red light (700 nm), activating system I, the degree of plant damage was very much less. A combination of the two light qualities during the PAN-treatment resulted also in a much smaller damage to plants than the 660 nm light alone. It is suggested that activation of PS II, resulting in a more reduced state of the electron transfer chain, renders the plant more susceptible to PAN than the activation of PS I, which causes a more oxidized state of the electron transfer chain. The present authors suggest that 'the light-PAN interaction in green tissues, causing tissue damage, seems to be a function of the photosynthetic reactions responsible for the formation of reduced compounds, such as sulfhydryl groups, and their subsequent removal by PAN via photo-oxidation.'

During spinach chloroplast treatment with PAN, light seems to be necessary in order to inhibit CO_2-fixation, ATP- and NADPH-formation (DUGGER et al., 1965 [72]). The HILL reaction and cyclic photophosphorylation in chloroplasts are inhibited also after a PAN-treatment in the dark (KOUKOL et al., 1967 [73]). The HILL reaction of chloroplasts, prepared from bean leaves treated with PAN, is already inhibited before visible damage appears on the leaves (DUGGER et al., 1965 [72]). The inhibition of the HILL reaction, contrary to that of the other photosynthetic reactions, could not be explained by oxidation of SH-groups. It might be due to NO_2, formed by the breakdown of PAN, because $NaNO_2$ at a similar concentration inhibits the HILL reaction (KOUKOL et al., 1967 [73]).

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O₃: Phase I

Inhibition of photosynthesis at sublethal concentrations, without the production of visible injury, is reported, e.g. for lime seedlings (TAYLOR *et al.*, 1961 [74]), *Pinus* species (MILLER *et al.*, 1969 [75]; BOTKIN *et al.*, 1971 [76]; BARNES, 1972 [77]), and many other plants such as oat, barley, wheat, corn, tobacco, bean, potato and tomato (HILL and LITTLEFIELD, 1969 [78]). This inhibition normally is fully annihilated after fumigation has stopped. Effects as mentioned are observed in treatments with very low concentrations (5–15 pphm) for a prolonged time, up to some months, or with higher concentrations (up to about 60 pphm) for a short time, up to some hours. A similar effect might be expected in the field, because in 'smoggy' atmospheres the O_3-level may range from 5–100 pphm (TRESHOW, 1970 [79]). In the Netherlands also levels above 10 pphm are noted during smog formation (WISSE and VELDS, 1970 [80]; GUICHERIT *et al.*, 1972 [81]). A decrease in photosynthesis is also found for *Euglena gracilis*, treated for a short period (DE KONING and JEGIER, 1968 [37; 82]).

Respiration under these conditions may be increased, e.g. for orange (TODD, 1958 [83]), lemon (DUGGER *et al.*, 1966 [65]) and *Pinus* species (BARNES, 1972 [77]), or decreased, e.g. in tobacco (MACDOWALL, 1965 [84]). In *Euglena gracilis*, stimulation is found after an exposure of 1 hr. at 1 ppm O_3, bubbled through the suspension (DE KONING and JEGIER, 1968 [37]).

Phase II

The most sensitive plant species, such as tobacco, spinach, bean, and pines, produce visible markings after an ozone exposure with 5–12 pphm for 2–4 hrs. (HILL *et al.*, 1970 [85]).

Photosynthesis in this phase is decreased, e.g. in *Pinus strobus* (BOTKIN, 1970 [76]), tobacco (MACDOWALL, 1965 [84]), coleus, tomato, and bean (TODD and PROPST, 1963 [86]) and *Lemna minor* (ERICKSON and WEDDING, 1956 [87]). Results suggest that there may be an extra impairment of photosynthesis above the loss of active photosynthetic area (TODD and PROPST, 1963 [86]; MACDOWALL, 1965 [84]).

Respiration is normally increased in this phase, e.g. in bean (TODD, 1958 [83]), coleus and tomato (TODD and PROPST, 1963 [86]), and tobacco (MACDOWALL, 1965 [84]), although for *Lemna minor* in the case of chlorophyll breakdown by O_3, respiration is also inhibited (ERICKSON and WEDDING, 1956 [87]).

Mode of action of O₃

O_3 enters the leaf through the stomata. In some cases the stomata may respond to ozone entrance with a closing reaction, as has been found for onion (ENGLE and GABELMAN, 1966 [88]), tobacco (LEE, 1965 [89]; MACDOWALL, 1965 [90]; RICH and TURNER, 1968 [91]), oats (HILL and LITTLEFIELD, 1969 [78]), and soybean (HOWELL and KREMER, 1972 [92]). In this way tolerant plants might be protected, because ozone uptake can be assumed to be proportional to stomatal conductance (RICH *et al.*, 1970 [93]). The nature of the closing reaction is not known. Two possible explanations are proposed. Firstly, ozone is known to af-

fect cell permeability and an increase of permeability in the guard cells might allow water to escape from them and cause closure of the stomata. Secondly, it is known that thiol oxidizers cause stomatal closure, probably by enzyme inactivation (MOURAVIEFF, 1971 [94]).

Inhibition of photosynthesis before visible damage occurs, may sometimes be explained by stomatal closure. However, not in all cases a positive correlation is found between leaf resistance (stomatal opening) and inhibition of photosynthesis or visible damage to plants such as beans (DUGGER et al., 1962 [95]), cotton (TING and DUGGER, 1968 [96]), and tobacco (TURNER et al., 1972 [97]).

Open stomata, without any barrier to gas exchange, do not necessarily imply ozone sensitivity. Other mechanisms of tolerance are also involved; TING and DUGGER (1968 [96]), for cotton leaves, found that leaf ontogeny and light intensity are important factors. Maximum susceptibility seems to occur at about 75% of full expansion, and at this stage there is a requirement of several hours light for visible damage to occur in susceptible leaves. Earlier, DUGGER et al. (1963 [98]) have shown for bean plants that after a 72 hr. dark period, after which the plants are no longer susceptible, the recovery of susceptibility started immediately after transfer to light. DUGGER et al. (1962 [99]) and LEE (1965 [89]), in experiments on pinto bean and tobacco plants respectively, observed that damage by ozone occurred if the sugar content of the leaves is in a certain low level range; above or below this range, the leaves appear protected. Exogenous supply of soluble sugar could prevent visible damage. TING and MUKERJI (1971 [100]) reported that the period of maximum susceptibility of cotton leaves corresponds to a minimum concentration of soluble sugars and free amino acids in the leaves. It is supposed that during the period of rapid growth, accompanied by low levels of soluble reserves, the compounds oxidized by ozone are not repaired at a rate sufficient to prevent ultimate leaf necrosis.

Once ozone has passed the stomata on the abaxial leaf side, which form the principal ports of entry (HEGGESTAD and MIDDLETON, 1959 [101]; RICH, 1963 [102]), it may dissolve in the water layer round the cells inside the leaf. GIESE and CHRISTENSEN (1954 [103]) and McNAIR SCOTT and LESHER (1963 [104]), in experiments with yeast and Escherichia coli cells respectively, found that the primary effect of ozone is on the boundary layers of the cell, since leakage of cell contents occurred. McNAIR SCOTT and LESHER (1963 [104]) suppose that ozone did not penetrate the bacterial cells, because the SH-concentration of the cells did not decrease until it leaked out. A primary effect of ozone on cell permeability is noted in the case of lemon leaves by DUGGER et al. (1966 [65]), while it may also be concluded for bean (TOMLINSON and RICH, 1967 [105]) and Pinus ponderosa (EVANS and MILLER, 1972 [106]) from chemical evidence. In all these cases it comes to increased cell permeability. McFARLANE (1966 [107]), however, reported a decreased permeability in tobacco leaf and potato tuber tissues induced by sublethal ozone concentrations.

Although the exact nature of ozone toxicity is not known, there is strong evidence that ozone, a powerful oxidant with a redox potential of +2.07 V (LATIMER and HILDEBRAND, 1951 [108]) oxidizes several reduced compounds in cells.

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Ozone may primarily attack cell membranes by oxidation of unsaturated fatty acids (UFA), which is reported for human red blood cells (GOLDSTEIN et al., 1969 [109]; BALCHUM et al., 1971 [110]) and spinach chloroplasts (MUDD et al., 1971 [111]). In bean leaves, however, this so-called lipid peroxidation is not detected until the leaves are visibly damaged (TOMLINSON and RICH, 1970 [112]).

Other targets sensitive to ozone oxidation are the sulfhydryl groups (SH) and the reduced pyridine nucleotides, such as NADH and NADPH. SH-groups are found to be sensitive in vitro and in vivo. Enzymes (TODD, 1958 [113]; ORDIN et al., 1969 [114]) and amino acids and proteins (MUDD et al., 1969 [115]) with SH-groups are oxidized by ozone. The SH-content of bean leaves is decreased by a relatively high O_3-concentration, e.g. 1 ppm applied for 30 min. (TOMLINSON and RICH, 1968 [116]), but not at 25 pphm applied for 3 hrs. (TOMLINSON and RICH, 1970 [117]). NADH and NADPH are irreversibly oxidized by ozone in in vitro systems (MUDD, 1965 [118]), while, in vivo, DE KONING and JEGIER (1969 [119]) presented evidence that Euglena gracilis cells treated with ozone in the light partly loose the ability for the production of these reduced cofactors.

When ozone reacts with the unsaturated lipids of the plasmalemma or subcellular membranes, different toxic breakdown products may ultimately be produced, such as free radicals (GOLDSTEIN et al., 1968 [120]), glycerolipids with short chain aldehyde substituents, hydrogen peroxide and malonaldehyde (MUDD et al., 1971 [111]), and ethylene (CRAKER, 1971 [121]). All these compounds have lytic properties. MUDD et al. (1971 [111]) showed that even in the presence of SH-compounds, ozone oxidizes the unsaturated fatty acids of spinach chloroplasts in vitro. They suggested that there probably is a primary attack of ozone on the unsaturated fatty acids, and that the sulfhydryl groups are partly oxidized by breakdown-products, such as H_2O_2. Not only photosynthesis, but also lipid and protein synthesis may be impaired by ozone. The impairment can be due to disturbance of the membrane integrity (UFA-oxidation), inactivation of enzymes (SH-oxidation) or irreversible oxidation of the cofactors.

It has been observed in studies with pinto bean leaves (TOMLINSON and RICH, 1971 [122]) and spinach chloroplasts (MUDD et al., 1971 [111]) that an ozone treatment may enhance the synthesis of steryl glucosides (sterol derivatives), while the synthesis of the essential galactodiglycerides is decreased. This may cause changes in cellular permeability, because sterols are generally assumed to control the permeability of plasma membranes in plants (GRUNWALD, 1968 [123]). Because several thiol oxidizers have a similar effect on the lipid composition (MUDD et al., 1971 [111]), the ozone effect in this case is suggested to be by inactivation of SH-enzymes operating in lipid synthesis. A possible explanation for impaired protein synthesis is found in ozone induced fragmentation of chloroplast ribosomes in pinto bean leaves, as noted by CHANG (1971 [124]); particularly the 23 S r-RNA component seems to be attacked (CHANG, 1972 [125]). It is suggested that ozone may destroy the integrity of polysome particles either by reaction with the sulfhydryl groups of ribosomal proteins or by impaired energy production, because NADPH may be oxidized by O_3.

LEDBETTER et al., (1959 [126]) observed that the palisade cells of most herbaceous and woody species are most sensitive to ozone injury, causing upper leafside flecking or stippling. HILL et al., (1961 [127]) reported that the chloroplasts were the first organelles to respond to ozone with disruption. The first ultrastructural change observed in bean leaves is in the stroma of the chloroplasts of the palisade cells (THOMSON et al., 1966 [128]). Primarily, there is a granulation in the stroma, followed by the formation of ordered arrays, granules, fibrils or plates. It is suggested that these structures are aggregates of protein molecules. At this stage there is no alteration in the mitochondria.

A secondary disruptive phase is characterized by the breakdown of the plasmalemma and other cellular membranes, while the mitochondria are swollen, and loosening of the grana membranes appears; finally the entire cellular content (including chloroplasts and mitochondria) accumulate in the centre of the cell. At this stage, damage should be irreversible. Recently it has been found by EVANS and MILLER (1972 [106]) that also in needles of *Pinus ponderosa*, treated with O_3, the first histological and histochemical changes, before visible damage occurs, appear in the photosynthetic tissue of the mesophyll cells. There is an aggregation of the chloroplasts in the cell periphery, and a carbohydrate accumulation outside the organelles. Later on, proteins and nucleic acids are aggregated or precipitated. Already CHRISTENSEN and GIESE (1954 [129]) have shown the susceptibility of nucleic acids in *in vitro* systems to ozone.

It may be supposed that the chloroplasts are first attacked by way of a lipid peroxidation mechanism, because it is known that chloroplasts are sensitive to peroxidation, presumably owing to their substantial UFA-content (HEATH and PACKER, 1965 [130]). Later on, the mitochondria may also suffer from lipid peroxidation since mitochondrial swelling (THOMSON et al., 1966 [128]; LEE, 1967 [131]) and inhibited oxidative phosphorylation (MACDOWALL, 1965 [84]; LEE, 1967 [131]) are observed. These are symptoms, also associated with peroxidation of mitochondria (TAPPEL and ZALKIN, 1959 [132]).

The often occurring increased respiration when the leaves are visibly damaged, could be partly explained by delayed starch transport which is observed, e.g., in tobacco (HANSON and STEWART, 1970 [133]).

Chlorophyll losses induced by ozone as found for *Lemna minor* (ERICKSON and WEDDING, 1956 [87]), tobacco (MACDOWALL, 1965 [84]), *Pinus ponderosa* (MILLER, 1965 [134]) and *Euglena gracilis* (DE KONING and JEGIER, 1968 [82]) may play a part in impairment of photosynthesis, although it is generally assumed not to be the primary mechanism of ozone toxicity.

1.2. SOME ASPECTS OF CURRENT VIEWS ON PHOTOSYNTHESIS AND RESPIRATION

In order to discuss possible modes of interference of O_3 with specific partial reactions of the chains of photosynthesis and respiration, it was thought useful to give a brief survey of recent concepts of the mechanisms of these processes.

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1.2.1. *Photosynthesis*

Photosynthesis can be described by the overall equation:

$$CO_2 + 2H_2O \xrightarrow{\text{light}} (CH_2O) + H_2O + O_2,$$
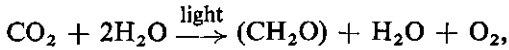
in which (CH_2O) represents the primary product of CO_2-reduction at the carbohydrate level.

Photosynthesis can be divided into two successive, main processes: the primary reactions and the carbon reduction pathway. The primary reactions consist of a light activated electron flow from water to NADP; in this part ATP, NADPH and O_2 are formed. The primary reactions are followed by the carbon reduction pathway, which is a light-independent biochemical process in which CO_2 is converted to carbohydrate. For each molecule of CO_2 to be reduced, 2 NADPH and 3 ATP are required.

(a) The primary processes. Light energy absorbed in chlorophyllous and cooperative pigments, embedded in the lamellar chloroplast thylakoids, is the driving force of the electron flow (fig. 1).

HILL and BENDALL (1960 [136]) have introduced the concept that two photochemical reaction systems act in series in the photosynthetic electron transport chain which now is generally accepted. The two photochemical reaction systems operating in two different photosynthetic pigment complexes, are called System I and II by DUYSENS *et al.* (1961 [137]). In green plants and green algae, system I (PS I) includes most of the chlorophyll a, a small portion of the chlorophyll b, and carotenoids; it transfers the absorbed light energy to the photochemical reaction centre I, currently denoted as P_{700} (KOK, 1961 [138]). System II (PS II) includes relatively a smaller portion of the chlorophyll a, and more chlorophyll b and carotenoids, as compared with PS I; it transfers the absorbed light energy to the photochemical reaction centre II, denoted as P_{680} (DÖRING *et al.*, 1969 [139]).

P_{700} and P_{680} are assumed to become excited by the light energy transferred to them, and become able to transfer electrons to subsequent components of the reaction chain. In this way, P_{680} receives electrons from a compound called Y and transfers them to a compound called Q. The exact nature of Q and Y is unknown. Y is supposed to play a role in the watersplitting system, which produces oxygen, H-ions and electrons. Q transfers electrons to a chain, in which cytochromes and some other compounds operate. Along this electron transport chain, the redox potential of the subsequent substances decreases, and reaches a level, insufficient for the reduction of carbon dioxide. At this point, the action of P_{700} is important. By uptake of light from the pigments of PS I, P_{700} becomes excited and able to transfer electrons from the mentioned electron transport chain to a compound Z, the exact nature of which is equally unknown. The redox potential of Z is sufficiently high to reduce Fd, FP and NADP. The ultimately produced NADPH acts as the primary reductant for CO_2. This electron transport from water, via P_{680}, Q, the electron carriers, P_{700}, Z, Fd, to NADP appears to be the main pathway of photosynthetic electron transport,
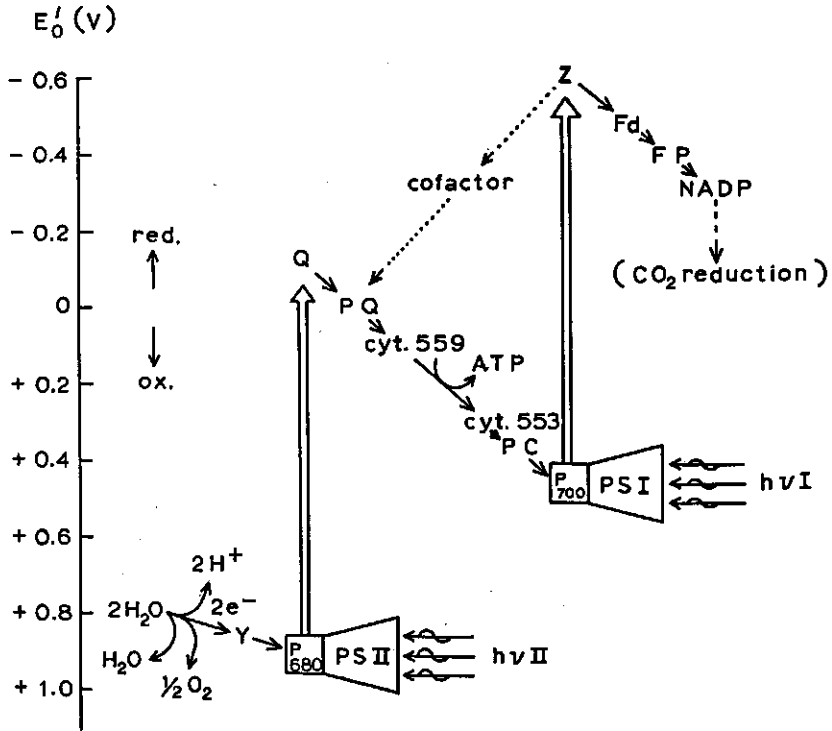
FIG. 1. Scheme for the primary reactions in photosynthesis. Abbreviations:
PQ: plastoquinone, PC: plastocyanin, Fd: ferredoxin, FP: flavoprotein, ATP: adenosine triphosphate, NADP: nicotinamide-adenine dinucleotide phosphate, E_0' (V): redox potential (volts). (Adapted, from VAN RENSEN, 1971 [135]).

as long as CO_2-assimilation proceeds. It generates O_2, NADPH and ATP. Along this pathway, ATP is produced by a process of non-cyclic photophosphorylation between Q and P_{700} (ARNON, 1959 [140]).

Under certain conditions (shortage of CO_2, shortage of oxidized NADP) an electron flow occurs, called cyclic electron transport, accompanied by cyclic photophosphorylation (ARNON et al., 1958 [141]), 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}. In this process, no oxygen and no NADPH are produced. The only detectable product is ATP.

Still another pathway of electron transport is the pseudo-cyclic electron transport, resulting in pseudo-cyclic photophosphorylation (ARNON et al., 1961 [142], 1964 [143]). This occurs when reduced ferredoxin does not reduce NADP, but is oxidized by oxygen. The conditions for this mechanism are high light intensities and shortage of CO_2. The pseudo-cyclic electron transport follows mainly the same pathway as the non-cyclic one, because the electrons are trans-

ported from water, via the electron carriers and ferredoxin to oxygen instead of to NADP and further into the CO_2-reduction cycle. It agrees with the cyclic one, because no oxygen is set free, no NADP is reduced and only ATP is formed.

(b) The carbon reduction pathways in photosynthesis. CO_2-reduction is a light-independent thermo-chemical process. Till now three different carbon reduction pathways are known: the CALVIN cycle, the glycolate pathway and the HATCH-SLACK pathway.

The CALVIN cycle (fig. 2) has been developed by CALVIN and his colleagues (CALVIN and BASSHAM, 1962 [145]; BASSHAM, 1964 [146]).

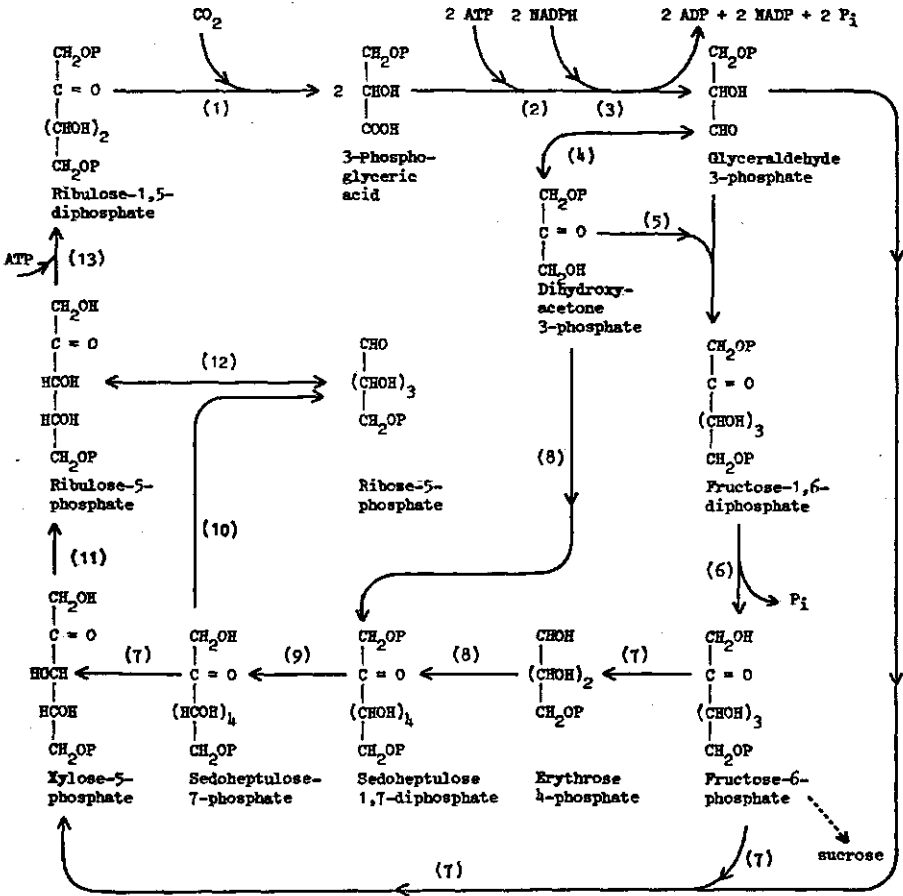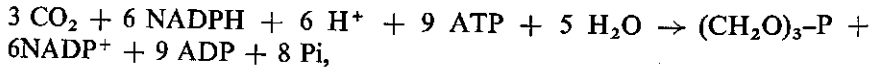FIG. 2. The CALVIN cycle. The enzymatic reactions are mediated by the following enzymes: (1) Ribulosediphosphate carboxylase, (2) Phosphoglycerate kinase, (3) Triosephosphate dehydrogenase, (4) Triosephosphate isomerase, (5) Aldolase, (6) Phosphatase, (7) Transketolase, (8) Aldolase, (9) Phosphatase, (10) Transketolase, (11) Epimerase, (12) Pentosephosphate isomerase, (13) Phosphoribulose kinase. (Adapted, from ZELITCH, 1971 [144]).

The photosynthetic carbon reduction cycle may be divided into three phases, viz., (a) a carboxylation phase, in which ribulose diphosphate accepts CO_2 to yield two molecules of 3-phosphoglyceric acid (fig. 2: 1); (b) a reductive phase, in which phosphoglyceric acid is reduced to triose phosphate (fig. 2: 2–4); (c) a regenerative phase, in which five triose phosphates are converted to three pentose phosphates, and the CO_2-acceptor, ribulose diphosphate, is regenerated (fig. 2: 5–13).

The net result of the cycle is:

$$3\ CO_2 + 6\ NADPH + 6\ H^+ + 9\ ATP + 5\ H_2O \rightarrow (CH_2O)_3\text{–P} + 6NADP^+ + 9\ ADP + 8\ Pi,$$

thus, for each molecule of CO_2 reduced, two molecules of NADPH and three molecules of ATP are required.

The CALVIN cycle is the most common pathway of carbon dioxide fixation in plants of temperate regions and in algae.
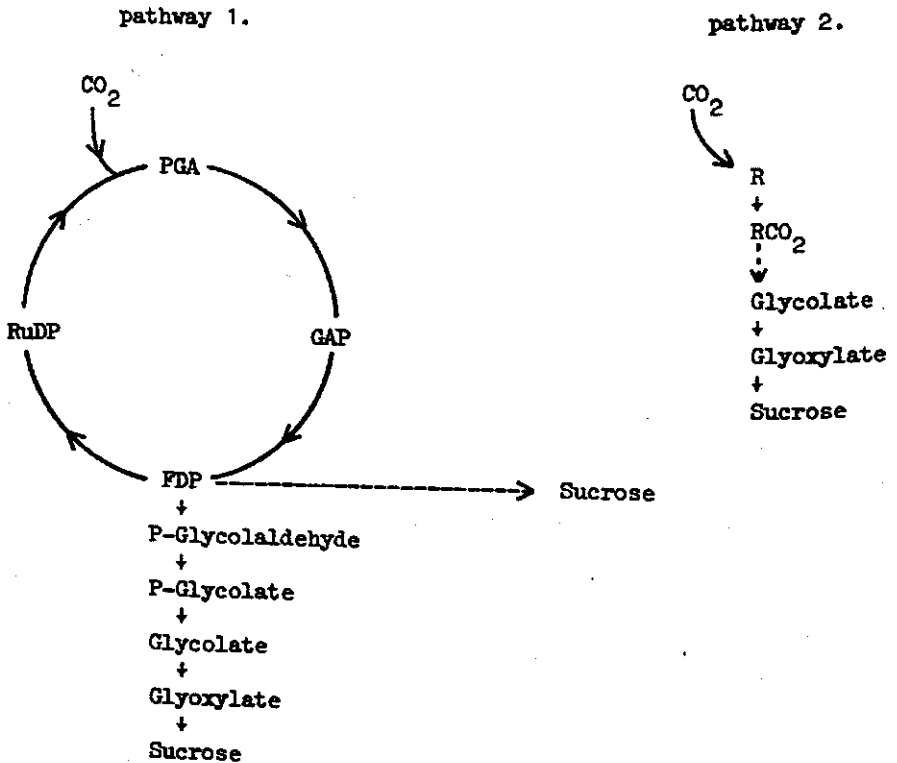

FIG. 3. Diagrammatic representation of the photosynthetic formation of glycolate (cf. also lit. cited). Abbreviations:
RuDP: ribulosediphosphate, PGA: phosphoglyceric acid, GAP: glyceraldehydephosphate, FDP: fructosediphosphate, R: CO_2 reducing substance.

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COOH
|
C - OPO$_3$H$_2$
‖
CH$_2$
Phosphoenolpyruvate (PEP)

CO$_2$

(2)

COOH
|
C = O
|
CH$_2$
|
COOH
Oxaloacetate

AMP + PP$_i$

ATP + P$_i$

(1)

NADPH+H$^+$

NADP$^+$

(3)

COOH
|
C = O
|
CH$_3$
Pyruvate

(4)

COOH
|
HOCH
|
CH$_2$
|
COOH
Malate

CO$_2$

RUDP

PGA

Calvin
cycle

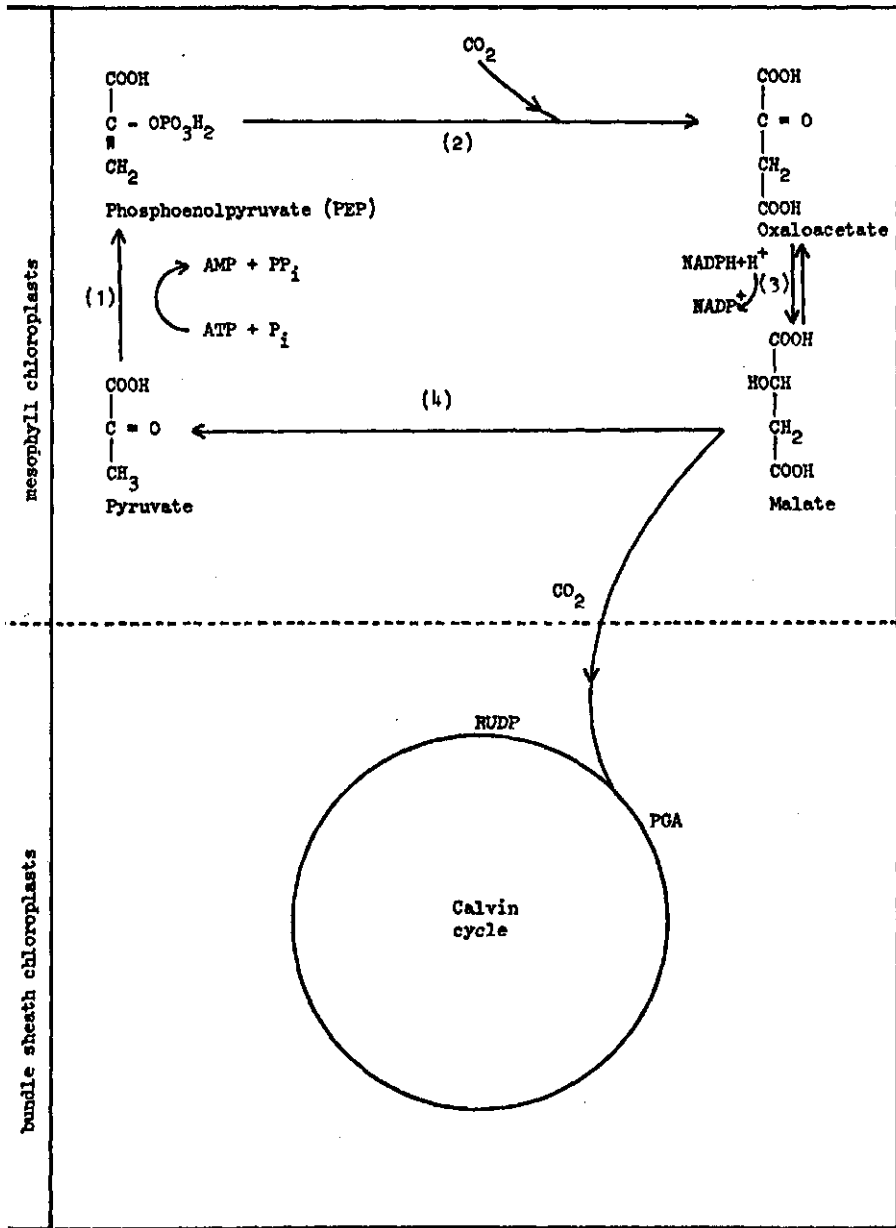mesophyll chloroplasts

bundle sheath chloroplasts

FIG. 4. The C$_4$-dicarboxylic acid pathway. The enzymatic reactions are mediated by the following enzymes:
(1) Pyruvate, P$_i$ dikinase, (2) Phosphoenolpyruvate carboxylase, (3) Malate dehydrogenase, (4) Malate enzyme. (Adapted, from HATCH and SLACK, 1970 [151]).

Under certain conditions (high light intensity, high O_2-concentration, and low CO_2-concentration) the glycolate pathway (fig. 3) has been proven to be important (TOLBERT, 1963 [147]). The source of the glycolate is not exactly known. One group of investigators (TOLBERT, 1963 [147]) supposes, that it originates as a by-product of certain sugardiphosphate conversions in the CALVIN cycle, e.g., from fructosediphosphate (fig. 3: pathway 1). Another group (ZELITCH, 1965 [148], 1971 [149]) suggests that glycolate synthesis is mediated by an independent carboxylation reaction (fig. 3: pathway 2), the nature of which is not yet exactly known; the carboxylating enzyme of this system should have a higher affinity to CO_2 than RuDP carboxylase from the CALVIN cycle. It is, however, possible that both pathways cooperate in glycolate synthesis. A considerable glycolic acid production under low CO_2-concentration during photosynthesis has been found for several green plants and algae, e.g., *Scenedesmus* (WILSON and CALVIN, 1955 [150]).

The HATCH-SLACK pathway or C4-dicarboxylic acid pathway (fig. 4) is the major pathway for carbon dioxide fixation by tropical grasses (e.g. in sugar cane, maize, sorghum) and some other plant species, such as *Amaranthus* and *Atriplex* (HATCH and SLACK, 1970 [151]).

This pathway may be divided into two stages: (1) The C4-pathway s.s., in which CO_2 is accepted by phosphoenolpyruvate and C4-dicarboxylic acids as oxaloacetate, malate and aspartate are formed; pyruvate and CO_2 are generated by malate; ultimately phosphoenolpyruvate is regenetated. This process should occur in the mesophyll cells of C4-plants, because the enzymes are located in the mesophyll chloroplasts; (2) The CO_2 generated from malate is refixed by the RuDP carboxylase reaction in the CALVIN cycle. The latter process should occur primarily in the bundle sheath chloroplasts.

The carboxylating enzyme PEP carboxylase has a much higher affinity to CO_2 than has RuDP carboxylase. The C4-pathway plants are characterized by having two types of chloroplasts, high rates of net photosynthesis per unit leaf area, and low rates of photorespiration (see below).

1.2.2. *Respiration*

Plant respiration may be divided into two different processes, viz., dark respiration and photorespiration.

(a) D a r k r e s p i r a t i o n. The overall reaction of respiration:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O + 672\ kcal,$$
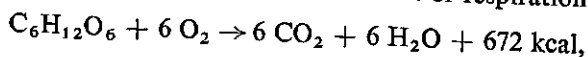
with hexose as substrate, is the same as the overall equation for CO_2-reduction in photosynthesis, but in opposite direction. Photosynthesis and respiration do not represent reversals of the same biochemical reactions, although both processes have enzymes and intermediates in common. Photosynthesis and respiration occur in different cell organelles (the chloroplasts and the mitochondria respectively), and therefore these processes are largely separated topographically. Respiration, e.g., provides the energy (ATP) for the essential biochemical reactions concerned with growth. The major pathway for aerobic respiration of

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carbohydrates is by way of glycolysis (EMBDEN-MEYERHOF pathway) and the tricarboxylic acid cycle (KREBS cycle).

Glycolysis (fig. 5) describes the initial stages of starch- or hexose hydrolysis, which occur in the cytoplasm by soluble enzymes.

This process may be subdivided into two major steps, viz., the conversion of glucose (or starch) by phosphorylation into fructose-1,6-diphosphate, and the conversion of this compound into two three-carbon compounds. The second step includes an initial oxido-reduction reaction, ultimately leading to the formation of two molecules of pyruvic acid and two molecules of ATP. Under aerobic conditions the pyruvic acid produced is completely oxidized to CO_2 and H_2O with synthesis of much more ATP. This occurs in the mitochondria. The first stage in the breakdown of pyruvic acid is an oxidative decarboxylation, catalyzed by pyruvate dehydrogenase and leading to the formation of acetyl coenzyme A (acetyl CoA). The overall equation for these reactions is:

$$CH_3.CO.COOH + CoA + NAD \rightarrow CH_3.CO.CoA + CO_2 + NADH$$
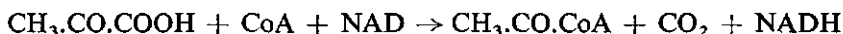
Pyruvic acid and acetyl CoA form the connecting links between glycolysis and the tricarboxylic acid cycle (KREBS cycle, fig. 6), in which the starting compound, oxaloacetic acid, is continually regenerated. This cycle was first demon-
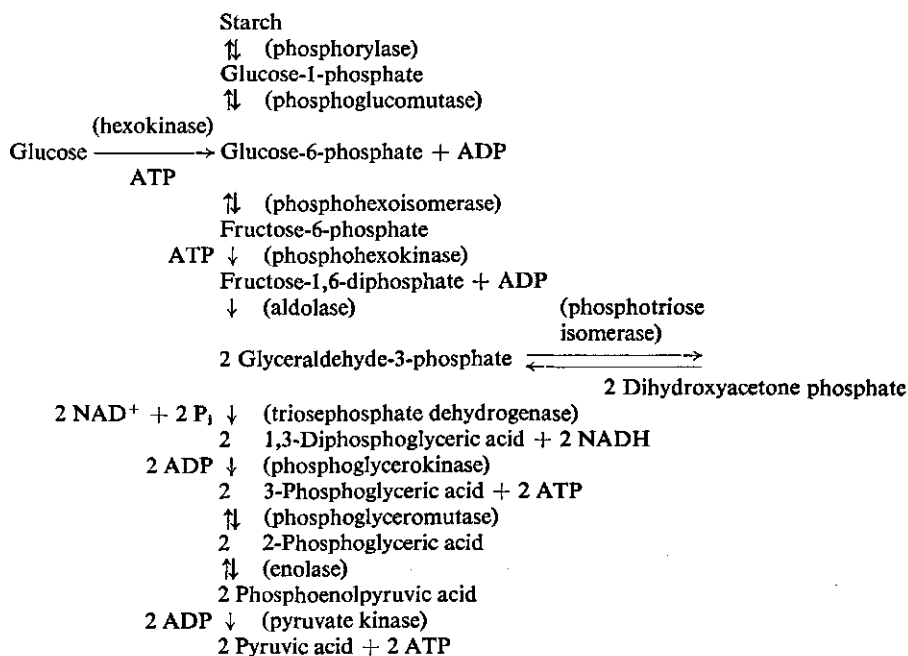
Starch
⇅ (phosphorylase)
Glucose-1-phosphate
⇅ (phosphoglucomutase)

(hexokinase)
Glucose ————————→ Glucose-6-phosphate + ADP
     ATP
⇅ (phosphohexoisomerase)
Fructose-6-phosphate
ATP ↓ (phosphohexokinase)
Fructose-1,6-diphosphate + ADP
↓ (aldolase)   (phosphotriose
                          isomerase)
2 Glyceraldehyde-3-phosphate ⇄—————————→
                                  2 Dihydroxyacetone phosphate
$2 NAD^+ + 2 P_i$ ↓ (triosephosphate dehydrogenase)
    2 1,3-Diphosphoglyceric acid + 2 NADH
2 ADP ↓ (phosphoglycerokinase)
    2 3-Phosphoglyceric acid + 2 ATP
⇅ (phosphoglyceromutase)
    2 2-Phosphoglyceric acid
⇅ (enolase)
2 Phosphoenolpyruvic acid
2 ADP ↓ (pyruvate kinase)
2 Pyruvic acid + 2 ATP

FIG. 5. Scheme for the initial transformation of starch or glucose by glycolysis. (Adapted, from GOODWIN and MERCER, 1972 [152]).

CH_3
C = O
CoA

COOH
C = O
CH_2
COOH
Oxaloacetic
acid

(1)

CoA

COOH
CH_2
HOC - COOH
CH_2
COOH
Citric acid

(2)
- H_2O

COOH
CH_2
C-COOH
CH
COOH
Cis-aconitic
acid

(9)
- 2H   NAD^+ → NADH + H^+

COOH
CH_2
HCOH
COOH
Malic acid

(8)

COOH
HC
CH
COOH
Fumaric acid

(7)
- 2H   FAD → FADH_2

COOH
CH_2
CH_2
COOH
Succinic
acid

+ H_2O (2)

COOH
CH_2
HC - COOH
HCOH
COOH
Iso-citric acid

NADP^+
+
NADPH+H^+   - 2H (3)

COOH
CH_2
HC - COOH
C = O
COOH
Oxalosuccinic
acid

CoA

(6)

GDP + P_i → GTP
ADP + GTP → ATP + GDP

COOH
CH_2
CH_2
CoA - C = O
Succinyl CoA

CO_2   CoA

(5)

-2H
NAD^+
+
NADH + H^+

COOH
CH_2
CH_2
C = O
COOH
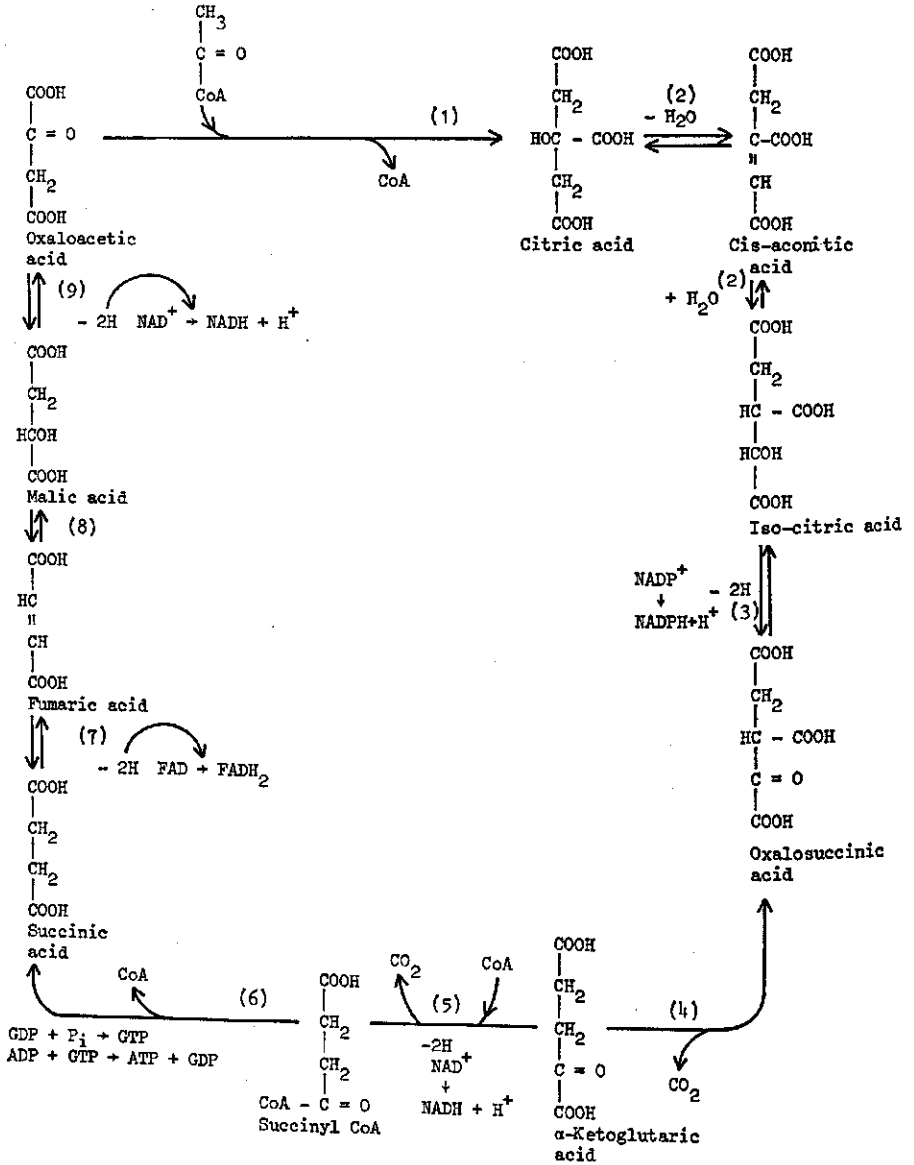α-Ketoglutaric
acid

(4)

CO_2

Fig. 6. The KREBS cycle. The enzymatic reactions are mediated by the following enzymes:
(1) Citrate synthetase, (2) Aconitase, (3) Isocitric acid dehydrogenase, (4) Carboxylase, (5) α-Ketoglutaric dehydrogenase, (6) Thiokinase, (7) Succinic dehydrogenase, (8) Fumarase, (9) Malic dehydrogenase. (Adapted, from DEVLIN, 1969 [153]).

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strated in animal muscle by KREBS and JOHNSON (1937 [154]), while its functioning in plants was proposed by CHIBNALL (1939 [155]).

The overall breakdown of pyruvic acid may be summarized as follows:

$$CH_3.CO.COOH + 3 H_2O \rightarrow 3 CO_2 + 10 H$$
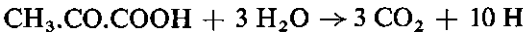
Under aerobic conditions the enzymes of the KREBS cycle cooperate with those of the electron transport system (fig. 7). The electron transport system consists of a sequential series of components transferring electrons. Most important to the living cell is the fact that the electrons taken up by hydrogen acceptors (NADP, NAD, FAD) in all oxidation steps of respiration are ultimately transferred via the electron transport system to O_2, and that energy production (ATP) takes place. The total yield of the oxidation of one molecule of glucose is 38 ATP.
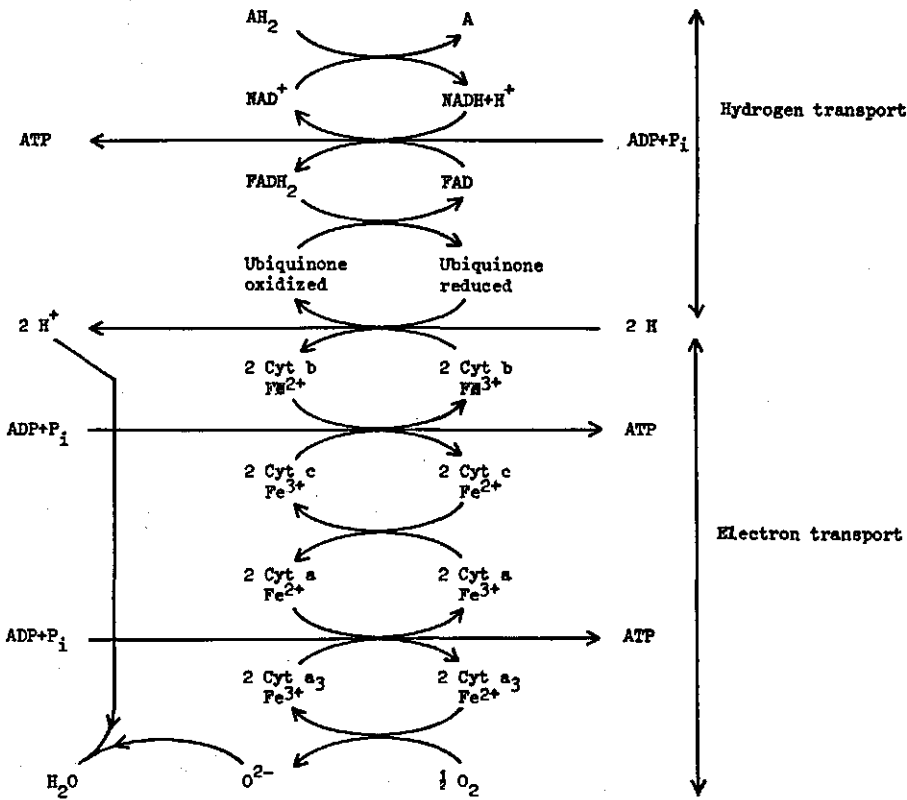

FIG. 7. Scheme of the electron transport pathway. A KREBS cycle intermediate (AH_2) is oxidized, releasing two hydrogen atoms possessing two electrons, which pass along a sequential series of cytochrome enzymes to oxygen. Three molecules of ATP are produced for each pair of electrons passing along this system. (Adapted, from LEHNINGER, 1971 [156]).
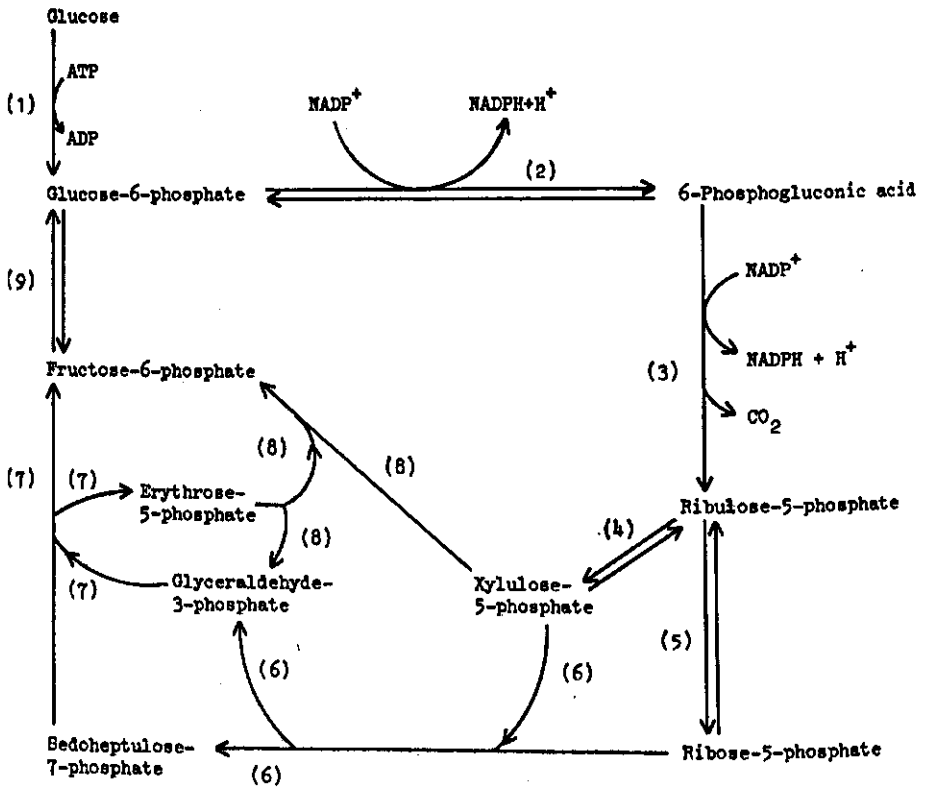
Glucose

FIG. 8. Scheme for the pentose phosphate pathway. The enzymatic reactions are mediated by the following enzymes:
(1) Hexokinase, (2) Glucose-6-phosphate dehydrogenase, (3) 6-Phosphogluconate dehydrogenase, (4) Epimerase, (5) Isomerase, (6) Transketolase, (7) Transaldolase, (8) Transketolase, (9) Hexose phosphate isomerase. (Adapted, from GOODWIN and MERCER, 1972 [158]).

Although the major pathway for the aerobic respiration of glucose is glycolysis followed by the KREBS cycle, an alternative pathway exists, which is probably located in the chloroplasts (KRAUSE and BASSHAM, 1969 [157]). This pathway is generally called the pentose phosphate pathway (fig. 8), and, like glycolysis uses glucose as starting material, but, unlike glycolysis, requires the presence of oxygen. This pathway produces NADPH molecules at two sites between hexose and pentose. NADPH and NADH, both electron acceptors, have quite different biochemical functions. Whereas NADH, as produced in glycolysis and the KREBS cycle, is for the greater part reoxidized by the respiratory chain, yielding ATP, NADPH is primarily occupied in synthesis processes, e.g. lipid synthesis (YUDKIN and OFFORD, 1971 [159]).

(b) Photorespiration. Contrary to the normal dark respiration consisting of glycolysis and the KREBS cycle, photorespiration is a light-induced process, which occurs in the chloroplasts and uses some early product of photosynthesis

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as a substrate (JACKSON and VOLK, 1970[160]). Glycolate is thought to be the original substrate, formed in the chloroplasts during photosynthesis, and it seems that, in glycolate metabolism, a cooperation exists between the chloroplasts and the peroxisomes, microbodies closely pressed against the chloroplasts. Ultimately, CO_2 is produced in photorespiration by the chloroplast (KISAKI and TOLBERT, 1969 [161]).

Normal dark respiration also occurs in the light, and the term photorespiration is sometimes used to indicate the total CO_2-evolution by the processes of dark respiration plus photorespiration.

1.3. SCOPE OF THE PRESENT INVESTIGATION

Among the various physiological and biochemical processes that may be affected by air pollutants in plants, photosynthesis and respiration deserve our special attention. Photosynthesis is the main energy accumulating process in plant life, while respiration is the breakdown process with concomittant formation of ATP and is especially important in dark reaction connected with 'growth'. The general experience is that photosynthesis and respiration have a high degree of sensitivity to air pollutants. The purpose of the present investigation was to contribute to unravel the mode of action of the air pollutant ozone. The effects of ozone on photosynthesis and respiration are studied by means of the WARBURG technique. The experiments were made with a unicellular alga, *Scenedesmus obtusiusculus* CHOD., because of the advantage of rapid cultivation possibilities. The use of algae, moreover, excludes the possible interference of stomatal reactions, as in higher plants. The development of suitable techniques for ozone production and treatment of the algae during the experiments appeared rather time consuming.

2. MATERIALS AND METHODS

2.1. OZONE GENERATION, ANALYSIS, SOLUBILITY AND TREATMENT
OF THE ALGAE

2.1.1. *Ozone generation*

In our experiments, after trying out several types of u.v. lamps, we have ulti-
mately used OZ 4 Watt bulbs (PHILIPS). These bulbs yield a relatively high and
constant ozone output. The 4 W bulbs have a tungsten filament and a small
amount of mercury. After the bulbs being switched on, the filament is heated
up, causing evaporation of the mercury. This results in a mercury vapour dis-
charge. The radiation so produced should, according to the PHILIPS documen-
tation, show a strong peak at 253.7 nm, but also a 184.9 nm short-wave emis-
sion. This 184.9 nm radiation, transmitted through the special glass of the bulb,
forms ozone from the oxygen of the surrounding air.

According to PAULING (1970 [162]) ozone is produced photochemically from
O_2 by light of the wavelength region 160–200 nm, while there is a destruction
of ozone by light of 240–360 nm wavelength.

A description of our O_3-generation system is given in figs. 9A and 9B. In order
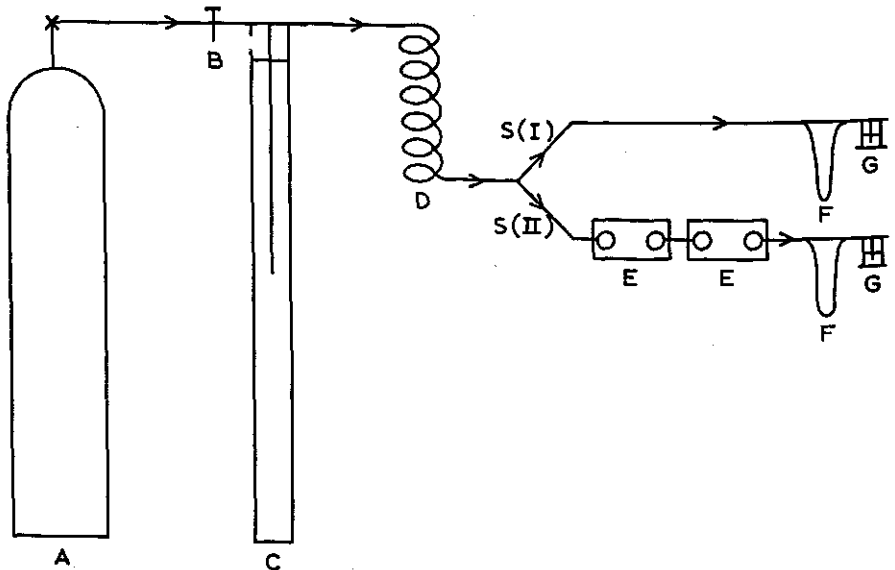to produce a high ozone level, it appeared important to cool the air from the

FIG. 9A. The air flow system. A = air bomb, B = flow-controller, C = water over-flow, D =
tap water cooling, E = ozone tubes, F = flow-meter, G = washing bottles, S(I) = stream
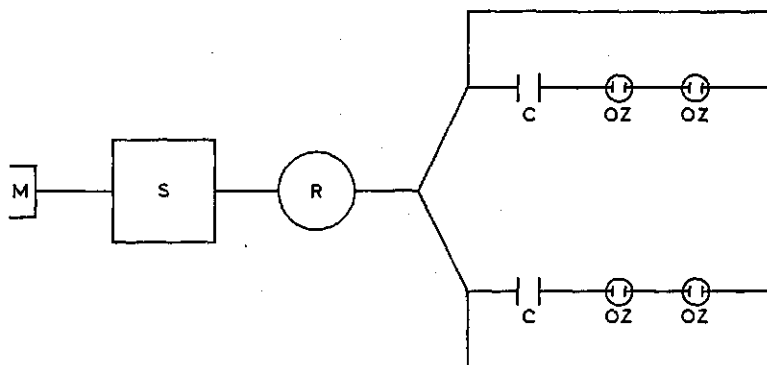I: pure air (blank), S(II) = stream II: air + ozone.

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FIG. 9B. The electric circuit for O_3 generation. M = mains, a.c. system; S = a.c. voltage stabiliser, output 220 V $\pm$ 1%; R = voltage regulator, output 200 V; C = a.c. condensor, 5 μF; OZ = ozone bulbs.

bomb by a tap-water cooled spiral (water temperature about 10–12 °C) before it passes the ozonisator. The ozone production with cooled air is significantly higher as compared with air directly from the bomb. Efforts to enhance the efficiency by cooling the ozonizer itself were negative, because the ozone output becomes lower.

2.1.2. *Ozone analysis*

All experiments have been made with an approximately constant ozone concentration in the air of about 150 ppm, although variations ($\pm$ 20 ppm) of unknown nature occurred. The air flow was always kept at a rate of 120 ml/min. The standard method for the determination of the ozone concentration before and after each experiment, was a neutral iodide procedure (BYERS and SALTZMAN, 1958 [163]). This method is quick and easy. The gas mixture was made to bubble from a capillary glass tip through 5 ml of a solution in an ordinary test tube (175 $\times$ 17 mm); the solution contains 0.08 M phosphate buffer pH 7.0 and 0.02 M potassium iodide (4.0 ml 0.1 M buffer + 1.0 ml 0.1 M KJ). The phosphate buffer consists of 61.2% 0.1 M Na_2HPO_4 and 38.8% 0.1 M KH_2PO_4. The exposure to the gas was 3 min. The reaction occurring is:

$$O_3 + 2 KJ + 2 H^+ \rightarrow O_2 + H_2O + 2 K^+ + J_2$$
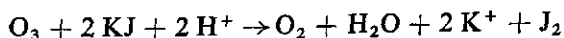
With real stoichiometry of the reaction, a certain amount of ozone should liberate the same molar amount of iodine.

At the end of the exposure time, suitable aliquots were taken from the solution and diluted with KJ-solution till the optical density of the liberated iodine at 350 nm can be accurately measured in glass boxes in a BECKMAN DU spectrophotometer. The optical density of a 1 molar solution of J_2 at 350 nm in a 1 cm light path is 26.5 $\times$ 10³, so the amount of liberated iodine in the original 5.0 ml solution is:

$$\frac{\text{optical density}}{26.5 \times 10^3} \times \frac{5.0}{1000} \times \text{dilution factor (moles)}$$

Suppose x μmoles of iodine are liberated, also x μmoles ozone were in the air bubbled through the solution. If the exposure time is 3 min. and the air flow 120 ml/min, 360 ml $= 36 \times 10^4$ μl air has bubbled through the solution. The ozone concentration may be expressed in ppm (v/v) with the aid of the standard molar volume (s.m.v.) for gases, e.g., by 760 mm Hg and 25°C the s.m.v. $= 24.45$ ltr. So, the ozone concentration amounts to: $(x \times 24.45)/(36 \times 10^4)$ ppm under the above mentioned conditions.

To test whether no iodine evaporates during the sampling time under our conditions, the gas mixture is bubbled through 5 ml KJ-solution with the addition of a surplus of thiosulfate. The remaining thiosulfate was titrated with an iodine solution of a known strength. It thus was found that the ozone concentration is underestimated with the standard KJ-procedure as compared with the titration procedure by about 25.0 %.

To test whether other oxidants, as impurities, are formed during our procedure of ozone generation, a more specific ozone test, the indigo-disulphonate (IDS) method, was used (BERGSHOEFF and SCHRIK-VROLIJK, 1970 [164]). This method is based on the bleaching activity of ozone on the blue IDS, according to:

$$C_{16}H_8N_2S_2O_8Na_2 + O_3 + H_2O \rightarrow 2\, C_8H_4NSO_5Na + H_2O_2$$
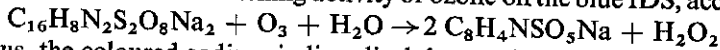
Thus, the coloured sodium indigo-disulphonate is converted into the colourless isatin-sulphonate.

The test solution of sodium indigo-disulphonate (0.01 %) in water is buffered at pH 6.85 with 0.1 M KH_2PO_4 + 0.1 M Na_2HPO_4. The gas mixture is permitted to bubble through 50 ml test solution in a fourfold absorber (BERGSHOEFF, 1966 [165]) at a constant rate of 120 ml/min by means of the normal air flow system mentioned earlier, and with the aid of a suction pump. Extinction changes at 610 nm measured at the BECKMAN DU spectrophotometer are compared with the iodine formation in a 50 ml KJ-test solution treated for the same duration in a fourfold absorber. The result is that with the IDS-method 82.3 % of the ozone is detected that is found with the KJ-method under these conditions. Since a stoichiometry-factor, F = 0.82, was found earlier (BERGSHOEFF and SCHRIK-VROLIJK, 1970 [164]) for the IDS-method, it may be concluded that during our ozone generation no other oxidants, such as NO_2 and H_2O_2, are formed.

2.1.3. Ozone solubility: A theoretical estimation of the ozone concentration in the algal suspension during the treatment

Let us assume the O_3-concentration in the air flow mixture to be 150 ppm. According to the HENRY formula for the solubility constant (K) of gases: K = p_a/x_a, in which p_a = the partial pressure of the gas (O_3) in the air flow mixture (in our case: $p_a = 150 \times 10^{-6} \times 760$ mm Hg) and x_a = the mole fraction of the gas (O_3) in the suspension,

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$$x_a = \frac{p_a}{K} = \frac{150 \times 10^{-6} \times 760}{K}.$$

The K-values for the solubility of ozone in the algal suspension at different temperatures may be taken to be equal to those for the solubility in water which are given in the International Criticle Tables (1928 [166]).
For 0°C: K = 1.475 × 10⁶, so:

$$x_a = \frac{150 \times 10^{-6} \times 760}{1.475 \times 10^6} = \frac{150 \times 760 \times 10^{-12}}{1.475}$$

To calculate the units of weight of O_3 per 1000 weights of H_2O, the following formula may be used:

$$\frac{x_a}{1-x_a} \cdot \frac{M_{O_3}}{M_{H_2O}} \cdot 1000 = \frac{150 \times 760 \times 10^{-12}/1.475}{1 - \dfrac{150 \times 760 \times 10^{-12}}{1.475}} \cdot \frac{48}{18} \cdot 1000 =$$

$= 20.61 \times 10^{-5}$ g O_3/1000 g H_2O = 206.1 µg O_3/1000 g $H_2O \approx$ 206.1 µg O_3/l H_2O

Equally we may calculate:
At 15°C: K = 2.191 × 10⁶, yielding an O_3 concentration of 138.6 µg/l H_2O
At 25°C: K = 3.475 × 10⁶, yielding an O_3 concentration of 87.2 µg/l H_2O
At 35°C: K = 6.220 × 10⁶, yielding an O_3 concentration of 48.9 µg/l H_2O
These concentrations are corrected for density of H_2O at different temperatures.

2.1.4. *Ozone treatment of the algae*

Comparisons were first made between treatment with ozone of a 50 ml algal suspension in (a) a glass washing bottle with an inlet tube with a capillary tip, (b) a glass washing bottle with a glass-sinter, (c) a glass spiral washing apparatus and (d) a glass tube (with a diameter of about 1.5 cm) with a glass plate inside, in a position which was very near to horizontal. The expected advantage of (b) is a better mixing of the gas in its passage through the suspension, of (c) and (d) it is the longer path through the suspension which could lead to better contact of the ozone with the algal cells and so to increase the toxic effect.

The biological test (inhibition of photosynthesis), however, showed that system (a) is one of the most efficient treatment procedures and because of its easy and quick operation this system was chosen as the standard method.

In each experiment the ozone exposure was measured against a blank (air exposure), which run at the same moment under equal conditions. Usually, the algae are suspended in tap water to 50 ml volume with a TROMMSDORFF density of 2.5 µl/ml, and brought into a 100 ml washing bottle. The bottle is placed in the wather bath of a WARBURG apparatus. The temperature of the bath is regulated in the normal way by heating and cooling (see under 2.3.). During the exposure to O_3, if not stated otherwise, the algae in the bottles are illuminated from below with incandescent lamps, with a light intensity on the bottom sur-

faces of the bottles of about 1.5–1.75×10^5 ergs/cm^2.sec (see under 2.3.). During the treatment, the bottles are shaken with the aid of the rocking framework of the WARBURG apparatus, because it was found that non-shaking is less efficient in producing the toxic effect (inhibition of photosynthesis). An important physico-chemical difficulty appearing during the treatment of the algae with ozone is the development of foam in the suspension. This phenomenon always appeared after about one and a half hour exposure. The foaming is probably produced by the leakage of surface active substances from the algal cells. It disturbs the results, because it causes a loss of algal cells adhering to the wall of the bottle just above the suspension surface which also implies that the cells foamed out of the suspension may be exposed to a higher ozone concentration.

To avoid foam development, a small dose of an antifoaming agent (silicone compound AF DOWN CORNING) was added to the suspension. It was checked that this antifoaming agent had no detrimental effect on photosynthesis and respiration of the algal cells.

In general, the ozone gasmixture leaving the algal suspension was made to bubble through a KJ-solution to remove the remaining ozone.

2.2 CULTIVATION OF THE ALGAE

The algae, *Scenedesmus obtusiusculus* CHOD. (cf. DAS, 1968 [167]), were kept as sterile stock cultures in tubes with 1.5% agar solidifying a medium which per litre 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 [168]), were supplied. The cells for the experiments were cultivated as follows. In a chamber sterilized with u.v. light the algae from an agar slant in a culture tube were transferred to about 15 ml sterilized culture medium with a sterile needle. This suspension was brought into a one litre erlenmeyer with about 300 ml culture medium, per litre containing:

KNO_3	1.0 g	$FeSO_4$	0.006 g
KH_2PO_4	0.136 g	Na-citrate	0.004 g
$MgSO_4$	0.5 g	EDTA	0.002 g

In addition, 1 ml 0.1 M $CaCl_2$ and 2 ml of a combined A_4 – and B_7 – solution of trace elements (ARNON, 1938 [168]) were supplied. Concentrated stock solutions of the salts were sterilized separately or in mixtures (the solution containing the trace elements and the iron).

The erlenmeyer flasks were placed on a rocking table and illuminated from below by fluorescent tubes. Room temperature was kept at 22 °C. The suspen-

sion was flushed with a stream of air containing 5% CO_2, through a capillary tube with a cotton wool filter. After 3–5 days, the algal cells were used for an experiment. Cell density then was about 2–5 µl cells/ml.

The algae were harvested by centrifugation, usually at 3000 rpm during 10 minutes, and the density of the suspension was determined with the aid of TROMMSDORFF tubes. Cells were suspended in the medium used in the experiments, and adjusted to the desired density value. The final densities of the suspensions are given in µl packed wet cells per ml.

2.3. MEASUREMENT OF PHOTOSYNTHESIS AND RESPIRATION BY MEANS OF GAS EXCHANGE

Measurements of gas exchange were made with a WARBURG apparatus. It had a thermostated waterbath of 100×30 cm, which could be kept at temperatures between 15°C and 35°C by an electric heater, controlled by a thermorelay with an accuracy of 0.05 °C, and a cooling system consisting of a regulated, continuous flow of tap water through a copper coil. The water in the thermostated bath was stirred by an electric pump. A rocking frame on which 11 manometers could be placed, was mounted in front of the bath. The reaction vessels had flat bottoms with a diameter of 5–6 cm and a total volume of about 50–60 ml. They were illuminated from below through the glass bottom of the bath by 6 incandescent lamps ('Splendor' 150 Watt). They were cooled by two small fans of 20 W each.

The intensity of visible light at the position of the bottom of the vessels in the bath 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 about 750 nm. The light intensity values given in the text of this paper are average values from 9 measurements at different sites of the bottom-surfaces of the vessels. The values are corrected for the infrared radiation transmitted by the SCHOTT RG 8 filter; under the conditions of our experiments this filter at the level of the vessels transmits about 66% of the total radiation of the lamps.

Different light intensities were obtained by neutral metal screens, placed between the lamps and the vessels on a framework below the waterbath. Unless stated otherwise, the unweakened light intensity in the range up to 690 nm was about $1.4–2.0 \times 10^5$ ergs/cm².sec.

In our experiments, WARBURG's carbonate-bicarbonate mixture no. 9 was used, consisting of 150 ml 0.1 M Na_2CO_3 and 850 ml 0.1 M $NaHCO_3$ per litre; the pH of this buffer is about 9.37. According to WARBURG (1928 [169]), this buffer is in equilibrium with a CO_2-concentration of 91×10^{-6} M at 25°C, which corresponds to about 0.22 vol. % 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), according to: $x = h.V_C$. The volume of the vessel was determined by weighing its content of mercury.

As a manometer liquid we used BRODIE solution consisting of 23 g NaCl, 5 ml Teepol as a detergent, 500 ml aqua dest. plus a small quantity of a coloured substance, such as the red 'Agfa' new coccin that secures good visibility for a long time. Ten meters pressure of this solution is assumed to equal 1 atmosphere (760 nm Hg).

In all gas exchange studies, 10 ml algal suspension were introduced into the vessels, kept at 25 °C. One manometer vessel was provided with distilled water and served as a thermobarometer.

In measurements of photosynthesis, usually 10 ml algal suspension, $T_D = 2.5$ were used; in those of respiration usually 10 ml algal suspension, $T_D = 10.0$ μl/ml.

Prior to using the incandescent lamps, as mentioned above, we used HPL lamps. The light of these lamps, however, caused inhibition of photosynthesis of untreated algae. The inhibition became stronger with increased exposure time. The effect could be avoided by filtering the light with a yellow filter, and it may be concluded that probably some long-wave u.v. is produced by these lamps, which is not filtered by the glass and the water phase and may inhibit photosynthesis.

2.4. DETERMINATION OF THE CHLOROPHYLL CONTENT
AND COMPOSITION OF THE ALGAE

We have used extraction with 96% ethanol. Usually, 15 ml algal suspension, with a T_D-value of 2.5, was centrifuged for 10 min. at 3000 rpm. After centrifugation, the algae are resuspended in 5 ml ethanol (96%) and shaken for extraction during 10 min. in a boiling waterbath in the dark to avoid chlorophyll bleaching in the light. The remnants of the cells are precipitated by centrifugation and the extract is diluted to 50 ml with 96% ethanol. The optical density of the chlorophyll solution was determined in glass boxes in a BECKMANN DU spectrophotometer. For computation of the chlorophyll a, chlorophyll b, and chlorophyll a+b contents, and the a/b ratio, the ratio $A_{max\ a}/A_{max\ b}$, that is the ratio of absorbances at the red maxima of chlorophylls a and b, was determined. Contents of chlorophylls a and b, and their sum then can be determined by multiplication, and the a/b ratio read or calculated from table data, as given by WINTERMANS (1969 [170]), which are derived from specific absorbance coefficients. In our experiments, $A_{max\ a}$ was determined at 665 $\pm$ 2 nm, while $A_{max\ b}$ was read at a constant distance of 16 nm from $A_{max\ a}$ and thus was established at 649 $\pm$ 2 nm. The final chlorophyll content is given in μg chlorophyll/μl algae/ml ethanol.

2.5. DETERMINATION OF LIPID CONTENT AND FATTY ACID COMPOSITION OF THE ALGAE

2.5.1. *Determination of lipid content*

For extraction of the algae, a modification of the BLIGH and DYER method (BLIGH and DYER, 1959 [171]) was used: 80 ml of algal suspension ($T_D = 2.5$ $\mu l/ml$) were centrifuged, and the algae resuspended in 50 ml methanol/chloroform in the ratio 2 :1 (v/v). The mixture was shaken, and the cell remnants were separated by filtering with the aid of a glass-filter with filterpaper, and a water current suctionpump. The remnants on the filterpaper were washed twice, viz., with 5 ml methanol/chloroform (2/1) and with 5 ml chloroform. About 30% distilled water was added to the combined filtrates. The ultimate methanol/water/chloroform mixture was shaken in a separatory funnel and set aside in a dark chamber at 5°C for separation. If the biphasic mixture (water/methanol and chloroform) was separated completely, the clear chloroform layer containing the lipid was removed to an evaporation vessel. Again, chloroform was added to the remaining water/methanol phase for repeated separation, sometimes followed by a third separation. All chloroform fractions were combined in the evaporation vessel and the chloroform evaporated under vacuum with the aid of a rotary evaporator. The lipid residue was taken up in a small amount of chloroform, brought into a small weighing tube, dried in an air stream and weighed.

2.5.2. *Determination of fatty acid composition*

A lipid extract obtained according to the procedure mentioned in 2.5.1. was used to determine fatty acid composition, as described by KUIPER and STUIVER (1972 [172]). About 10 mg lipid was taken up into 2 ml chloroform, and deacylated by shaking with 2 ml KOH for 15 min. at 37–38 °C. After this hydrolyzing procedure, a mixture of 0.5 ml 1.5 n HCl and 3 ml hexane was added and intensely shaken in a tube with a small diameter (0.5 cm), to separate the fatty acids. If the biphasic mixture was separated completely, the upper, hexane layer, containing the liberated fatty acids, was taken up in small tubes and dried in an air-stream. Ultimately, the fatty acids were methylated with 1 ml 10% BCl_3 in methanol for 2 min. in a boiling waterbath, and analyzed on a 'Victoreen' gas chromatograph with a hydrogen flame ionization detector. The column was composed of 15% diethylene glycol succinate on Anakrom 60/70 as a stationary phase.

2.5.3. *Lipid peroxidation*

To test whether lipid peroxidation occurred in the algae during ozone treatment, the appearance of malondialdehyde, a breakdown product of unsaturated fatty acid hydroperoxides, was estimated by the use of thiobarbituric acid (TBA) as described by HEATH and PACKER (1968 [173]). A 3 ml algal suspension ($T_D = 2.5$ $\mu l/ml$) was incubated in a test tube with 3 ml 0.5% TBA in 20% trichloroacetic acid (TCA) for 30 min. at 95°C in a waterbath. After cooling

and centrifugation to yield a clear supernatant, the extinction (E) of the solution was measured at 532 and 600 nm. From the value of $E_{532\,nm} - E_{600\,nm}$, the level of malondialdehyde was estimated by using the extinction coefficient of $155\,mM^{-1}\,cm^{-1}$ (HEATH and PACKER, 1968 [173]).

2.6. DETERMINATION OF PROTEIN CONTENT AND AMINO ACID COMPOSITION OF THE ALGAE

2.6.1. *Determination of protein content*

The KJELDAHL method was used for the estimation of the total nitrogen content of the algae. The procedure described by HUNTJENS (1971 [174]) was followed, and carried out at the laboratory of Microbiology of the Agricultural University, Wageningen; it is a modification of the method described by CHIBNALL *et al.* (1943 [175]). An amount of 165 ml of an algal suspension ($T_D = 2.5\,\mu l/ml$) was centrifuged, the cells were taken up in a small amount of demineralized water to transfer them to a longnecked 100 ml KJELDAHL flask. About 1 g of a mixture, containing $Na_2SO_4 + CuSO_4.5\,H_2O + Se$ in a ratio 190:3:4, was added, followed by 10 ml concentrated H_2SO_4; a digestion-catalyst mixture without algae was introduced into a similar flask, and operated as a blank. The flasks containing the algal mixture, were placed on a gas-heated stand and the mixture was boiled gently in such a way that foam development was not too extensive, while the flask was regularly shaken. During this procedure the colour of the digest changes from green to brown and ultimately to black. Finally, after digestion, a clear liquid remains, and the heating was continued for another 30 minutes. About 50 ml distilled water were added to the cooled digest, and after renewed cooling the solution was brought into a flask, connected with a distillation apparatus. After addition of excess NaOH solution (about 50 ml, 0.2 n) to the distillation flask, ammonia was distilled off, collected in excess boric acid (about 10 ml, 2%) followed by titration with 0.0211 n H_2SO_4 using methylred-bromocresolgreen as an indicator (green → colourless → red). The N-content can be calculated from:

$$mg\,N = a \times b \times 14,$$
with $a =$ ml H_2SO_4 used by titration
$b =$ strength of the H_2SO_4 solution
$14 =$ atomic weight of nitrogen,

and the protein content from:

$$mg\,protein = mg\,N \times 6.25.$$

2.6.2. *Determination of the amino acid composition*

Again, 165 ml algal suspension ($T_D = 2.5\,\mu l/ml$) were centrifuged and the algae resuspended in about 50 ml 6 n HCl. Hydrolysis was carried out in a sealed glass tube in vacuo for 24 hr. at 110°C. The hydrolyzed sample was

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allowed to evaporate under vacuum until HCl was completely removed and the dry residue was dissolved in a small amount (5–10 ml) of 0.2 M citrate, pH 2.2. The turbid solution was filtered with the aid of a bacterial filter, and a clear, yellow filtrate was obtained.

The amino acids in the citrate solution were analyzed with the aid of an automatic 'Biocal' 200 amino acid analyzer. The procedure was again carried out at the laboratory of Microbiology of the Agricultural University, Wageningen, as described by VAN EGERAAT (1972 [176]), following the procedure of MOORE and STEIN (1954 [177]). The acid and neutral ninhydrin-positive compounds were eluted from a column, containing a 55 × 0.9 cm Biorad A6 spherical ion-exchange resin, and using a citrate buffer of pH 3.25 for elution. The alkaline ninhydrin-positive compounds were eluted from a column, containing a 25 × 0.9 cm Biorad A5 ion-exchange resin, and eluted by using a citrate buffer of pH 5.28. The ninhydrin solution contained: 750 ml methyl cellosolve; 250 ml sodium acetate buffer (4n, pH 5.51); 20 g ninhydrin; and 400 mg $SnCl_2.2 H_2O$.

The identity of the ninhydrin-positive compounds in the sample were determined by comparison with analyses obtained from calibration mixtures of authentic ninhydrin-positive compounds. The relative amounts of the various ninhydrin-positive compounds in the sample were determined by measuring the areas enclosed by the corresponding peaks on the chromatogram, as compared with the areas found when known concentrations of authentic ninhydrin-positive compounds were used.

2.7. ELECTRON MICROSCOPE STUDIES

A small amount of an algal suspension (about 20 ml; $T_D = 2.5$ μl/ml) was centrifuged and the algae killed and fixed with potassium permanganate in water at 0 °C during 15 min., followed by washing. The suspension then obtained was centrifuged at a moderate speed. The supernatant was removed and the centrifuge tube with the pellet brought to 45 °C in a waterbath. Meanwhile, a 2 % agar solution had been prepared and was maintained also at 45 °C in the waterbath. A small quantity of the agar solution was then added to the pellet and thoroughly mixed by stirring. While keeping the mixture at 45 °C, a small quantity was sucked up in a pipette with an internal diameter of 0.5 mm. The mixture inside the pipette solidifies immediately, forming a rod. After being removed from the pipette, this rod was cut into small pieces. These pieces were stained in 2 % uranyl acetate during 2 hrs. After washing with water and dehydration in an alcohol series, the pieces were embedded in an epon araldite mixture via propylene oxide. Ultrathin sections were made on an LKB Ultrotome III, using glass knives, and examined in a PHILIPS EM 300 transmission electron microscope. The procedure was carried out at the Technical and Physical Engineering Research Service by HENSTRA and coworkers.

3. RESULTS

3.1. EFFECTS OF PRE-EXPOSURES TO OZONE ON THE RATES
OF PHOTOSYNTHESIS AND RESPIRATION*

3.1.1. *Inhibition of photosynthesis and respiration in relation to exposure time and suspension temperature*

Table 1 and fig. 10A show that the inhibition of photosynthesis (measured at light saturation and at 25 °C) as compared with a sample, similarly treated with air, in general increases with the duration of the exposure to ozone, and with the temperature during exposure. At all temperatures the effect has the tendency to flatten off with increased duration of exposure (see also figs. 10C I and 10C II), while the effect increases over the whole temperature range used (see also figs. 10B I and 10B II). The highest inhibition percentage is observed for the average value of the 4 hr. and 35 °C series and amounts to 63.8 %. Minor irregularities were observed for the 1 hr. exposure series, and for the 15 °C temperature series at different exposure times.

In order to study the temperature effect during ozone exposure in more detail, we have tried to evaluate Q_{10}-values for inhibition as well as for remaining
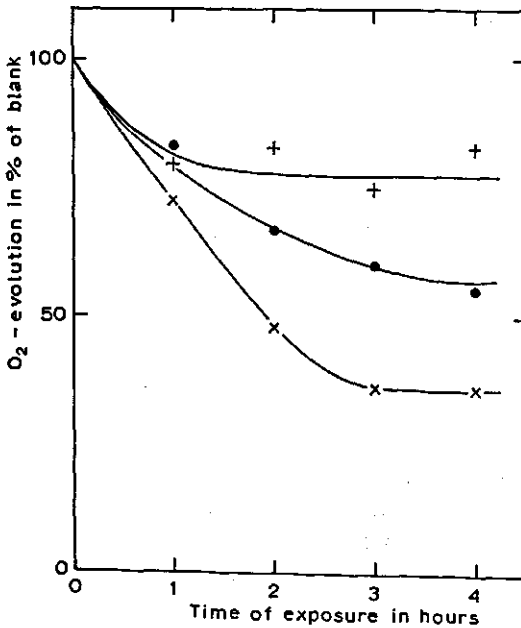

FIG. 10A. Inhibition of photosynthesis, measured at 25 °C and at saturating light intensity, after ozone exposures during different times and at different temperatures; $+ - + = 15$ °C, $\bullet - \bullet = 25$ °C, $\times - \times = 35$ °C.

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* Respiration was measured as dark respiration. Not in all cases dark respiration was measured and thus a strict distinction between true and apparent photosynthesis could not always be made.

TABLE 1. Photosynthesis as affected by exposure time and temperature during exposure to ozone. O_2-evolution for the blank and the ozone-treated samples measured at 25°C and at saturating light intensity. Data presented in μl O_2/25 μl algae per hour. (cf. also fig. 10A).

Exposure time	15°C				25°C				35°C			
Temperature	Blank	O_3	Percent activity	Percent inhibition	Blank	O_3	Percent activity	Percent inhibition	Blank	O_3	Percent activity	Percent inhibition
1 hr.	674.0 (3)	538.4 (3)	79.9	20.1	809.2 (3)	673.2 (3)	83.2	16.8	874.4 (3)	634.5 (3)	72.6	27.4
2 hr.	779.3 (6)	646.4 (6)	82.9	17.1	814.4 (8)	543.1 (8)	66.7	33.3	749.9 (3)	357.0 (3)	47.6	52.4
3 hr.	787.2 (3)	590.4 (3)	75.0	25.0	808.7 (3)	486.4 (3)	60.1	39.9	737.2 (3)	268.5 (3)	36.4	63.6
4 hr.	840.7 (3)	695.1 (3)	82.7	17.3	721.0 (3)	399.1 (3)	55.4	44.6	924.8 (3)	334.8 (3)	36.2	63.8

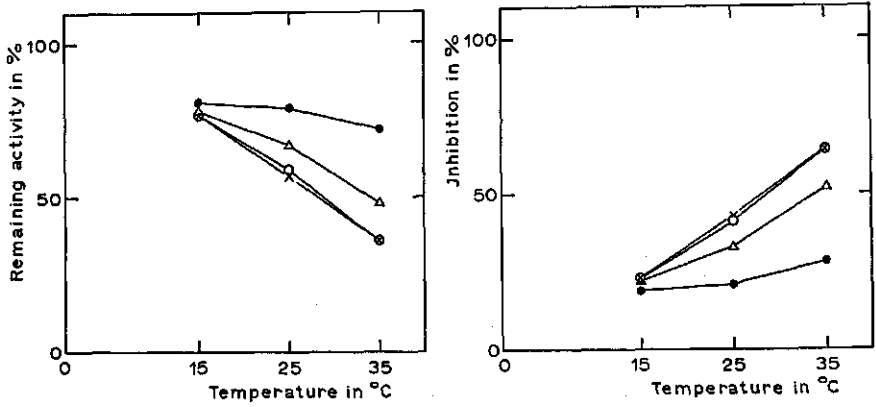Numbers in parentheses: number of experiments.

FIG. 10B I. Remaining activity (left) and inhibition (right) of O_2-evolution in % of the blank, after ozone exposures at different temperatures and during different times (data used, as obtained from the curves in fig. 10A); ● – ● = 1 hr., △ – △ = 2 hr., ○ – ○ = 3 hr., × – × = 4 hr.
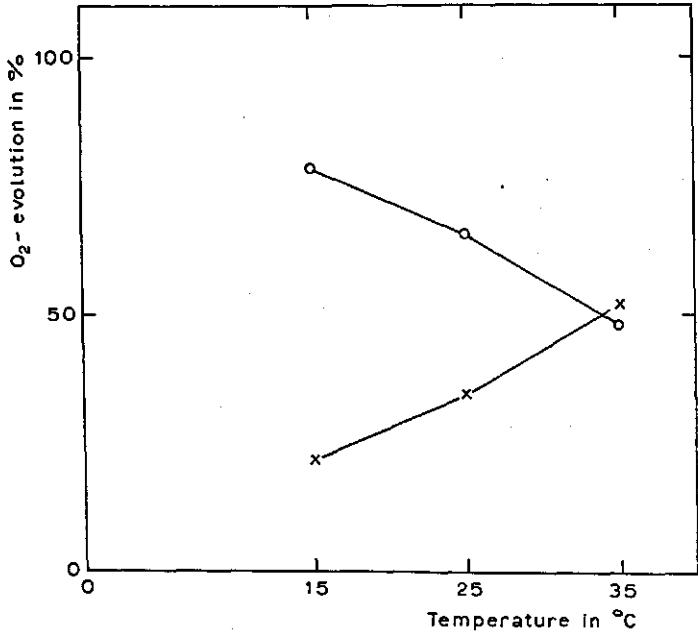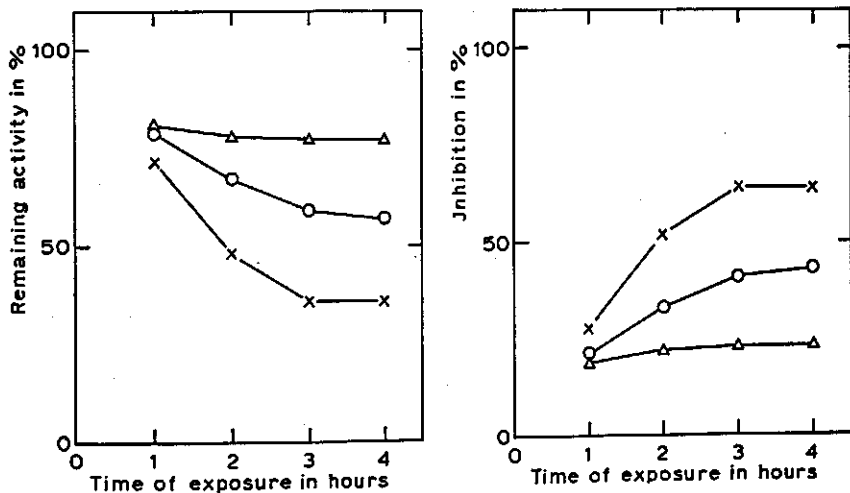

FIG. 10B II. O_2-evolution in % of the blank, after ozone exposures at different temperatures and averaged over 4 exposure times (data used, as obtained from the curves in fig. 10A); ○ – ○ = remaining activity (%), × – × = inhibition (%).

FIG. 10C I. Remaining activity (left) and inhibition (right) of O_2-evolution in % of the blank, after ozone exposures during different times at different temperatures (data used, as obtained from the curves in fig. 10A); $\triangle - \triangle = 15°C$, $O - O = 25°C$, $\times - \times = 35°C$.
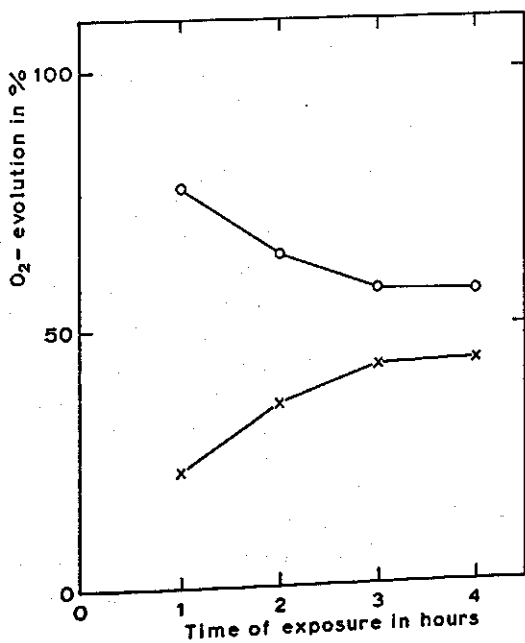

FIG. 10C II. O_2-evolution in % of the blank, after ozone exposures at different exposure times and averaged over 3 temperatures (data used, as obtained from the curves in fig. 10A); $O - O$ = remaining activity (%), $\times - \times$ = inhibition (%).

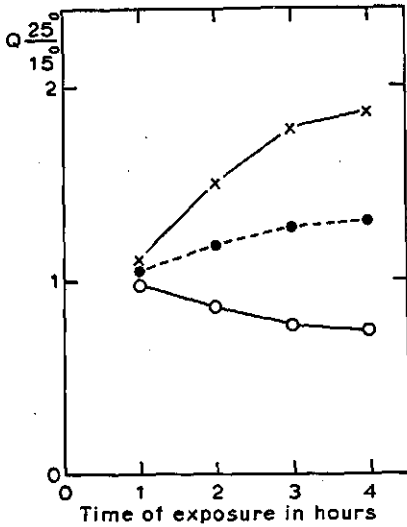FIG. 10D. Q $^{25}/_{15}$ values of remaining activity (O – O) and inhibition (×–×) of photosynthesis; the dotted line (● – – ●) represents the average value for remaining activity and inhibition (data used, as obtained from the curves in fig. 10A).
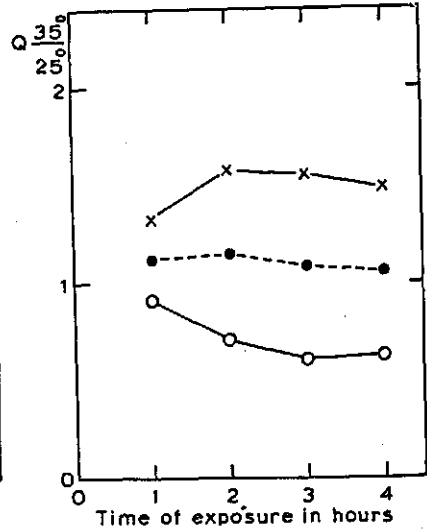
FIG. 10E. Q $^{35}/_{25}$ values of remaining activity (O – O) and inhibition (×–×) of photosynthesis; the dotted line (● – – ●) represents the average value for remaining activity and inhibition (data used, as obtained from the curves in fig. 10A).

activity of photosynthesis (figs. 10D and 10E). The first fact to be clear is that at 15°C inhibition has reached its final (fairly low) level already after 1 hour (see figs. 10A and 10C I). On the contrary, at the higher temperatures, causing lower ozone concentrations in the suspensions (see 2.1.3.), inhibition still increases considerably, at the least up to three hours exposure and the more so, the higher the temperature of the suspension is. This entails that Q 25/15 increases strongly with time; the values found are between about 1.10 and 1.90 from 1 to 4 hrs. Q 25/15 of the remaining activity is, as might be expected from the course of Q_{10} of the inhibition, below unity and decreases further in the course of time. The numerical decrease is from slightly below 1 to about 0.7 which is a smaller shift than the one observed for inhibition (here the corresponding figure is ∼ 0.59, in the reverse direction).

Since at 25°C and at 35°C the time course of inhibition is much more similar than at any of these temperatures and 15°C, the change of Q 35/25 with time is much less dramatic than that for Q 25/15, the values are between 1.30 and 1.60 with the largest change between 1 and 2 hours. In this respect the situation more or less corresponds to the one for Q 25/15 where the change of Q_{10} between 1 and 2 hrs. is also largest, viz., from 1.10 to 1.50. For Q 35/25 the values slightly decrease between 2 and 4 hours, contrary to the situation for Q 25/15. Q 35/25 of the remaining activity much resembles that of Q 25/15;

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Q 35/25 runs from 0.90 to 0.60 between 1 and 4 hours of pre-exposition in the light to ozone.

It would seem that the numerical values of the Q_{10}'s obtained are affected by the percentual values of inhibition and remaining activity as found in the experiments. The rather obvious differences between Q 25/15 and Q 35/25 for inhibition may thus be due to two coupled facts, as observed, viz., 1) the relatively low level of inhibition at 15°C, and 2) the fact that this level is reached already after 1 hr. contrary to what happens at higher temperatures.

Since there, of course, is a kind of physiological coupling between inhibition and remaining activity, which should also express itself in their temperature dependencies, we have tentatively tried to express this supposed relationship in the 'average' for the Q_{10}-values for both regions after different times of exposure. This 'average' runs smoothly in both cases, showing slight increase for Q 25/15 and slight decrease for Q 35/25. Ideally, one probably might have expected a horizontal line at the 1.0 level, the deviations may be mainly due to the reasons mentioned above under 1) and 2). This may (perhaps in part) explain the nearer approach to this 'ideal' for Q 35/25.

It is remarkable to see that the effect of ozone on photosynthesis is affected by temperature, in such a strong way that at the highest ozone concentration, which prevails at the lowest temperature (15°C) of the suspension, the toxic effect of ozone is smallest owing to the low temperature of the suspension. On the contrary, at higher suspension temperatures resulting necessarily in lower ozone concentrations in the suspension, the effect is higher.

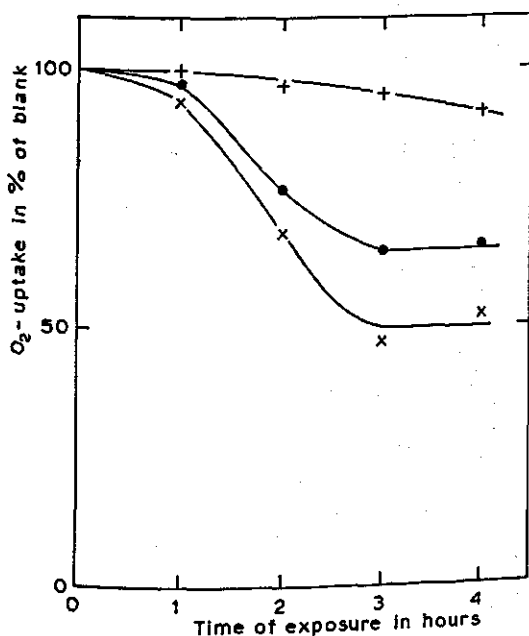Table 2 and fig. 11A indicate that the inhibition of respiration increases rather

FIG. 11A. Inhibition of respiration, measured at 25°C after ozone exposures during different times and at different temperatures; + – + = 15°C, ● – ● = 25°C, × – × = 35°C.

TABLE 2. Dark respiration as affected by exposure time and temperature during exposure to ozone. O_2-uptake for the blank and the ozone-treated samples measured at 25 °C in the dark. Data presented in µl O_2/25 µl algae per hour. (cf. also fig. 11A).

Exposure time	Temperature	15 °C				25 °C				35 °C			
		Blank	O_3	Percent activity	Percent inhibition	Blank	O_3	Percent activity	Percent inhibition	Blank	O_3	Percent activity	Percent inhibition
1 hr.		50.5 (3)	50.3 (3)	99.6	0.4	60.0 (3)	58.4 (3)	97.3	2.7	54.4 (3)	50.9 (3)	93.6	6.4
2 hr.		59.9 (3)	58.1 (3)	97.0	3.0	56.9 (8)	43.6 (8)	76.6	23.4	47.6 (3)	32.6 (3)	68.5	31.5
3 hr.		58.6 (3)	55.9 (3)	95.4	4.6	66.8 (3)	43.2 (3)	64.7	35.3	59.8 (3)	28.4 (3)	47.5	52.5
4 hr.		57.5 (3)	52.6 (3)	91.5	8.5	66.5 (3)	43.7 (3)	65.7	34.3	48.3 (3)	25.3 (3)	52.4	47.6

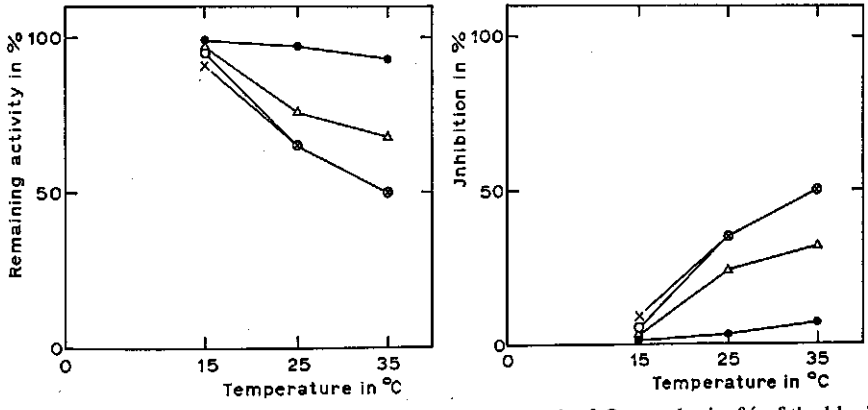Numbers in parentheses: number of experiments.

FIG. 11B I. Remaining activity (left) and inhibition (right) of O_2-uptake in % of the blank, after ozone exposures at different temperatures and during different times (data used, as obtained from the curves in fig. 11A); ●–● = 1 hr., △–△ = 2 hr., ○–○ = 3 hr., ×–× = 4 hr.
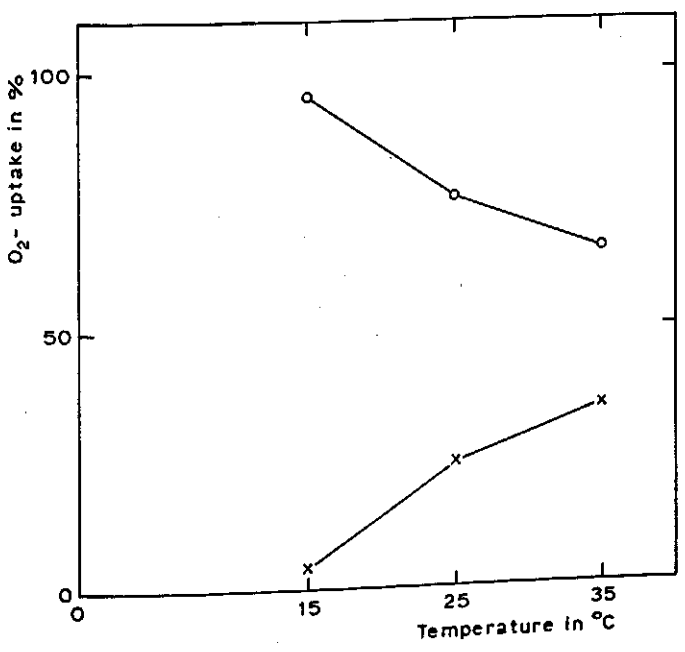

FIG. 11B II. O_2-uptake in % of the blank, after ozone exposures at different temperatures and averaged over 4 exposure times (data used, as obtained from the curves in fig. 11A); ○–○ = remaining activity (%), ×–× = inhibition (%).

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regularly with the duration of the contact to ozone and with the temperature of the suspension during ozone exposure. The highest inhibition percentage was found for the average value of the 3 hr. and 35 °C series and amounts to 52.5 %.
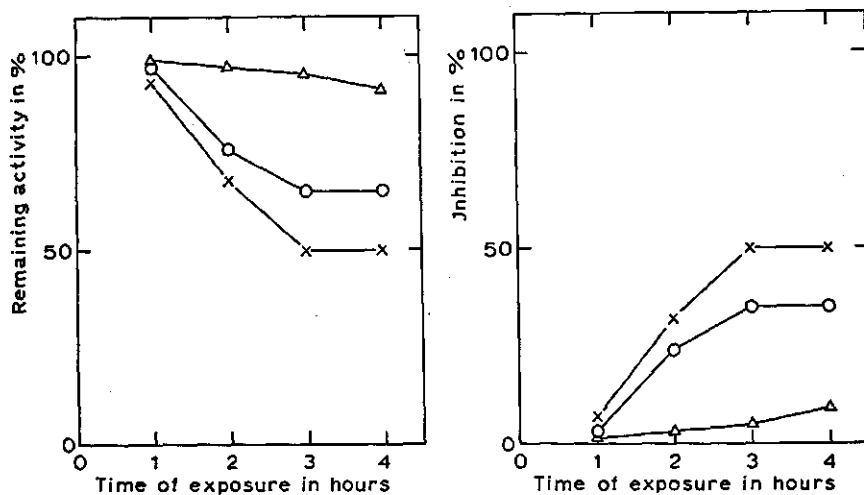

FIG. 11C I. Remaining activity (left) and inhibition (right) of O_2-uptake in % of the blank, after ozone exposures during different times at different temperatures (data used, as obtained from the curves in fig. 11A); $\triangle - \triangle = 15$°C, $O - O = 25$°C, $\times - \times = 35$°C.
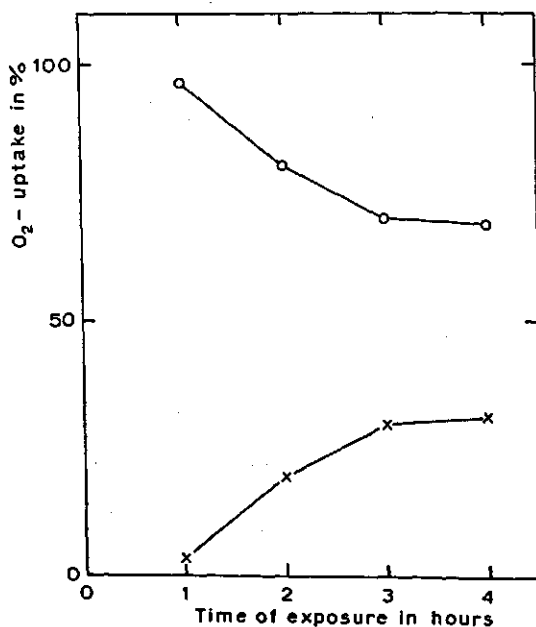

FIG. 11C II. O_2-uptake in % of the blank, after ozone exposures at different exposure times and averaged over 3 temperatures (data used, as obtained from the curves in fig. 11A); $O - O =$ remaining activity (%), $\times - \times =$ inhibition (%).

The 25°C and 35°C series show a time-rate saturation effect after 3 hrs. exposure, while the 15°C series does not. The overall temperature effect in the exposure-time series was always higher with increasing temperature (see also figs. 11B I and 11B II), while in general the effect has the tendency to flatten off with increased duration of ozone exposure (see also figs. 11C I and 11C II).

As in the case of photosynthesis, we have made a more extensive evaluation of the temperature effect on Q_{10}-values for inhibition and remaining activity of respiration (figs. 11D and 11E). It may be noted that inhibition of respiration in the 15°C series, yielding the highest ozone concentration in the suspension (see 2.1.3.), is very small, contrary to the higher temperature series, in which the effect increases sharply after 1 hr. (see figs. 11A and 11C I). This results in high
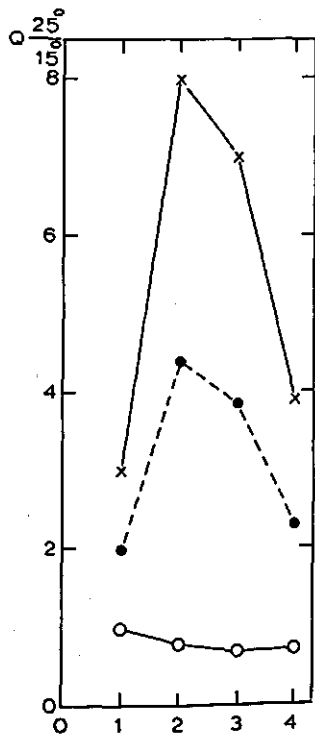

Time of exposure in hours

FIG. 11D. Q $^{25}/_{15}$ values of remaining activity (O – O) and inhibition ($\times$–$\times$) of respiration; the dotted line ($\bullet$ –– $\bullet$) represents the average value for remaining activity and inhibition (data used, as obtained from the curves in fig. 11A).

FIG. 11E. Q $^{35}/_{25}$ values of remaining activity (O – O) and inhibition($\times$–$\times$) of respiration; the dotted line ($\bullet$ –– $\bullet$) represents the average value for remaining activity and inhibition (data used, as obtained from the curves in fig. 11A).

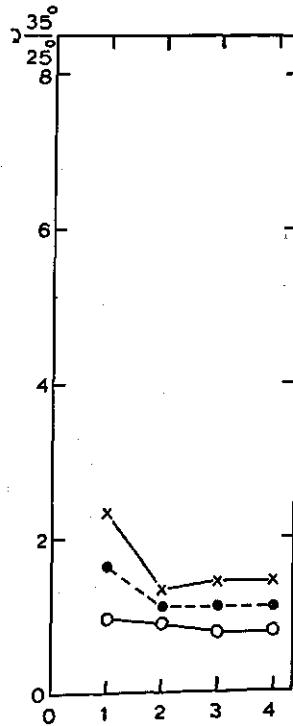Q 25/15 values ranging from 3.0 up to 8.0 from 1 to 2 hrs. and declining to about 4.0 at 4 hours. Q 25/15 of the remaining activity is below unity and decreases in the course of time from slightly below 1 to about 0.8 which is a much smaller shift than the one observed for inhibition, in the reverse direction.

The change of Q 35/25 with time is much smaller than that of Q 25/15 by way of the more corresponding time courses of inhibition at 25°C and at 35°C. The Q 35/25 values are between ∼ 1.3 and 2.3, with the largest change between 1 and 2 hrs. Q 35/25 of the remaining activity resembles much that of Q 25/15; Q 35/25 runs from slightly below 1 to 0.8 between 1 and 4 hours of exposure in the light to ozone.

The numerical values of the Q_{10}'s and therefore the differences between Q 25/15 and Q 35/25 for inhibition are affected by the facts 1) the low level of inhibition at 15°C, which shows an increase with time up to 4 hours, and 2) the strong increase of inhibition at 25°C and 35°C after 1 hr. of exposure up to 3–4 hours; especially the strong difference between 15°C and 25°C is very striking and, obviously, is largely responsible for the observed Q 25/15 values for respiration.

We have looked tentatively at an 'average' value for the Q_{10}'s of inhibition and remaining activity. It is questionable whether one should ideally expect also here a horizontal line at the 1.0 level, this seems uncertain owing to the very low values for inhibition at 15°C, and generally, to the reasons mentioned under 1) and 2). This may also explain the much nearer approach to the 'ideal' for Q 35/25 than for Q 25/15.

Also for respiration it appeared that under relatively high ozone concentrations in the suspension at low temperature, a stronger toxic effect of ozone is not manifest at 15°C. On the contrary, at higher suspension temperatures with necessarily lower ozone concentrations in the suspension, the effect is higher. As in the case of photosynthesis this indicates that the increase of the rate of the damaging reaction with increase in temperature strongly overcompensates the lower solubility of ozone.

It should be observed that, in the present paper, photosynthesis and respiration have always been measured at 25°C, and, as far as photosynthesis is concerned, in the temperature series at light saturation. Therefore, the temperature effects measured are concerned with the pretreatment of the algal suspensions with ozone, at various temperatures. Thus, the effects of temperature represent effects on the damaging of the photosynthetic apparatus by the pretreatment with ozone, in most cases in the light (saturating intensities), during different periods (1–4 hours). It should already be mentioned here (cf. also 3.1.5.) that the effect is, for the durations of treatment applied, mainly irreversible. It, thus, can be stated that what is measured at 25°C afterwards, is the reduced capacity of the cells for photosynthesis and respiration, owing to the preceding ozone treatment.

It thus seems, that the Q_{10}-values of 'inhibition', i.e. reduction in capacity, have a more realistic value than those of 'remaining activity', since the results register the effect of a previous, 'inhibiting' treatment with ozone. For photo-

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synthesis, the Q_{10}-values appear quite plausible, viz., from ~ 1.9 for Q 25/15 to ~ 1.5 for Q 35/25 after 4 hrs. of pretreatment. Figs. 10B I and 10C I indicate that the inhibition has reached final values after about 3 hours of pretreatment with ozone. Respiration behaves somewhat differently, resulting in very high Q_{10}-values for the lower region (see above). It should be emphasized again that the values have all been measured at 25°C, in dark, and that they reflect the effect of pretreatments in the light at different temperatures and different durations. The accuracy of the high Q_{10}-values in the lower region may be small owing to the low inhibition values obtained at 15°C, much smaller still than for photosynthesis. It may be observed that it is a fairly general experience that photosynthesis is more sensitive to harmful effects than respiration.

Some more general remarks may be added to the preceding discussion. It should be pointed out that understanding of temperature relationships ultimately can only be promoted by physiological analysis, viz. by investigating in how far the Q_{10} or Q_5 for a certain temperature region can be experimentally modified by changing the conditions of the environment or of the object (age, pretreatment, etc.). Very few cases have been analysed in this way so far (c.f. WASSINK, 1972 [178]). In two cases, at least, this could be satisfactory explained by the assumption that, in principle, the temperature curve is exponential, but that, with increasing temperature, in increasing parts of the object processes with low Q_{10}-values, e.g. diffusion, will act as rate limiting processes. This causes an increasing depression of an exponential slope, thus often suggesting linearity of the overall rate measured, with temperature (WASSINK, 1972 [178]).

Taking into consideration what has been said above, it may, nevertheless be tempting to add a few words on temperature effects of photosynthesis as obtained in direct measurements. BALDRY et al. (1966 [179]) have studied temperature relationships in photosynthesis of pea chloroplasts. They observed that Q_{10} runs from very high values at low temperatures to low values at high temperatures, and collected similar data from authors working with intact cells (l.c. Table I). In a subsequent paper, SELWYN (1966 [180]) showed that the curves of BALDRY et al. can be described, starting from a mechanism including feedback features. The author correctly remarks that 'a kinetic correlation of this sort does not prove that this is the mechanism which is actually involved'. It will only be possible to give preference to certain types of explanation above others when a further physiological analysis of the kind suggested above provides definite indications into one direction or another. As far as the extremely high Q_{10}-values at low temperatures are concerned (BALDRY, l.c., Table I), we may remark that they are not confined to photosynthesis. They are, e.g., also observed for water permeability and protoplasmic streaming (WASSINK, 1972 [178]), and it is suggestive to relate them to a sort of 'stiffness' of the membrane structure, viz. to its capacity to form 'statistic pores' at a sufficient rate. It may be added that the Q_{10}-values in BALDRY's Table I, plotted against temperature altogether show a remarkably consistent course.

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3.1.2. *Inhibition of photosynthesis as affected by light intensity during the exposure period*

Table 3 and fig. 12 indicate that the inhibition of photosynthesis increases with light intensity during the exposure to ozone. The inhibition of the rate of photosynthesis as measured subsequently at 25 °C and under light saturation was 32.9% after a 2 hr. dark exposure which amounts to about 50% of that by a 2 hr. full light exposure (66.1%). It seems that the effect was nearly light saturated at low light intensity during exposure to ozone, and it may be stated that the overall effect in this case consists for about 50% of dark oxidation effects and for 50% of photo-oxidation effects.

TABLE 3. Photosynthesis as affected by different light intensities during 2 hr. exposure to ozone at 35 °C. O_2-evolution for the blank and the ozone treated samples measured at 25 °C and at saturating light intensity; Data presented in $\mu l\ O_2/25\ \mu l$ algae per hour; cf. also fig. 12.

Light intensity	Blank	O_3	Percent activity	Percent inhibition
L_1	895.3 (4)	303.3 (4)	33.9	66.1
L_2	865.4 (4)	353.1 (4)	40.8	59.2
L_3	914.1 (4)	451.7 (4)	49.4	50.6
D	778.1 (4)	522.1 (4)	67.1	32.9

Numbers in parentheses: number of experiments.
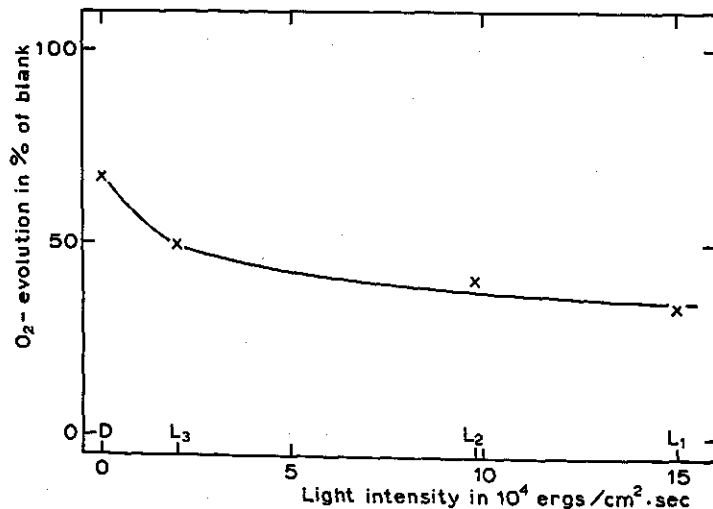$L_1 = 15.0 \times 10^4, L_2 = 9.8 \times 10^4, L_3 = 2.5 \times 10^4$ ergs/cm². sec, D = dark.

FIG. 12. Inhibition of photosynthesis, measured at 25 °C and at saturating light intensity, after a 2 hr. ozone exposure at different light intensities at 35 °C.
D = dark, $L_3 = 2.5 \times 10^4, L_2 = 9.8 \times 10^4, L_1 = 15.0 \times 10^4$ ergs/cm². sec.

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3.1.3. *Effect of ozone on the photosynthesis-light intensity curve, as measured after exposure to ozone*

Table 4, 5 and 6 and figs. 13, 14 and 15 demonstrate that the inhibition effect of ozone on photosynthesis is more pronounced in the light saturated part of the photosynthesis-light intensity curve than in the light limited part. Algae exposed during 2 hr. at 15°C, 25°C and 35°C respectively, to ozone in the light

TABLE 4. Photosynthesis, measured at different light intensities, as affected by 2 hr. exposure to ozone at 15°C. O_2-evolution for the blank and the ozone treated samples measured at 25°C and presented in $\mu l\ O_2/25\ \mu l$ algae per hour; cf. also fig.13.

Light intensity	Blank	O_3	Percent activity	Percent inhibition
L_1	784.1 (3)	628.9 (3)	80.2	19.8
L_2	767.0 (3)	633.1 (3)	82.5	17.5
L_3	576.4 (3)	494.6 (3)	85.8	14.2
L_4	269.6 (3)	273.5 (3)	101.4	−1.4

Numbers in parentheses: number of experiments.
$L_1: 9.3 \times 10^4$, $L_2: 5.9 \times 10^4$, $L_3: 2.0 \times 10^4$, $L_4: 0.7 \times 10^4$ ergs/cm². sec.

TABLE 5. Photosynthesis, measured at different light intensities, as affected by 2 hr. exposure to ozone at 25°C. O_2-evolution for the blank and the ozone treated samples measured at 25°C and presented in $\mu l\ O_2/25\ \mu l$ algae per hour; cf. also fig. 14.

Light intensity	Blank	O_3	Percent activity	Percent inhibition
L_1	810.5 (8)	533.5 (8)	65.8	34.2
L_2	820.5 (8)	552.9 (8)	67.4	32.6
L_3	512.5 (8)	408.6 (8)	79.7	20.3
L_4	286.2 (8)	234.9 (8)	82.1	17.9

Numbers in parentheses: number of experiments.
$L_1: 9.3 \times 10^4$, $L_2: 5.9 \times 10^4$, $L_3: 2.0 \times 10^4$, $L_4: 0.7 \times 10^4$ ergs/cm². sec.

TABLE 6. Photosynthesis, measured at different light intensities as affected by 2 hr. exposure to ozone at 35°C. O_2-evolution for the blank and the ozone treated samples measured at 25°C and presented in $\mu l\ O_2/25$ ul algae per hour; cf. also fig. 15.

Light intensity	Blank	O_3	Percent activity	Percent inhibition
L_1	750.9 (3)	351.5 (3)	46.8	53.2
L_2	748.7 (3)	362.5 (3)	48.4	51.6
L_3	465.1 (3)	268.2 (3)	57.7	42.3
L_4	248.1 (3)	185.0 (3)	74.6	25.4

Numbers in parentheses: number of experiments.
$L_1: 9.3 \times 10^4$, $L_2: 5.9 \times 10^4$, $L_3: 2.0 \times 10^4$, $L_4: 0.7 \times 10^4$ ergs/cm². sec.
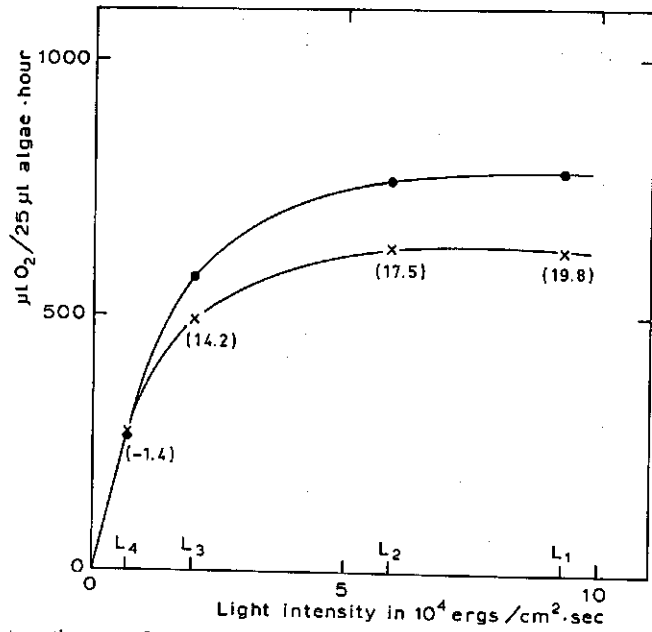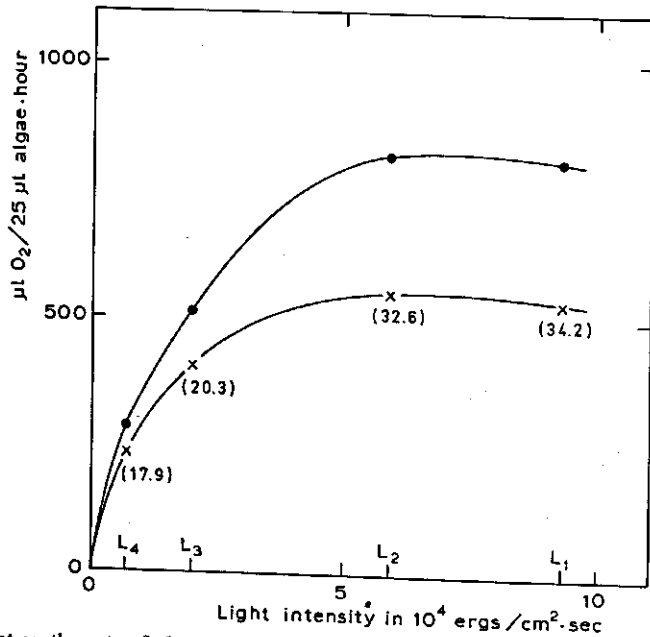
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FIG. 13. Effect on the rate of photosynthesis, measured at 25 °C and at various light intensities, after a 2 hr. ozone treatment at 15 °C in the light; data corrected for dark O_2-uptake. $\times - \times$: Ozone treatment, $\bullet - \bullet$: Blank (air treatment). Numbers in parentheses: % inhibition of photosynthesis.

FIG. 14. Effect on the rate of photosynthesis, measured at 25 °C and at various light intensities, after a 2 hr. ozone treatment at 25 °C in the light; data corrected for dark O_2-uptake. $\times - \times$: Ozone treatment, $\bullet - \bullet$: Blank (air treatment). Numbers in parentheses: % inhibition of photosynthesis.
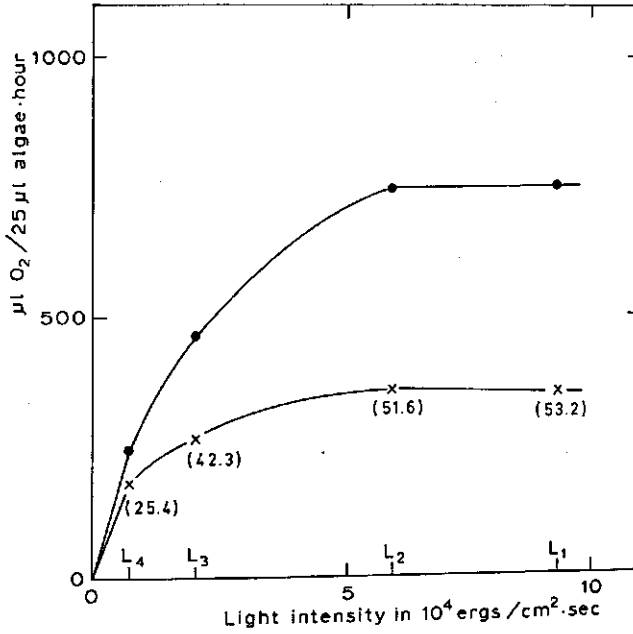
Fig. 15. Effect on the rate of photosynthesis, measured at 25 °C and at various light intensities, after a 2 hr. ozone treatment at 35 °C in the light; data corrected for dark O_2-uptake. × – × Ozone treatment, ● – ●: Blank (air treatment). Numbers in parentheses: % inhibition of photosynthesis.
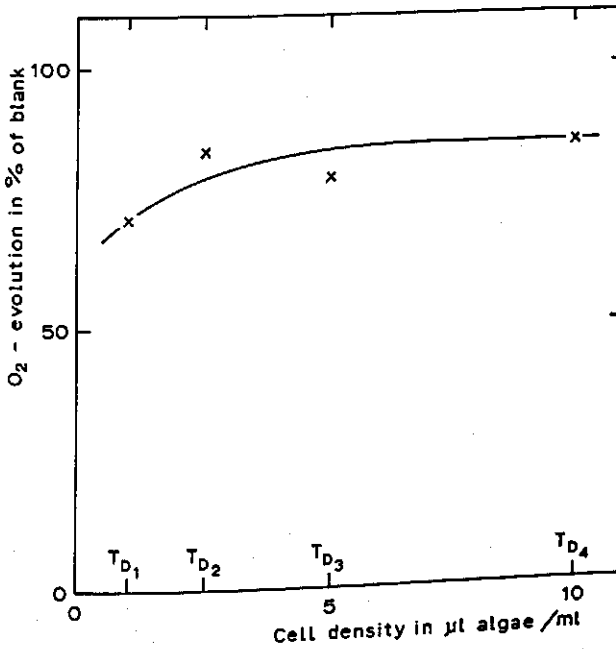

Fig. 16. Inhibition of photosynthesis, measured at 25 °C and at saturating light intensity, after a 4 hr. exposure at 35 °C in the dark at various suspension densities.
T_{D1}: 1.0 µl/ml; T_{D2}: 2.5 µl/ml; T_{D3}: 5.0 µl/ml; T_{D4}: 10.0 µl/ml.

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TABLE 7. Photosynthesis as affected by different suspension densities during 4 hr. exposure to ozone in the dark at 35 °C. O_2-evolution for the blank and the ozone treated samples measured at 25 °C and at saturating light intensity; presented in µl $O_2/25$ µl algae per hour; cf. also fig. 16.

Density	Blank	O_3	Percent activity	Percent inhibition
T_{D_1}	582.2 (3)	415.2 (3)	71.3	28.7
T_{D_2}	614.2 (4)	516.9 (4)	84.2	15.8
T_{D_3}	594.6 (4)	468.9 (5)	78.9	21.1
T_{D_4}	566.7 (4)	480.3 (4)	84.8	15.2

Numbers in parentheses: number of experiments.
Exposed an 80 ml (instead of 50 ml) suspension with densities:
T_{D_1}: 1.0, T_{D_2}: 2.5, T_{D_3}: 5.0, T_{D_4}: 10.0 µl/ml.

TABLE 8. Photosynthesis as affected by a pure air post-treatment after exposure to ozone in the light at 35 °C. O_2-evolution for the blank and the ozone treated samples measured at 25 °C and at saturating light intensity; presented in µl $O_2/25$ µl algae per hour; cf. also fig. 17.

Treat-ment	Without post-treatment				With post-treatment			
	Blank	O_3	Per-cent activity	Per-cent inhibi-tion	Blank	O_3	Per-cent activity	Per-cent inhibi-tion
A *	798.6 (4)	705.9 (4)	88.4	11.6	778.5 (4)	702.5 (4)	90.2	9.8
B **	845.5 (3)	504.6 (3)	59.7	40.3	897.4 (4)	427.8 (4)	47.7	52.3
C ***	726.3 (3)	336.6 (3)	46.3	53.7	721.9 (3)	385.6 (3)	53.4	46.6

Numbers in parentheses: number of experiments.

* Treatment A	without post-treatment	blank: 1 hr. air O_3: 1 hr. O_3
	with post-treatment	blank: 1 hr. air + ½ hr. air O_3: 1 hr. O_3 + ½ hr. air
** Treatment B	without post-treatment	blank: 2 hr. air O_3: 2 hr. O_3
	with post-treatment	blank: 2 hr. air + ½ hr. air O_3: 2 hr. O_3 + ½ hr. air
*** Treatment C	without post-treatment	blank: 3 hr. air O_3: 3 hr. O_3
	with post-treatment	blank: 3 hr. air + 1 hr. air O_3: 3 hr. O_3 + 1 hr. air

were inhibited in the rate of photosynthesis; the inhibition increases with the temperature during the previous exposure to ozone. In each temperature series the rate of subsequently measured photosynthesis at different light intensities at 25 °C appears to be more inhibited with increasing light intensity during the measurement, until light saturation was reached. The overall effect of ozone on photosynthesis seems to be an inhibition in both the light limited and light saturated part of the photosynthesis-light intensity curves.

3.1.4. *Inhibition of photosynthesis as affected by the suspension density of the algae during exposure to ozone in the dark*

Table 7 and fig. 16 indicate that there hardly is a regular relation between suspension density and the effect of ozone on the rate of photosynthesis subsequently measured (at light saturation). The differences are small, only at the lowest density ($T_D = 1$ $\mu l/ml$) the inhibition percentage appears somewhat higher.
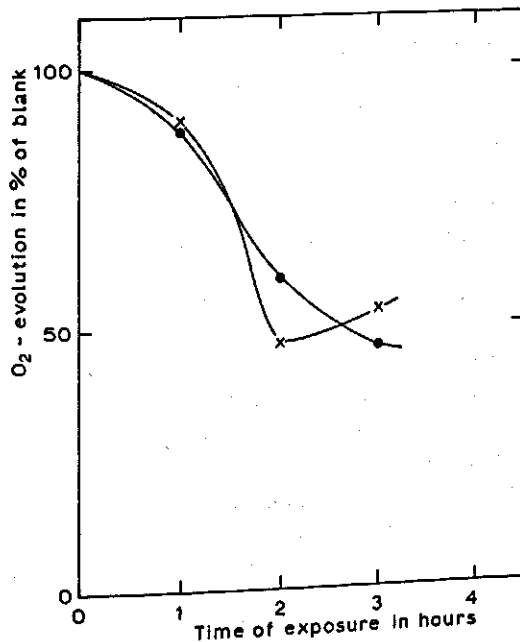

FIG. 17. Effect of a pure air treatment after an ozone exposure, both in the light at 35 °C, on photosynthesis, as measured at 25 °C and at saturating light intensity.
At 1 hr. exposure: ● = 1 hr. O_3 and × = 1 hr. $O_3 + \frac{1}{2}$ hr. air.
At 2 hr. exposure: ● = 2 hr. O_3 and × = 2 hr. $O_3 + \frac{1}{2}$ hr. air.
At 3 hr. exposure: ● = 3 hr. O_3 and × = 3 hr. $O_3 + 1$ hr. air.

3.1.5. *Effect of a short refreshing period with pure air, after an ozone treatment, on the rate of photosynthesis*

Table 8 and fig. 17 show that there is no clear regular refreshing effect of a pure air post-treatment on the rate of subsequently measured photosynthesis at light saturation. If the algae are treated for 1 hr. with ozone, a ½ hr. post-treatment causes a very slight recovery of photosynthesis. On the other hand, a ½ hr. post-treatment after a 2 hr. exposure with ozone causes even a stronger inhibition of photosynthesis. Finally in the case of a 3 hr. ozone exposure followed by a 1 hr. post-treatment there is some recovery of the photosynthesis.

3.1.6. *Conclusions*

a. Photosynthesis and dark respiration are both inhibited by a preceding ozone exposure of the algal suspension (figs. 10A and 11A).

b. The inhibition of photosynthesis and dark respiration increases with the exposure time to ozone (figs. 10C I, 10C II and 11C I, 11C II).

c. The inhibition of photosynthesis and dark respiration increases with the temperature of the algal suspension during ozone exposure (figs. 10B I, 10B II and 11B I, 11B II).

d. The Q_{10}'s for remaining activity of photosynthesis and respiration are below unity, decreasing with exposure time, and ranging from $\sim$ 1.0–0.7 for Q 25/15 and from $\sim$ 1.0–0.6 for Q 35/15. The Q_{10}'s for inhibition of photosynthesis and respiration show rather high values, especially those of Q 25/15 for respiration, which so up to 8.0. These relations have been discussed at some length in 3.1.1.

e. The inhibition of photosynthesis increases with the light intensity applied during the ozone exposure (fig. 12).

f. The inhibition of photosynthesis, induced by an ozone exposure, is more pronounced in the light saturated part of the photosynthesis-light intensity curve than in the light limited part, in the entire temperature range (cf. the percentages along the curves in figs. 13, 14, 15).

g. The inhibition of photosynthesis, induced by an ozone exposure in the dark, is hardly affected by the suspension density of the algae during the exposure period; only with very thinly populated suspensions ($T_D = 1.0$ µl/ml) a somewhat higher inhibition is obtained (fig. 16).

h. The inhibition of photosynthesis, induced by an ozone exposure, was not significantly affected by a post-treatment with pure air (fig. 17).

TABLE 9. Loss of chlorophyll and change of the ratio a/b in relation to inhibition of photosynthesis, induced by an ozone exposure time series in the dark and in the light at 35°C (cf. also figs. 18 and 19).

Exposure in the light (L) or in the dark (D)	Rate of photosynthesis				Chlorophyll a + b			Chlorophyll a			Chlorophyll b			a/b ratio		
	Blank	O_3	Per-cent activity	Per-cent inhibition	Blank	O_3	Per-cent decrease	Blank	O_3	Per-cent decrease	Blank	O_3	Per-cent decrease	Blank	O_3	Difference
1 hr. L	827.9(3)	625.9(3)	75.6	24.4	10.037(3)	9.407(3)	6.3	7.421(3)	6.805(3)	8.3	2.616(3)	2.601(3)	0.6	2.89(3)	2.84(3)	0.05
1 hr. D	622.3(3)	484.5(3)	77.9	22.1	10.408(3)	9.824(3)	5.6	7.889(3)	7.122(3)	9.7	2.519(3)	2.701(3)	-7.2	3.23(3)	2.68(3)	0.55
2 hr. L	829.3(3)	330.1(3)	30.8	69.2	10.930(3)	8.561(3)	21.7	8.734(3)	6.486(3)	25.7	2.196(3)	2.075(3)	5.5	4.01(3)	3.19(3)	0.82
2 hr. D	715.2(3)	512.9(3)	71.7	28.3	10.924(3)	9.628(3)	11.9	8.747(3)	7.575(3)	13.4	2.177(3)	2.054(3)	5.6	4.08(3)	3.71(3)	0.37
3 hr. L	826.6(3)	424.8(3)	51.4	48.6	10.812(3)	8.946(3)	17.3	8.585(3)	6.849(3)	21.2	2.227(3)	2.097(3)	5.8	3.84(3)	3.25(3)	0.59
3 hr. D	611.9(4)	437.5(4)	71.5	28.5	10.566(4)	10.284(4)	2.7	8.029(4)	7.784(4)	3.1	2.537(4)	2.502(4)	1.4	3.20(4)	3.15(4)	0.05
4 hr. L	833.6(4)	357.5(3)	42.9	57.1	9.349(3)	6.563(3)	29.8	6.635(3)	4.383(3)	33.9	2.713(3)	2.179(3)	19.7	2.49(3)	2.07(3)	0.42
4 hr. D	700.8(4)	415.5(4)	59.3	40.7	10.298(4)	9.291(4)	9.8	7.875(4)	7.112(4)	9.7	2.423(4)	2.178(4)	10.1	3.33(4)	3.38(4)	-0.05

O_2-evolution for the blank and the ozone trials measured at 25°C and at saturating light intensity; presented in μl/25 μl algae per hour. Chlorophyll contents presented in μg chlorophyll/μl algae per ml ethanol.
Numbers in parentheses: number of experiments.

3.2. Effects of ozone on chlorophyll content and composition of the algae

3.2.1. *Decrease of chlorophyll content and change of the chlorophyll a/b ratio in relation to inhibition of photosynthesis, induced by an ozone exposure series in the light and in the dark*

Table 9 and fig. 18 show that a treatment in the light results in a substantial loss of chlorophyll, mainly of chlorophyll a, accompanied by an inhibition of photosynthesis; the inhibition of photosynthesis and the loss of chlorophyll are, on the whole, higher when the exposure time to ozone increases.

For the dark exposure series (fig. 19) there seems to be no significant correlation between the inhibition of photosynthesis and the loss of chlorophyll; the inhibition of photosynthesis increases with time, while the loss of chlorophyll does not increase regularly with time and rarely exceeds 10%.

The chlorophyll a/b ratio decreases owing to ozone exposures in the light and in the dark, because the chlorophyll a content decreases much more strongly than the chlorophyll b content (cf. Table 9).

3.2.2. *Conclusions*

a. The decrease of the chlorophyll content, induced by ozone exposure, is more pronounced after an exposure period in the light than after one in the dark (cf. figs. 18 and 19).

b. The decrease of the chlorophyll content, following ozone exposure in the light, increases with the exposure time to ozone (fig. 18).
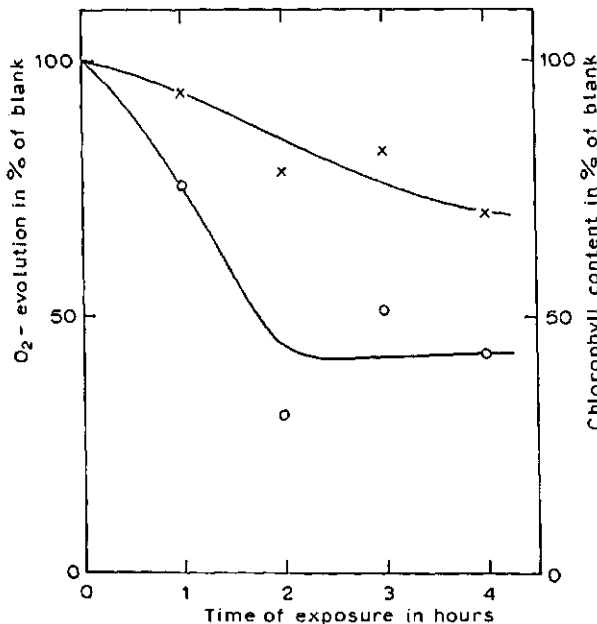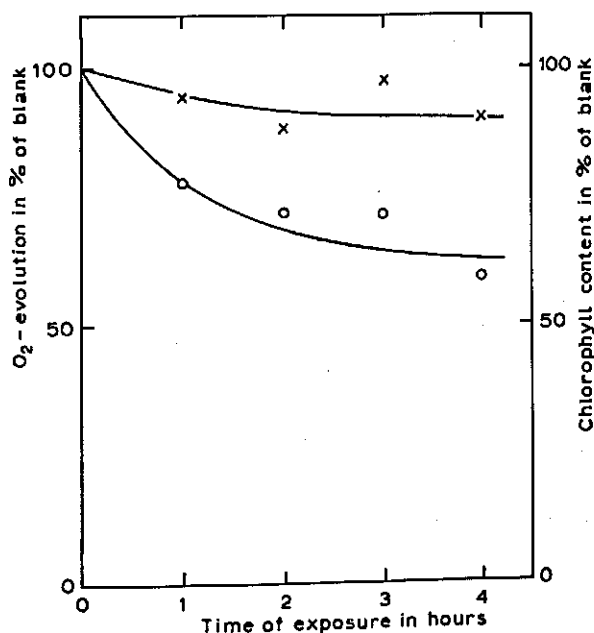

Fig. 18. Decrease of total chlorophyll content in relation to inhibition of photosynthesis, as induced by an ozone exposure time series at 35 °C in the light; O – O = inhibition of photosynthesis, ×–× = decrease of chlorophyll content.

FIG. 19. Decrease of total chlorophyll content in relation to inhibition of photosynthesis, as induced by an ozone exposure time series at 35°C in the dark; O – O = inhibition of photosynthesis, X – X = decrease of chlorophyll content.

c. Preferentially the chlorophyll a content is diminished and the ratio chlorophyll a/b decreased by ozone exposure (cf. Table 9).

3.3. PRELIMINARY STUDIES ON THE EFFECT OF OZONE ON LIPID CONTENT AND FATTY ACID COMPOSITION

3.3.1. Decrease of lipid content and alteration of fatty acid composition as induced by a 4 hr. ozone exposure in the light at 35°C

An 80 ml algal suspension ($T_D = 2.5$ µl/ml) treated for 4 hr. with ozone in the light at 35°C, showing a 75.6% inhibition of photosynthesis, has a 66.7% decrease of lipid content (the blank sample contains 3.3 mg lipid, while the

TABLE 10. Fatty acid composition change by an ozone exposure period.

Fatty acid	Composition (%)		Difference (%)
	Blank	O₃	
Saturated			
16/0 (palmitic)	11.2	15.2	+ 4.0
18/0 (stearic)	11.7	12.7	+ 1.0
Unsaturated			
18/1 (oleic)	24.1	23.0	– 1.1
18/2 (linoleic)	20.6	21.6	+ 1.0
18/3 (linolenic)	32.4	27.5	– 4.9

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ozone treated sample contains 1.1 mg lipid).

Table 10 show the altered pattern of fatty acid composition in these algae. There seems to be a substantial loss of the most unsaturated fatty acid, linolenic acid.

3.3.2. *Lipid peroxidation test*

Attempts have been made to detect malondialdehyde production, as a measure of lipid peroxidation in ozone-treated algae with the aid of the colorimetric method, using thiobarbituric acid. Algal samples exposed for 4 hr. at 35°C in the light and showing a 58.7% inhibition of photosynthesis and a 38.2% decrease of the chlorophyll content, did not show malondialdehyde production.

3.3.3. *Conclusions*

a. There is a substantial lipid loss in ozone-exposed algae.
b. Malondialdehyde production, as a measure of lipid peroxidation, could not be detected in ozone-exposed algae.
c. Indications are that the apparent lipid loss, induced by an ozone exposure, is mainly due to oxidation of the unsaturated fatty acid, linolenic acid (Table 10).

3.4. PRELIMINARY STUDIES ON THE EFFECT OF OZONE ON PROTEIN CONTENT AND AMINO ACID COMPOSITION

3.4.1. *Decrease of protein content and alteration of amino acid composition, as induced by a 4 hr. ozone-exposure in the light at 35°C*

A 165 ml algal suspension sample ($T_D = 2.5 \, \mu l/ml$) treated for 4 hr. with ozone in the light at 35°C, showing a 62.4% inhibition of photosynthesis and a 27.7% decrease of the chlorophyll content, had a decrease of protein content of only 6.1% (the blank sample contained 61.9 mg protein, while the sample treated with ozone for 4 hrs. contained 58.1 mg protein).

Figs. 20 and 21 and table 11 show the altered pattern of amino acid composition of these algae. There appears to be a relatively important gain in phenylalanine (about 15% of the original content), and smaller losses of several other components, at least in absolute figures. With respect to the initial content, the loss in serine in percents is relatively important, viz. from 4.7 to 3.9%, i.e. about 17% of the original content. Moreover, an unidentified alkaline component, present in relatively moderate amount, had totally disappeared after the ozone treatment.

3.4.2. *Conclusions*

a. There seems to be only a small loss of protein in ozone-exposed algae.
b. Indications are that the protein loss, induced by an ozone exposure, is mainly due to oxidation of the acid component serine, and to a total loss of an unidentified alkaline component (cf. figs. 20 and 21).
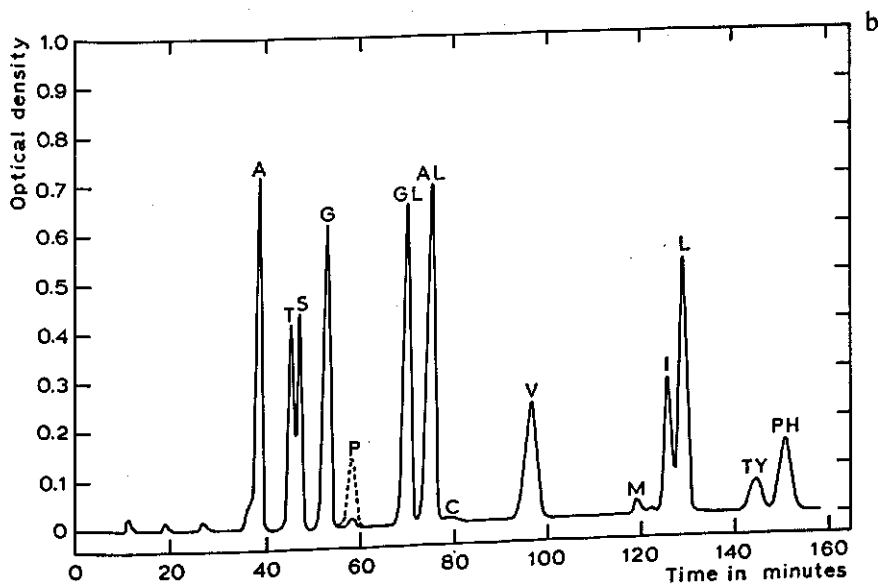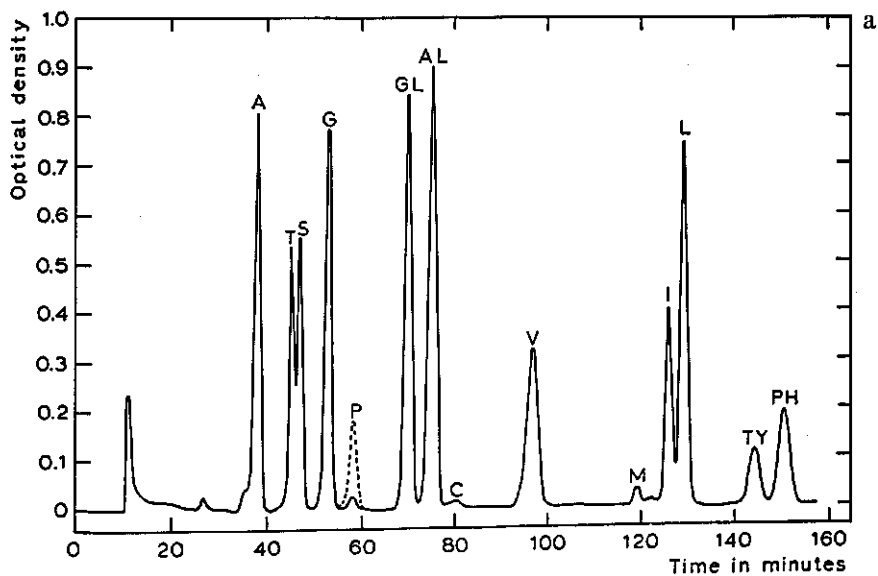
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FIG. 20. Chromatogram of the acid and neutral amino acids present in *Scenedesmus obtusius-culus*. a, air exposed algae; b, ozone exposed algae.
A, aspartic acid; T, threonine; S, serine; G, glutamic acid; P, proline; GL, glycine; AL, alanine; C, cystine; V, valine; M, methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylala-nine.
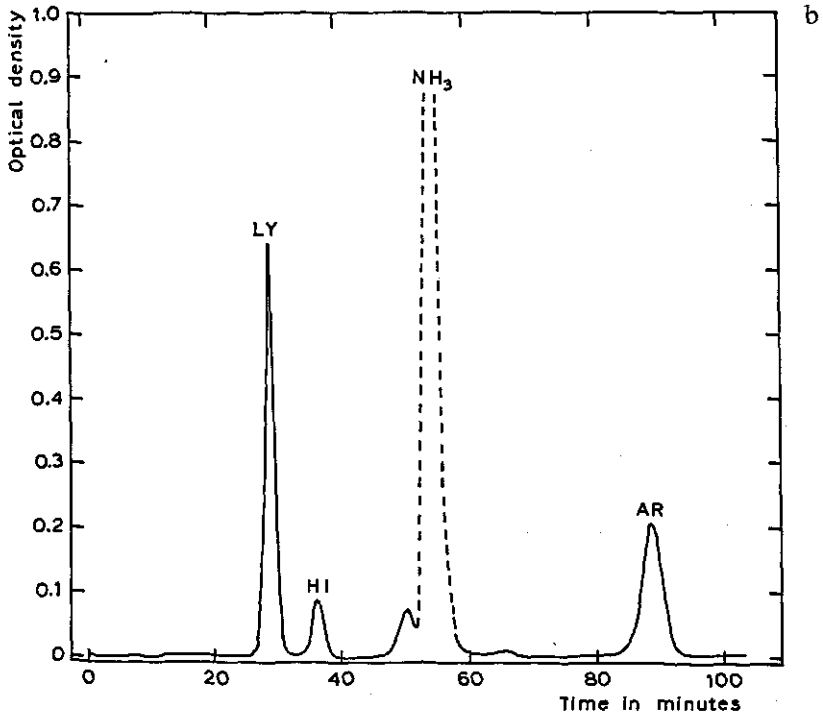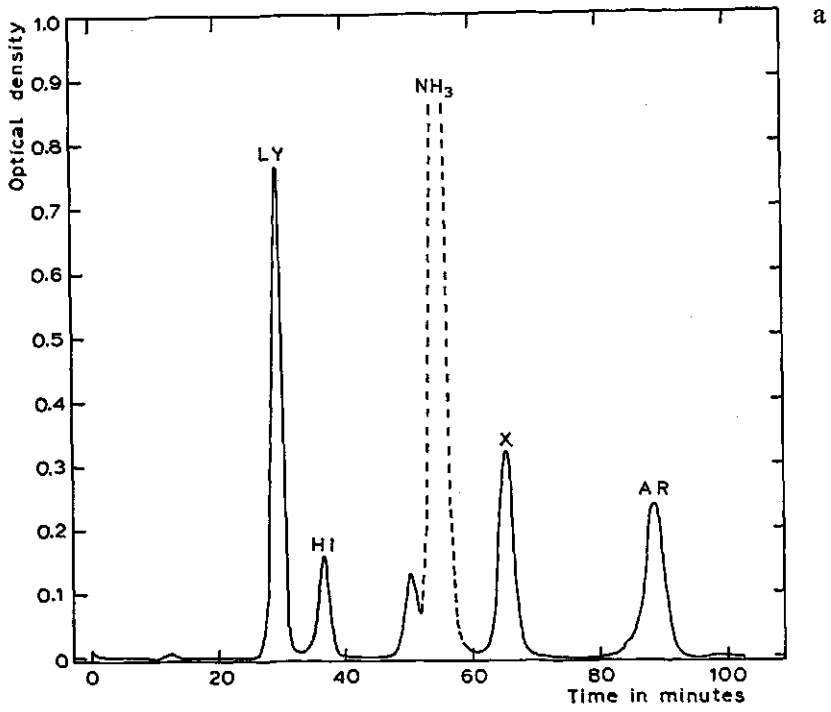
FIG. 21. Chromatogram of the alkaline amino acids present in *Scenedesmus obtusiusculus*.
a, air exposed algae; b, ozone exposed algae. LY, lysine; HI, histidine; AR, arginine; X, unknown component.

TABLE 11. Amino acid change by an ozone exposure period (cf. also figs. 20 and 21).

Amino acid	Blank (Mole %)	O₃ (Mole %)	Difference
Neutral + acid components:			
Phenylalanine	26.3	30.3	+4.0
Tyrosine	1.5	1.4	−0.1
Leucine	6.5	6.6	+0.1
Iso-leucine	3.0	3.0	0.0
Methionine	0.3	0.3	0.0
Valine	4.9	4.6	−0.3
Cystine	0.5	0.3	−0.2
Alanine	9.7	9.1	−0.6
Glycine	8.3	8.7	+0.4
Proline	4.3	4.4	+0.1
Glutamic acid	7.5	7.3	−0.2
Serine	4.7	3.9	−0.8
Threonine	5.1	4.6	−0.5
Aspartic acid	7.8	7.4	−0.4
Alkaline components:			
Arginine	2.7	3.1	+0.4
Histidine	1.0	0.7	−0.3
Lysine	3.3	4.3	+1.0
Unknown component	2.6	− −	−2.6

3.5. PRELIMINARY STUDIES ON THE EFFECT OF OZONE ON THE ULTRASTRUCTURE OF THE ALGAE

3.5.1. *Damage of the ultrastructure, as induced by a 4 hr. ozone exposure in the light at 35°C*

Plate 1 (blank) and Plate 2 (O₃-exposed) show the changes in the ultrastructure of the algae by an ozone exposure. The damage involves a disruption of the plasmalemma and the chloroplast envelope, while there is a widening effect on the chloroplast membrane system. Moreover, there often is the formation of electron-thin ring systems around starch granules.

3.5.2. *Conclusions*

The ultrastructural changes of the algae, induced by an ozone exposure, are:
a. a disruption of the plasmalemma and the chloroplast envelope.
b. a loosening of the chloroplast membrane system.
c. the formation of electron-thin ring systems around starch granules.

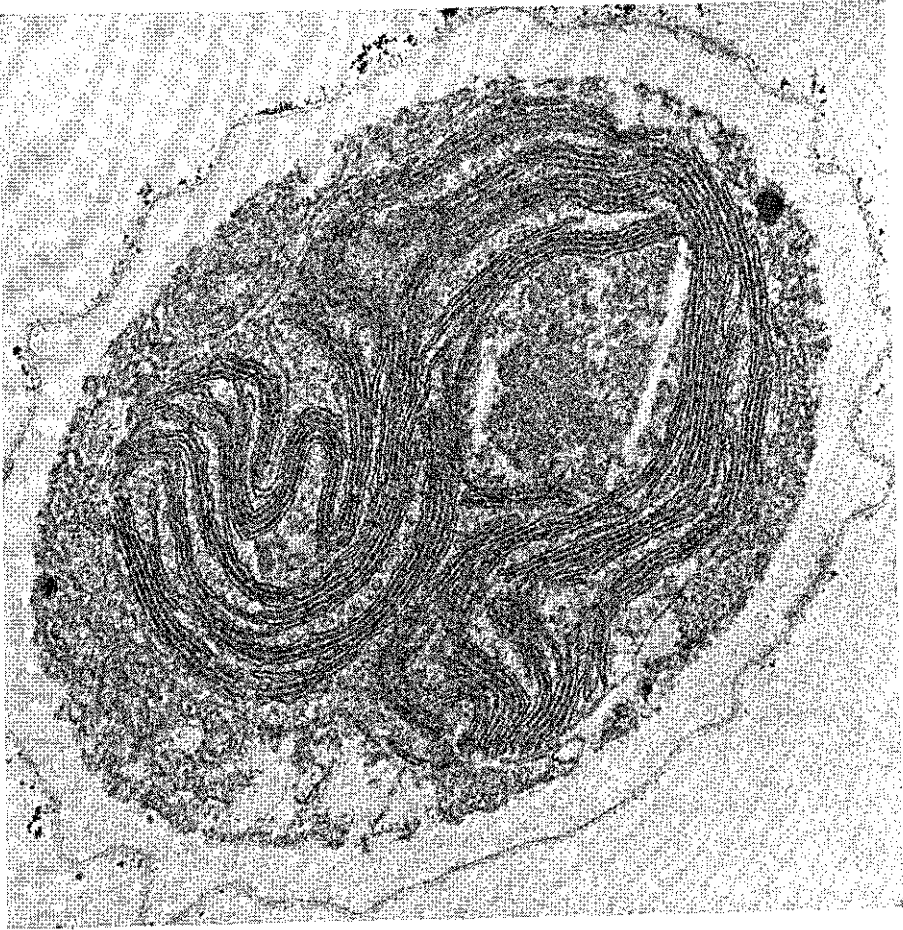Plate 1. Ultrastructure of an air exposed cell of *Scenedesmus obtusiusculus*; × 66,600.

Plate 2. Ultrastructure of an ozone exposed cell of *Scenedesmus obtusiusculus*; × 66,600.

4. GENERAL DISCUSSION

As may be concluded from research data about the effect of all kinds of air polluting compounds on living plant material, the photosynthesis and respiration processes of the cells are very sensitive to pollution of the air (section 1.1.4.). This is no wonder, because the photosynthesis and respiration apparatus, located in the chloroplasts and mitochrondria, are concentrated in the leaves, which are the most active parts of the plants in gas exchange with the environment.

It has been one of the aims of this study to obtain a better understanding of the mode of action of ozone on photosynthesis and respiration.

Most data on the effects of ozone on photosynthesis and respiration have been obtained in studies with higher plants or isolated organelles. The studies of DE KONING and JEGIER (1968 [37, 82], 1969 [119]) and our experiments are concerned with gas exchange of unicellular organisms, viz., *Euglena gracilis* and *Scenedesmus obtusiusculus*, respectively.

In our experiments photosynthesis and dark respiration are inhibited after ozone exposure. Contrary to this, DE KONING and JEGIER (1968 [37]) stated stimulation of respiration (10%), accompanying a decrease of photosynthesis (15%) in *Euglena gracilis* cells after a 1 hr. exposure to 1 ppm O_3. Maybe, a difference in starch content and starch hydrolysis between *Euglena* and *Scenedesmus* cells may explain the different behaviour.

Our results suggest that the primary attack of ozone in photosynthesis and respiration is on membrane structure, and possibly on dark enzyme activity. This may be concluded from the observations that:

a. the most pronounced effect is on the light saturated part of the photosynthesis-light intensity curve,

b. the inhibitory effect on photosynthesis and respiration is stronger at a higher temperature during exposure,

c. the inhibitory effect on photosynthesis is stronger at a higher light intensity during exposure,

d. there is an ultrastructural loosening effect on the chloroplast membrane system and a disruption of the plasmalemma and the chloroplast envelope,

e. a decrease of lipid and protein content is detected,

f. in a preliminary experiment (unpublished) it was found that light cells are more sensitive than dark cells from continuous algae cultures. KOK (1952 [181]) already found that cells at the end of the light period contained relatively high amounts of carbohydrates and lipids.

The higher inhibition of photosynthesis after an ozone exposure at a higher light intensity may, possibly in part, be explained by photo-oxidation of the chlorophyll; the chlorophyll decrease seems to be higher after exposure to ozone in light than after exposure in darkness.

In our experiments a significant loss of chlorophyll is induced by ozone, which mainly concerns chlorophyll a; contrary to this, in *Euglena gracilis* a preferen-

tial loss of chlorophyll b was noted (DE KONING and JEGIER, 1968 [82]). We may consider this chlorophyll bleaching not of primary importance, although a greather loss of chlorophyll may play a part in the impairment of photosynthesis.

It may be supposed that ozone causes leaking out of certain organic material by attacking the cell surface or plasmalemma, because after a certain exposure period, foaming of the algal suspension occurs. A similar leaking effect was already detected in *Escherichia coli* cells (MCNAIR SCOTT and LESHER, 1963 [104]). Cell permeability was noted to be influenced by ozone in lemon (DUGGER *et al.*, 1966 [65]), bean (TOMLINSON and RICH, 1967 [105]), *Pinus ponderosa* (EVANS and MILLER, 1972 [106]), tobacco, and potato (MCFARLENE, 1966 [107]).

The oxidation of unsaturated fatty acids, which was reported for human red blood cells exposed to ozone (GOLDSTEIN *et al.*, 1969 [109]), and spinach chloroplasts (MUDD *et al.*, 1971 [111]) with accompanying malondialdehyde production, is also detected in *Scenedesmus* cells, however, without production of appreciable amounts of malondialdehyde.

As primary targets for ozone toxicity in the algal cells are to be considered: the unsaturated fatty acids in the membranes, the SH-groups of enzymes (MUDD *et al.*, 1969 [115]; TOMLINSON and RICH, 1968 [116]) and the reduced cofactors NADH and NADPH (MUDD, 1965 [118]; DE KONING and JEGIER, 1969 [119]).

The ultrastructural changes, consisting of a breakdown of the plasmalemma and other cellular membranes together with a loosening effect on the chloroplast membrane system, as observed in the ozone-treated algal cells, were also noted in the palisade cells of bean leaves after ozone exposure (THOMSON *et al.*, 1966 [128]). They observed a primary granulation in the stroma, which might be caused by protein aggregation. A similar phenomenon was not observed in the algal cells.

It may be of appreciable interest to study in future experiments the sensitivity of the various membrane components and enzymes in the algal cells to ozone and other air pollutants, in relation to their overall effects on photosynthesis and respiration.

SUMMARY

In the present study the mode of action of the air pollutant ozone was investigated by studying its effects on photosynthesis, respiration and some biochemical and structural properties of the unicellular alga, *Scenedesmus obtusiusculus* CHOD.

In chapter 1, an effort was made to review the extensive literature on the effects of the most important air pollutant gases SO_2, HF, NO_2, PAN and O_3 on the processes of photosynthesis and respiration, while possible modes of action of each of these gases are discussed.

In chapter 2, section 2.1., the procedure for ozone exposure is extensively described; a lot of time was required to obtain a reliable experimental design.

In chapter 3, section 3.1., it is shown that photosynthesis and dark respiration are inhibited by previous exposure of the cell suspensions to ozone; the inhibition is increased with the duration of exposure and with temperature during exposure. The effect of an ozone treatment is increased by light during the exposure, and, consequently, photosynthesis appears more inhibited in as much as the light-intensity during the preceding exposure to ozone was higher. Photosynthesis is most inhibited in the light saturated part of the light intensity curve, which indicates that the inhibition of dark reactions of photosynthesis is more pronounced than that of the primary photoreactions. The degree of inhibition of photosynthesis is practically independent of cell density during a preceding exposure to ozone in the dark. The inhibition of photosynthesis, induced by an ozone exposure in the light, can be somewhat recovered by an after-treatment with pure air; however, the recovery effect was not alway very clear.

In chapter 3, section 3.2., it is shown that the chlorophyll content decreases by exposure to ozone; the decrease is more pronounced after exposure in the light than after exposure in the dark. Preferentially, chlorophyll a is destroyed, which implies that the ratio chlorophyll a/b decreases.

In chapter 3, section 3.3., it appeared in a preliminary study that there is a substantial loss of lipid in ozone-exposed algae, and there are indications that this is mainly due to oxidation of the unsaturated fatty acid, linolenic acid. Malondialdehyde production, often accompanying lipid peroxidation, was not detected in the ozone exposed algae.

In chapter 3, section 3.4., it is shown in a preliminary study that there is a very small loss of protein content in ozone-exposed algae, which may be ascribed mainly to oxidation of the acid component serine, and of an unidentified alkaline compound.

In chapter 3, section 3.5., in a preliminary study, it is described that, in ozone exposed algae, ultrastructural changes are observed, e.g., a disruption of the plasmalemma and the chloroplast envelope, a widening of the chloroplast membrane system, and the formation of electron-thin ring systems around starch granules.

ACKNOWLEDGEMENTS

SAMENVATTING

In dit onderzoek werd het werkingsmechanisme van ozon (O₃) bestudeerd door het effect ervan na te gaan op fotosynthese, ademhaling en enige biochemische en structurele eigenschappen van het ééncellige groenwier, *Scenedesmus obtusiusculus* CHOD.

In hoofdstuk 1 werd een uitgebreid literatuuroverzicht gegeven betreffende het effect van de belangrijkste gasvormige luchtverontreinigende componenten SO₂, HF, NO₂, PAN en O₃ op de processen van fotosynthese en ademhaling, terwijl bovendien de kennis omtrent het werkingsmechanisme van deze stoffen wordt besproken.

In hoofdstuk 2 (sectie 2.1.) wordt de behandelingsprocedure met ozon uitvoerig uiteengezet, omdat er tamelijk veel tijd is besteed aan het ontwikkelen van een betrouwbare proefopzet.

In hoofdstuk 3 (sectie 3.1.) wordt besproken, dat de fotosynthese en de donker-ademhaling geremd worden door ozon; de remming neemt toe met de behandeltijd en is sterker als de temperatuur van de suspensie tijdens de behandeling hoger is. Het effect van een ozonbehandeling is sterker als de behandeling in het licht wordt uitgevoerd en tevens blijkt de fotosynthese sterker geremd te zijn bij een hogere lichtintensiteit gedurende de behandeling. De fotosynthese is sterker geremd in het lichtverzadigde gedeelte van de fotosynthese-lichtintensiteitcurve dan in het lichtgelimiteerde gedeelte, hetgeen erop duidt dat de remming van donkerreacties van de fotosynthese belangrijker is dan de remming van de primaire lichtreacties. De remming van de fotosynthese is vrijwel onafhankelijk van de dichtheid van de celsuspensie gedurende de behandeling. De remming van de fotosynthese door ozon kan voor een gering deel hersteld worden door een nabehandeling met zuivere lucht; het herstel treedt echter niet in alle gevallen op.

Hoofdstuk 3 (sectie 3.2.) laat zien dat het chlorofylgehalte van ozon-behandelde algen lager is; de afname is sterker na een lichtbehandeling dan na een donkerbehandeling. De afbraak van chlorofyl a is sterker dan die van chlorofyl b, waardoor de verhouding chlorofyl a/b afneemt.

Voorlopige waarnemingen hebben nog het volgende aangetoond:
In hoofdstuk 3, sectie 3.3., werd gevonden, dat het lipidegehalte van ozonbehandelde algen aanzienlijk daalt; dit verlies wordt waarschijnlijk hoofdzakelijk veroorzaakt door de oxydatie van het onverzadigde vetzuur linoleenzuur. Malondialdehydeproduktie, een indicatie voor lipideperoxydatie, kon in ozonbehandelde algen niet aangetoond worden.

In hoofdstuk 3, sectie 3.4., werd een geringe afname van het eiwitgehalte in ozon-behandeld materiaal gevonden, hetgeen waarschijnlijk hoofdzakelijk toegeschreven kan worden aan oxydatie van de zure component serine en van een onbekende basische component.

In hoofdstuk 3, sectie 3.5., werd door elektronenmicroscopisch onderzoek

gevonden, dat de volgende ultrastructurele veranderingen optreden in ozon-
behandelde algen: breukvorming in het plasmalemma en in de chloroplasten-
membranen, een verwijding van het membraansysteem van de chloroplast en de
vorming van een elektronen-transparante, ringvormige laag om de zetmeel-
korrels.

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