POLYPHOSPHATE FORMATION IN *CHLORELLA* IN RELATION TO PHOTOSYNTHESIS

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Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te Eindhoven, 21 October 1921, is goedgekeurd door de promotor Dr. E. C. WASSINK, hoogleraar in het Plantenphysiologisch Onderzoek.

De Rector Magnificus der Landbouwhogeschool,
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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR W. F. EIJSVOOGEL,
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CHAPTER I

INTRODUCTION

The underlying question which has led to the series of investigations reported in this paper was thus formulated by VOGLER: "Is it possible to irradiate photosynthetic organisms in the absence of carbon dioxide and to store at least a portion of the radiant energy within the cell in a form which can later be used for CO_2 fixation in the dark?" (70). This question arose from an investigation of the connection between energy-producing and energy-consuming metabolism, the latter being inconceivable without such an energetic link. As far as respiration is concerned, this problem has been elucidated to a great extent by important discoveries about the gradual oxidation through stepwise dehydrogenation, and the coupling of these processes with phosphorylation. Thus immediate degradation of the chemical energy of the substrate is prevented by its transformation into chemical energy of special organic phosphate compounds which then are available for synthetic processes in the cell. Such 'high-energy phosphates' are represented by the symbol ~ph; the energy they contain is often indicated as 'phosphate bond energy'. A survey in which this concept about the rôle of phosphate bond energy in metabolism was discussed and its importance stressed, was given by LIPMANN (39).

Soon afterwards, these considerations were applied to autotrophic organisms. The importance of these organisms for the generation of organic matter needs no comment here. They derive chemical energy from a great variety of sources and utilize it for the formation of organic material. With the chemo-autotrophic organisms it is often possible to relate substrate and oxidation product in a stoichiometric way. But unlike similar reactions *in vitro*, part of the chemical energy of oxidation is saved and found in reduced, organic material. VOGLER and UMBREIT (71) published the results of experiments with sulfur bacteria, *Thiobacillus thiooxidans*, showing separation in time between sulfur oxidation and CO_2 assimilation. Furthermore, they found that the oxidative phase (in the absence of CO_2) was accompanied by the uptake of orthophosphate in the bacteria. When oxidation was stopped by withdrawal of oxygen, and CO_2 admitted, an uptake of CO_2 was observed, accompanied by a release of orthophosphate into the medium. Although the quantities of phosphate involved were small in comparison with the amounts of O_2 and CO_2 respectively that were taken up by the bacteria, the results were considered as an argument in favour of the concept that energy transfer was mediated by energy-rich phosphates and that chemical energy could be stored in such compounds. In the discussion of these results it was suggested that this concept could be extended to photoautotrophic organisms, and the question quoted above was raised (70).

The problem was taken up by EMERSON, STAUFFER and UMBREIT (19), who investigated the relation between phosphorylation and photosynthesis in *Chlorella*, in order to test a hypothesis they thus formulated: "The function of light energy in photosynthesis is the formation of 'energy-rich' phosphate bonds. According to this view, the light energy absorbed by the chlorophyll system is converted, more or less immediately, into 'energy-rich' phosphate bonds (in the sense of LIPMANN) which furnish the energy for the remainder of the photosynthetic process."

If so, storage of energy in phosphate compounds could be expected when

cells were illuminated in the absence of CO_2 (see above), and this was first looked for. Orthophosphate content of *Chlorella* suspensions was determined after various periods of incubation in light and darkness, in the presence and in the absence of CO_2. Some changes were observed, but the effects of 'light' and 'no carbon dioxide' were hardly appreciable. Afterwards, the organic phosphates, soluble in trichloroacetic acid (TCA), were fractionated with barium salts. Although total organic phosphate remained rather constant, the distribution of phosphate over the fractions was significantly different in either light or darkness, presence or absence of CO_2. RABINOWITCH (50), appreciating these results as interesting, rejected that they could be considered as valid arguments for a hypothesis as outlined by EMERSON *et al.* (19). This criticism can be thus summarized: From an energetic point of view it appears that the quantity of chemical energy, available in energy-rich phosphate bonds is small as compared with the quantity of chemical energy required to reduce CO_2 to carbohydrate. If each quantum of light led to the formation of one molecule of energy-rich phosphate, more quanta would be needed per molecule of CO_2 than actually is assumed. If each quantum of light would produce more than one molecule of energy-rich phosphate, the quantum number would remain within reasonable limits, but it is difficult to visualize such a multiple phosphorylation as the effect of a primary photochemical process.

As the hypothesis in its original form was an extension to photo-autotrophic organisms of concepts regarding CO_2 assimilation in chemo-autotrophic ones, it is necessary to mention here that the original experiments with *Thiobacillus thiooxidans* (70, 71) were not wholly confirmed by other investigators. BAALSRUD and BAALSRUD (4) made thorough investigations with three species of *Thiobacillus*, and confirmed that substrate oxidation can occur in the absence of CO_2. Substrate oxidation was accompanied by a slow uptake of orthophosphate but this occurred both in the presence and absence of CO_2. There was no evidence for the building of 'reducing power' during substrate oxidation in the absence of CO_2, nor could enhanced CO_2-fixation be observed when CO_2 was admitted after such substrate oxidation. After completion of substrate oxidation, orthophosphate was released, irrespective of the presence or absence of CO_2. Their conclusion was that, although for reasons of comparative biochemistry "it was eminently reasonable to invoke high energy phosphate compounds as intermediaries" in energy transfer, the available experimental material was insufficient to support the hypothesis set forth by VOGLER and UMBREIT. The connection between oxidation and assimilation in *Thiobacillus* thus must be assumed to be much closer than has been assumed for some time (4).

Other investigations, however, have shown without doubt that phosphate metabolism is important in photosynthesis.

The application of tracer technique in photosynthesis research has led to results which are important in the present issue. Something like separation between illumination and CO_2 assimilation was established when it was found that cells illuminated in the absence of CO_2 were able to fix more CO_2 in darkness afterwards than cells that had been photosynthesizing, or cells that had been kept in dark throughout. There is disagreement, however, whether this dark uptake represents a delayed, real assimilation (with reduction of the fixed CO_2) or only carboxylation of an acceptor, formed in the light. This gives rise to an organic acid with the tracer atom in the COOH group. For a discussion of this question, see *e.g.* (6).

An important rôle of phosphate in photosynthesis was certainly indicated by the finding that among the first products of CO_2 assimilation, phosphate containing compounds are formed (see [1, 6]).

Several investigators have studied the incorporation of phosphate into green cells, often using labeled phosphate. Reviews of this work are available (1, 6), so only a brief discussion will follow here.

GEST and KAMEN (21), working with labeled phosphate, established that the uptake and turnover of phosphate are much greater in the light than in the dark (organisms studied: *Rhodospirillum*, *Chlorella*, *Scenedesmus*). Phosphate entered soon into organic compounds, insoluble in TCA. SIMONIS and GRUBER published two papers on the correlation between phosphate metabolism and photosynthesis in *Helodea densa* (57, 58). In experiments lasting up to 60 minutes, total uptake of phosphate was only slightly enhanced in light as compared with darkness. Tracer P entered only very slowly into TCA-insoluble compounds. In light more of the phosphate taken up was present as organic phosphate, and less as orthophosphate than in the dark control. The incorporation of phosphate into organic compounds was greater in the presence of CO_2 than in its absence. Previous feeding of *Helodea* leaflets with glucose resulted in an important increase in cellular orthophosphate and TCA-soluble organic phosphates, but only so in the light. HOLZER (25) observed a slow uptake of orthophosphate by *Chlorella* in the light. No significant changes were found in TCA-soluble phosphate compounds others than orthophosphate. Several findings regarding the metabolism of phosphate in *Chlorella* at the start of an illumination are important. KANDLER (30) observed a transient drop in orthophosphate content of *Chlorella* suspensions when light was given. The stationary level, however, was only slightly below the dark level. When the suspensions were darkened subsequently, a transient rise in orthophosphate was noticed, followed by a return to the stationary dark level. These observations were confirmed in our laboratory (74). According to KANDLER, these variations in orthophosphate reflect an increased phosphorylation in light, soon followed by an increased utilization of the formed energy-rich phosphate compounds. STREHLER (62, 63) observed a transient rise in ATP content of *Chlorella* suspensions at the start of an illumination after previous anaerobic incubation in the dark. His curves are more or less mirror images of those published by KANDLER (30). In several respects these induction effects of phosphate metabolism are comparable to those known from other phenomena connected with photosynthesis (see 74, 75). Both KANDLER and STREHLER suggest that, primarily, a 'reductant' is formed in the light and that reoxidation of part of this reductant is coupled to the formation of energy-rich phosphate (ATP), which is then used in the reduction of CO_2.

Now, leaving aside for the moment several details to be dicussed lateron, there is evidence for the formation of energy-rich phosphate in the light and for its rapid utilization. On the other hand, the fact that the steady state levels of orthophosphate and of ATP in light differ only slightly from those in darkness (30 resp. 62), and STREHLER's observation (62) that the steady state level of ATP in the light is independent of CO_2, do not point to storage of 'reducing power' or of energy-rich phosphate.

WASSINK and coworkers, among whom is the present author, are the only ones who report a long lasting uptake of phosphate in light in the absence of CO_2 (76, 78, 79, 85, 86). That the phosphate compounds formed under these conditions in *Chlorella* do not represent readily available 'reducing power'

follows from their stability in darkness, in the presence of respiratory CO_2.

It was, therefore, of interest, to attempt to isolate and identify the phosphate compounds formed. Furthermore, the influence of various conditions on the relation between accumulation of phosphate and photosynthesis was studied. A number of observations made in this laboratory regarding the phosphate metabolism of *Chlorella* which have been published previously (78, 79, 86) will be discussed briefly. A preliminary report of some further results has been given recently by the author (85).

CHAPTER II

THE UPTAKE OF PHOSPHATE BY *CHLORELLA* IN LIGHT AND IN DARKNESS

Part of the data contained in this Chapter have been published in a previous paper from our laboratory (78), and constitute the basis of further observations along similar lines. Some modifications of the technique used are included in the following description.

§ 1. METHODS

Chlorella vulgaris, 'strain A', has been grown in a modified KNOP solution, relatively poor in phosphate, and containing: 900 mg $NaNO_3$, 250 mg KH_2PO_4, 50 mg $MgSO_4$, 2 ml 0.3% $FeSO_4$, 1 ml of a solution of mixed trace elements, and distilled water up to 1000 ml. Cultures were inoculated with *Chlorella*, from an agar slant, in a 1000 ml Erlenmeyer flask containing 500 ml of autoclaved culture medium. The cultures were grown for 3–4 days under continuous illumination from fluorescent tubes ('daylight' and 'warm white', 40 W, Philips), and flushed with air containing 5% CO_2, while temperature varied from 20-25° C.

Lateron, the growing technique described by WASSINK *et al.* (77) was adopted, and better and more regular crops were obtained. Fixation of phosphate was not influenced by this change. Cells were then grown as follows:

a) The inoculum from agar slants was brought into 100 ml bottles with about 50 ml solution, containing: 1.26 g KNO_3, 1.22 g KH_2PO_4, 2.46 g $MgSO_4$.7 aq., 0.03 g $FeSO_4$, 1.00 g Na-citrate, 15 g glucose, and water up to 1000 ml. Continuous light was given for 3–6 days. These bottles were not aerated.

b) Five ml of heavy algal suspension from these bottles were used as inoculum into a 1000 ml Erlenmeyer flask containing 500 ml of culture medium, made according to WARBURG's prescription with slight modifications, and containing per litre 1.00 g $Ca(NO_3)_2$, 0.25 g KNO_3, 0.25 g KH_2PO_4, 0.50 g $MgSO_4$.7 aq., 0.15 g NaCl, 0.02 g $FeSO_4$ and 0.04 g Na-citrate.

c) Continuous illumination was replaced by an alternation of light and darkness (14 hours light, 10 hours dark). The Erlenmeyer flasks were continuously shaken, and illuminated from below by fluorescent tubes at a distance of about 10 cm. Air containing 5% CO_2 was continuously passing through. Cells were usually harvested at the end of a light period.[1])

For an experiment the cells were centrifuged, washed once in a phosphate-free medium, containing K_2SO_4, and suspended in the experimental medium, containing 2.6 g K_2SO_4 and 45 mg KH_2PO_4 in 1000 ml distilled water. With dilute HCl or H_2SO_4, pH was adjusted to 4.0. In later experiments, when cells were grown in the presence of $Ca(NO_3)_2$, precipitates containing Ca and P were often present, and interfered with the accurate estimation of cell density in TROMMSDORFF tubes. Moreover, the phosphate content was increased to undesirable levels when these precipitates entered into the suspension medium. Therefore, the harvested

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[1]) Recent data from TAMIYA *et al.* (65) indicate that differences in photosynthetic capacity of *Chlorella* cells can be observed when cells, harvested at the end of a dark period are compared with cells collected at the end of a light period. According to TAMIYA, highest photosynthetic capacity is found in 'dark' cells, i.e. harvested at the end of a dark period. With our 'light' cells we found good photosynthesis, amounting to 20–30 mm^3 CO_2/mm^3 cells/hour, at 25°C.

culture was brought first to about pH 5, centrifuged, washed in distilled water, and suspended in the usual way.

Suspensions containing 4–5 mm^3 wet packed cells/ml were put into 50 ml cylindrical vessels. These vessels were placed in a thermostate with glass walls, kept at about 27°C, and illuminated with a 100 W incandescent lamp from either side of the bath. The cylinders were continuously flushed either with CO_2-free air, or air containing 5% CO_2. Air was freed from CO_2 by pumping it through strong KOH; in later experiments a tube of soda lime was inserted after the KOH. Air with 5% CO_2 was obtained from a steel flask.

For extraction and determination of total orthophosphate in the suspension, the procedure of LOWRY and LOPEZ (42) was followed. To 1 ml cold 20% trichloroacetic acid (TCA), 3 ml suspension were added. After a suitable time of contact, 16 ml 0.1 molar Na-acetate were added, and the mixture was centrifuged. Samples of 5 or 10 ml were pipetted and used for the determination of orthophosphate. WASSINK *et al.* (78) give data showing that the time of contact with cold TCA is of no great importance as long as it is between 1 and 15 minutes. Generally, 3 minutes exposures were applied, and comparable results were obtained when either cold TCA or TCA at room temperature were used. A second extraction of the cells with TCA did not significantly alter the results. Measurements of transmission were first made with a 'Lumetron 400 A' colorimeter, using a red filter. Lateron, an 'Objecta' colorimeter was used, read at 700 mμ.

Although the treatment with cold TCA is generally adopted for the extraction of orthophosphate and organic phosphates from tissues, the assumption that organic phosphates are not attacked by this compound may not be entirely justified. KANDLER (30) indicates that, besides orthophosphate and well-known phosphate compounds such as sugar-esters, ATP *etc.*, the TCA extract contains 'structure phosphate', very loosely bound to the protoplasm. Such very labile compounds will already be hydrolyzed by cold TCA. Also SIMONIS and GRUBER (57) point to the possibility that very labile compounds may be determined as orthophosphate. Although we used the extraction method and orthophosphate determination of LOWRY and LOPEZ, designed for the determination of orthophosphate in the presence of very labile organic phosphates, it remains possible that part of the orthophosphate thus determined was not present in this form before the extraction. In former publications on the subject (78, 79, 86), we therefore consistently used the designation 'TCA-soluble' or 'TCA-labile' phosphate instead of 'orthophosphate'. This, however, may give rise to confusion. Sometimes the designation 'TCA-soluble phosphate' is also used as an abbreviation for 'TCA-soluble organic phosphate', as, for example, by BROWN and FRENKEL (6). These authors seem to have misunderstood us where they write (l.c., p. 49):

"They" (*i.e.* WASSINK *et al.*, [79]) "fractionated (5 min. hydrolysis with *N* HCl at 100°C) the TCA soluble phosphate into a labile and a stable fraction and found that the conversion of the labile fraction into the stable fraction was reduced in the presence of carbon dioxide. Addition of glucose had quantitatively the same effect as carbon dioxide in diminishing the conversion of labile into stable phosphate."

However, in the cited paper of WASSINK *et al.* (79) no mention was made of hydrolysis of the TCA extract with hot HCl. In the discussion section of the mentioned paper the terms 'TCA-labile' and 'TCA-stable' do not refer to the degree of stability of phosphate compounds in contact with hot HCl, but to their stability in contact with cold TCA. No misunderstanding would probably have arisen if 'orthophosphate' had been written instead of 'TCA-labile' or 'TCA-soluble' phosphate, and 'bound phosphate' instead of 'TCA-insoluble' or 'TCA-stable' phosphate. In this paper, therefore, I shall follow the usual practice, and write 'orthophosphate' for all the phosphate which is determined as orthophosphate in the extract, obtained from *Chlorella* with cold TCA.

§ 2. THE UPTAKE OF PHOSPHATE AS INFLUENCED BY LIGHT AND CARBON DIOXIDE

In experiments with suspensions of *Chlorella* as described, changes in the orthophosphate content of the TCA extract were observed after 1–3 hours. These changes were small in darkness, both in the presence and in the absence of CO_2. In light much larger changes were found, and in all cases there was a decrease of orthophosphate as a result of illumination. The fixation of phosphate in the light was strongly decreased by carbon dioxide. Figs. 1 and 2 illustrate these changes, and Table I gives average results of a number of experiments (see also [78]). It is apparent that the fixation of phosphate continues for hours,

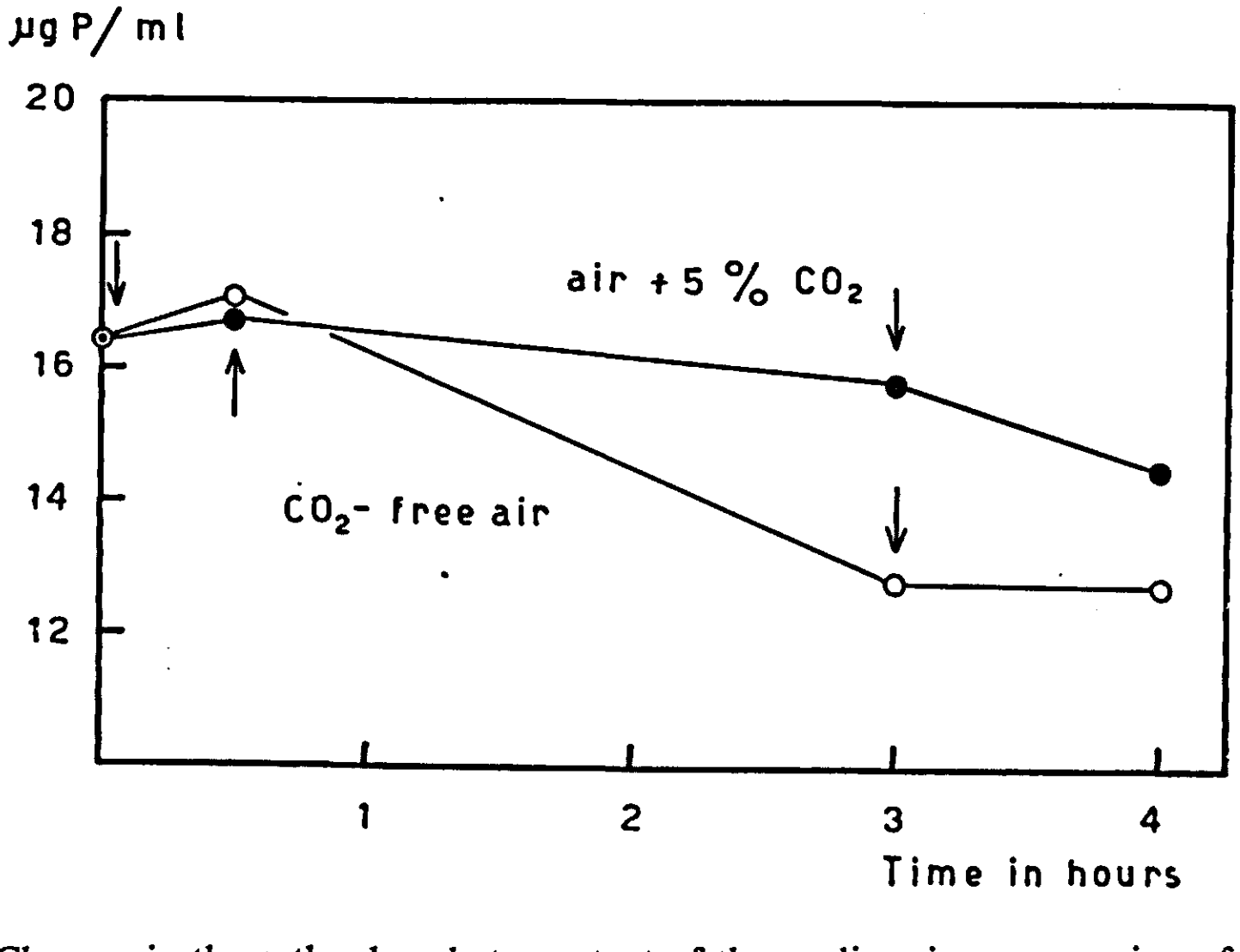

FIG. 1. Changes in the orthophosphate content of the medium in a suspension of *Chlorella*, in light and darkness, in the presence and absence of CO_2. ~ 4 mm³ cells/ml; pH ± 4.0. ↑ Shift to light, ↓ shift to darkness. From (78).

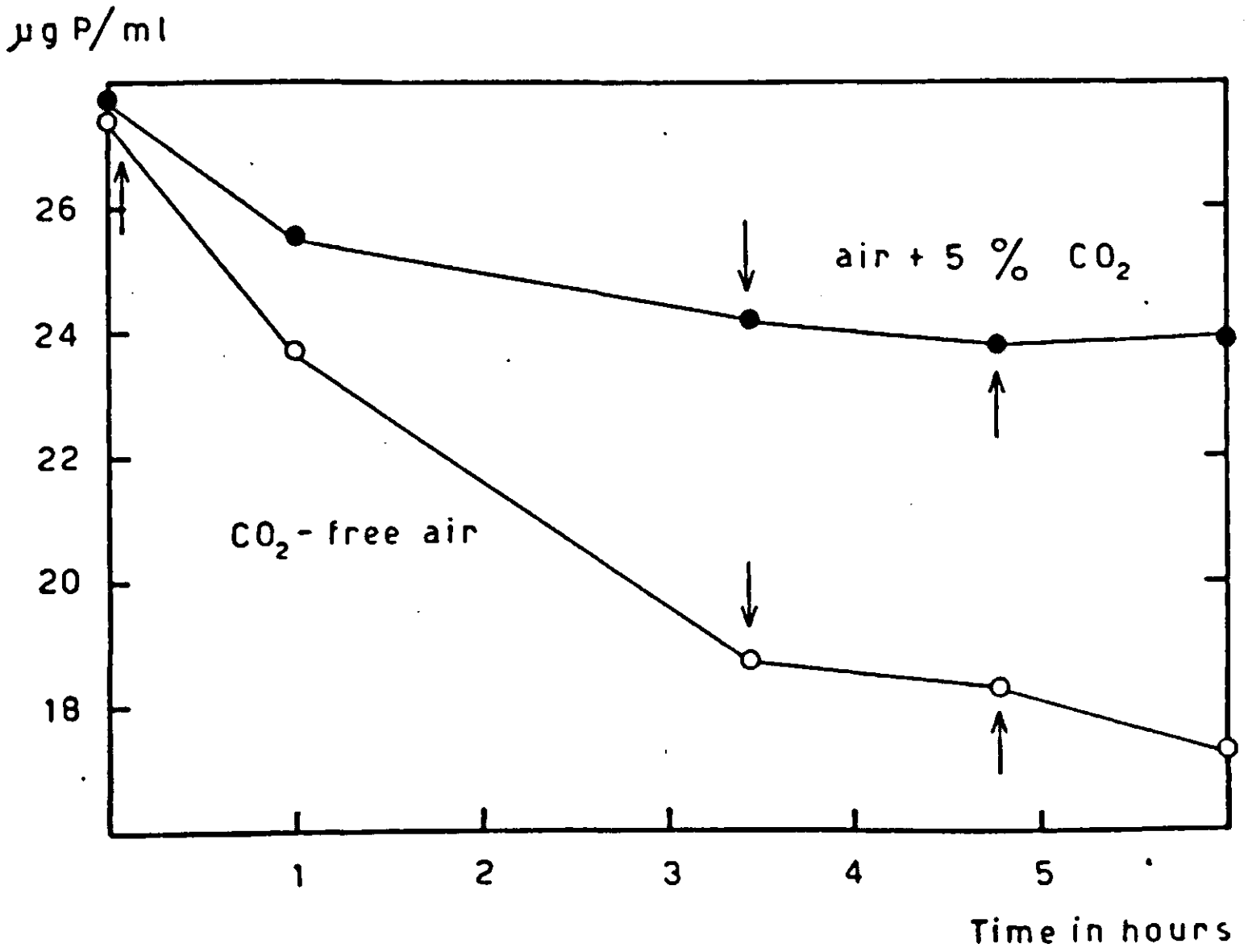

FIG. 2. Changes in orthophosphate in a suspension of *Chlorella*, in light and darkness, in the presence and absence of CO_2. pH ± 4.0. ~ 4 mm³ cells/ml. ↑ Shift to light, ↓ shift to darkness. From (78).

although the rate decreases gradually. No breakdown of newly formed phosphate compounds is observed in darkness (fig. 2, see also Chapters I and VI).

TABLE I

Decrease in orthophosphate in suspensions of *Chlorella* as a result of various treatments. Summarized results of a number of experiments. From (78).

Treatment	Time of exposure in minutes	Phosphate converted, μg P/ml	Number of observations
Light; air + 5% CO_2 . .	60	1.3 ± 0.2 [1])	18
	120	2.15 ± 0.25	18
	180	2.6 ± 0.25	16
Light; CO_2-free air . . .	60	2.5 ± 0.25	24
	120	4.2 ± 0.3	24
	180	5.7 ± 0.45	17
Dark; air + 5% CO_2 . .	120	0.55 ± 0.15	8
Dark; CO_2-free air . . .	60	0.2 ± 0.25	11
	120	0.25 ± 0.2	10

[1]) Standard error of the mean.

CHAPTER III

ANALYSIS OF THE CELLULAR PHOSPHATES FORMED IN LIGHT

§ 1. EXTRACTION WITH HOT HYDROCHLORIC ACID

A brief communication has already been published concerning the properties of phosphate compounds formed by *Chlorella* in light (86). It was established that the orthophosphate which was converted in the light, had become insoluble in cold TCA, but could, by extraction with hot 1 N HCl, be recovered from the TCA-extracted cells (to be denoted as residue I). Residue I was taken up in 5 ml 1 N HCl and heated for 5 minutes in a boiling waterbath, centrifuged, and washed with 3 ml 1 N HCl at room temperature. The combined HCl extracts were analyzed for orthophosphate and total phosphate. The residue of the HCl extraction (residue II) was submitted to wet destruction with a mixture of nitric, sulfuric and perchloric acids. Its phosphate content was only slightly influenced by illumination, so it is evident that the HCl extract contained the phosphate taken up or converted in the light. Satisfactory balances were obtained, as shown in fig. 3, and Table II (p. 78), partly reproduced from (86). Control determinations of total phosphate of the suspension showed that no phosphate was lost during the successive extractions; recovery averaged 100%.

The 5 minute extraction with hot HCl caused considerable hydrolysis so that a great part of the phosphate was determined as orthophosphate. Especially most of the phosphate compounds formed during the illumination in CO_2-free air, were already hydrolyzed during this extraction with HCl. The phosphate compounds formed during illumination in air containing 5% CO_2, were hydrolyzed only for about 50% during 5 minute HCl extraction. This indicates that the presence of CO_2 influences phosphate fixation not only quantitatively but also qualitatively. The latter may mean the formation of different compounds in the two cases under discussion, or the formation of similar compounds in different proportions.

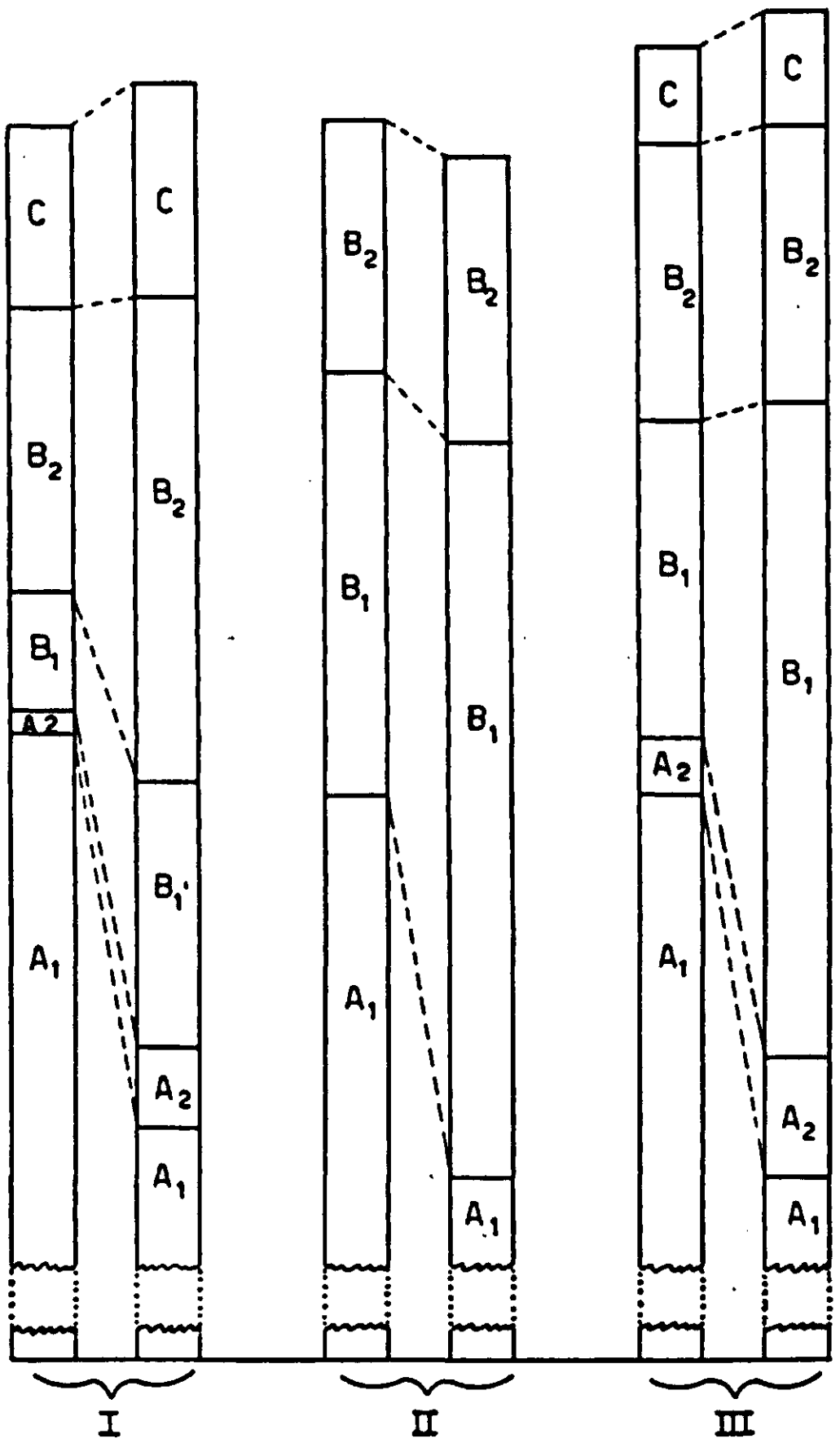

FIG. 3. Fractionation of *Chlorella* suspensions, before (left columns) and after (right columns) illumination in CO_2-free air during $3\frac{1}{2}$ hours. pH ± 4.0.
A: TCA extract; A_1 = orthophosphate;
A_2 = bound phosphate;
B: Residue I extracted with 1 N HCl at 100°C;
B_1 = hydrolyzed during extraction;
B_2 = not hydrolyzed during extraction;
C: residue II: total P remaining after extractions A and B;
Columns I: extracted 2 min. with hot HCl; II: idem 5 min.; III: id. 7 min.

Interest now was centered on the labile phosphate formed in the absence of CO_2, since it possibly was an energy-rich phosphate (*e.g.* ATP), which in general are easily hydrolyzed in hot HCl. In another series of experiments, the time of extraction in HCl was varied. It was found that extraction for less than 2 minutes results in a markedly incomplete extraction. Some data on experiments, comparable with those from (86), but with HCl extraction times of 2 and 7 minutes, are also given in Table II, and illustrated in fig. 3. Indeed, a hydrolysis curve made out of these data shows that the phosphate, accumulated in light in the absence of CO_2 is entirely hydrolyzed in 7 minutes in hot 1 N HCl (fig. 4). The distinction between 'HCl-labile' and 'HCl-stable' phosphates, as used by WINTERMANS and TJIA (86), becomes less appropriate with these data at hand,

TABLE II

Changes in the distribution of phosphates in a suspension of *Chlorella* as a result of illumination in the absence of CO_2, in μg P/ml. After 2 extractions with cold 5 % TCA, the residue (I) was suspended in 1 N HCl and heated for 2,5 and 7 minutes respectively at 100° C. Data from representative experiments.

	Time 0	$3\frac{1}{2}$ hours light CO_2-free air	Difference
1. TCA extract			
orthophosphate	18.1	9.8	−8.3
bound phosphate	0.0	1.3	+1.3
residu I, 2 min. hot HCl, total P	8.0	15.3	+7.3
hydrolyzed in 5 minutes	1.9	4.7	+2.8
not hydrolyzed in 5 minutes	6.1	10.6	+4.5
residue II	2.9	3.7	+0.8
Sum (direct determination: 29.3)	29.0	30.1	+1.1
2.[1]) TCA extract			
orthophosphate	33.0	25.3	−7.7
bound phosphate	−	−	−
residue I, 5 min. hot HCl, total P	10.0	17.1	+7.1
hydrolyzed in 5 minutes	6.4	12.9	+6.5
not hydrolyzed in 5 minutes	3.6	4.2	+0.6
residue II	−	−	−
Sum	43.0	42.4	−0.6
3. TCA extract			
orthophosphate	14.4	9.0	−5.4
bound phosphate	1.0	1.5	+0.5
residue I, 7 min. hot HCl, total P	6.6	11.6	+5.0
hydrolyzed in 7 minutes	3.5	8.5	+5.0
not hydrolyzed in 7 minutes	3.1	3.1	0.0
residue II	1.4	1.8	+0.4
Sum (direct determination: 23.7)	23.4	23.9	+0.5

[1]) Taken from (86).

since certain compounds belong to the first or to the second category, depending on the duration of extraction with hot HCl. Their conclusion, however, that phosphate taken up in light in the absence of CO_2, is stored in (a) labile compound(s), remains valid. As to the phosphate taken up in light in the presence of CO_2, no hydrolysis curve comparable with that from fig. 4 could be determined, owing to the unfavourable relation between the magnitude of the experimental error and the effect to be studied.

It has been previously reported (78) that with our way of TCA extraction no significant difference exists between orthophosphate and total phosphate in the TCA extract. This is in contrast with the findings of several authors who found phosphates other than orthophosphate in the TCA extract of *Chlorella*. EMERSON *et al.* (19) extracted several times, each extraction lasting for 12 hours, and found several organic phosphates in the extracts. HOLZER (25) extracted for 1 hour, and found ortho- and metaphosphate in the TCA extract. GEST and KAMEN (21), however, after extraction of *Chlorella* for 1 hour with 5–6 % TCA, only found orthophosphate in the extract, so that their results are comparable

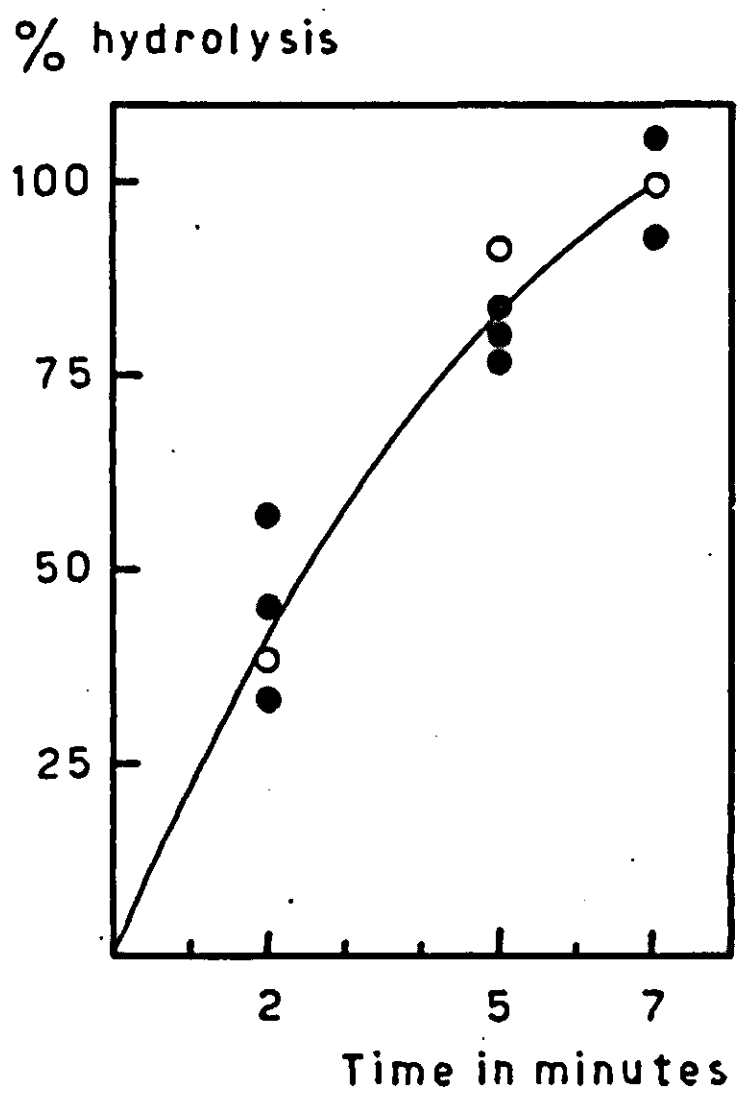

FIG. 4. Phosphate compounds formed by *Chlorella* in light, in CO_2-free air, and soluble in hot HCl. Percentage hydrolysis as a function of time of treatment with hot HCl. (Open circles correspond to experiments shown also in Table II).

with ours (86). In the more recent experiments reported in this §, however, a small quantity of bound phosphate was regularly found in the TCA extract. After illumination in CO_2-free air this bound phosphate increased, accounting for about 15% of the converted phosphate (see Table II, 1 and 3, and sections A_2 in fig. 3). The difference with (86) remains unexplained.

§ 2. PAPER CHROMATOGRAPHY OF THE HYDROCHLORIC ACID EXTRACT

When it had been established that (a) labile phosphate compound(s) which accumulated in the light could be liberated without complete hydrolysis by a brief HCl extraction as described in § 1 of this Chapter, this extract was submitted to paper chromatography.

The separation of various phosphate compounds by paper chromatography has been described by several authors (5, 12, 15, 22, 46). We used Whatman nr. 1 paper, washed with HCl and distilled water. The paper was not washed with 8-hydroxyquinoline, as recommended by HANES and ISHERWOOD (22) for removal of traces of heavy metals, but EDTA (Versene) was added to some batches of solvent mixture. When EDTA was omitted, however, the chromatograms did not show serious defects. A suction filter constructed from lucite (perspex) sheet, as described by HANES and ISHERWOOD (22) proved to be convenient for the necessary washings of the paper.

Various water-miscible solvents were used. First some used by HANES and ISHERWOOD (22), *viz.*: n-propanol 60/conc. ammonia 30/water 10 parts, and: tert. butanol 80 ml/water 20 ml + 4 g picric acid. The latter solvent seemed somewhat over-saturated with picric acid at the temperature at which our chromatograms were run (about 12 °C). Lateron, the solvents described by BANDURSKI and AXELROD (5) were used, *i.e.* the acid solvent: methanol 80/ formic acid (88% w/w) 15/water 5, and the basic solvent: methanol 60/ammonia (28% w/w) 10/water 30.

After development of a chromatogram, the paper was dried in an air stream, then sprayed with the reagent recommended by HANES and ISHERWOOD (22),

and heated for 5–10 minutes at about 85 °C. Orthophosphate becomes soon visible as a yellow spot (*cf.* [5]). After heating, other compounds appear as faint, yellow spots, and in this stage contact prints can already be made successfully on photographic paper. The spots were made more clearly visible by spraying with 1 % ascorbic acid, or by applying ultra-violet light. Background coloration may occur also, and is noticed especially when the paper is not entirely dry during the ultraviolet irradiation. Background coloration can be overcome by exposure of the chromatogram to ammonia vapour, as found by BANDURSKI and AXELROD (5).

Chlorella suspensions of the usual pretreatment (p. 73) were extracted before and after illumination in CO_2-free air, first with TCA, and then with 1 N HCl at 100 °C for 100–120 seconds. The HCl extracts were concentrated *in vacuo* at room temperature; 1–3 mm^3 of the concentrate were applied to the filterpaper. Samples of organic and inorganic phosphates were applied separately on the same paper for comparison. Partial hydrolysis of the cellular phosphates takes place during the extraction, as described, and very probably also during the concentration of the extract, so that a heavy orthophosphate spot was always observed in the *Chlorella* extracts. It proved to be impossible, moreover, to obtain chromatograms of the HCl extracts without part of the phosphate remaining at the starting point. Addition of EDTA to the solvent gave some, but no definite improvement. Between the orthophosphate spot and the initial spot (often apart from the latter), the *Chlorella* extracts showed trails of variable length and intensity.

EBEL and VOLMAR (18) (see also EBEL [15]) have observed an influence of the molecular weight of the inorganic phosphates on the R_f values, both in alkaline and acid solvents. High molecular weight polymers of phosphoric acid, the so called GRAHAM salts, did not move at all. Orthophosphate had a greater R_f value than pyrophosphate and tripolyphosphate. As no other compounds were known with $R_f = 0$ in both alkaline and acid solvents, it seemed possible that our *Chlorella* extract contained highly polymerized phosphates.

The chromatographic behaviour of 'metaphosphate' was studied with a preparation (MAY and BAKER) of the free acid, from which solutions containing 1 mg P/ml were made. The acid solution showed appreciable hydrolysis in a few days; after neutralization with NaOH the solution is more stable. The fresh solution, chromatographed with the solvents given by BANDURSKI and AXELROD (5), showed 2 spots, one not moving at all, in accordance with (18), and one having R_f values like pyrophosphate.[1]) Chromatograms of older solutions showed orthophosphate spots and also trails from the initial spot to the orthophosphate spot, probably indicating compounds with intermediate molecular weight (degradation products) in accordance with EBEL's suggestion (12) regarding the degradation of GRAHAM salt. So it seemed possible that polymers of phosphoric acid would be responsible for the results obtained with the HCl extracts. Indeed, both extraction and concentration of the extracts might cause hydrolysis, by which orthophosphate and low-weight polymers in variable proportions may arise. During paper chromatography such a mixture would behave as we found

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[1]) In our experiments the R_f of pyrophosphate in the alkaline solvent was found to be 0.35, a value very near 0.33 and 0.36 as found by EBEL and VOLMAR (18), although we used the solvent described by BANDURSKI and AXELROD (5). The very low R_f value of 0.05 for pyrophosphate in the alkaline solvent found by the latter authors, was not reproduced in our experiments.

with the *Chlorella* extracts. To test this possibility it was necessary to apply an extraction method which would cause less hydrolysis than that using hot HCl.

§ 3. THE OCCURRENCE AND EXTRACTION OF POLYPHOSPHATES

a. Introduction

Phosphoric acid can occur in various degrees of polymerization. These polymer compounds can be derived from H_3PO_4 by dehydration, and can be prepared by heating of orthophosphates. The lowest member of the group, pyrophosphoric acid, $H_4P_2O_7$, can be represented as $2\ H_3PO_4–H_2O$. Related to this compound are other linear polymers, such as triphosphoric acid, tetraphosphoric and higher acids (see fig. 5), which generally can be represented as: $(H_3PO_4)_n–(H_2O)_{n-1}$. Another way of writing this formula would be: $(HPO_3)_n.H_2O$, and it will be clear that for high values of n, this formula closely approaches $(HPO_3)_n$. A small group of compounds has the exact formula $(HPO_3)_n$. These are the cyclic condensed phosphates, from which tri- and tetrametaphosphate are known, and represented in fig. 5. For a general discussion see SCHMIDT (52)

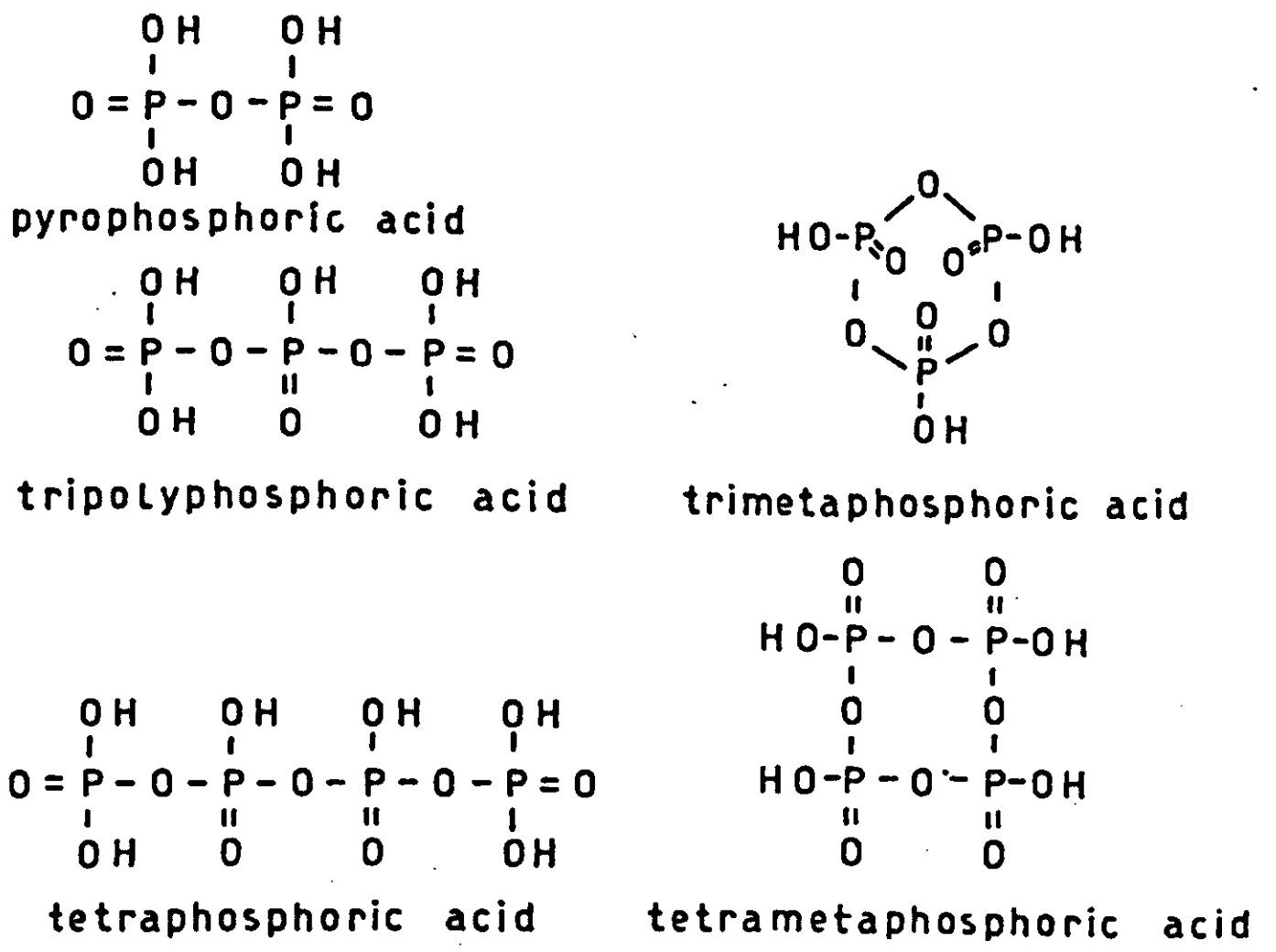


FIG. 5. The lower condensed phosphates (after [52]).

and EBEL (15). Concerning the nomenclature, it seems advisable to restrict the term 'metaphosphate' to the lower, cyclic polymers, having the exact composition $(HPO_3)_n$. The linear polymers are best indicated as 'polyphosphates'. Now, if mixed ring- and chain structures were found, they would also have the exact formula $(HPO_3)_n$, as pointed out in (52). Concerning the phosphate polymers found in living organisms, not much is known about their structure. They often have high molecular weights (*cf.* [52]). Their chemical properties are more in a line with the well-known properties of the polyphosphates than with those of the metaphosphates (52). EBEL (11) therefore, advocates the use of the term polyphosphates instead of metaphosphate for the high molecular polymers of phosphoric acid in yeast and other organisms. Because in most of the literature

the term 'metaphosphate' has been used, the older term will still be retained in connection with a discussion of the literature. Generally, however, the term polyphosphates will be used in this paper.

The occurrence of polymers of phosphoric acid in various lower organisms has been known for a long time. In a general review of the biochemistry of this group of compounds, SCHMIDT points out that metaphosphate in yeast was found already at the end of the previous century (52). Extensive studies on yeast metaphosphate were made by WIAME (80, 82), SCHMIDT *et al.* (53, 54), EBEL (11–14), YOSHIDA (87, 88). In fungi (*Aspergillus*) MANN (43) found metaphosphate in considerable quantities, together with pyrophosphate, and its presence was also reported in *Neurospora* (28). Among bacteria examples are: *Corynebacterium diphtheriae* (14) and several *Mycobacteria* (83). Among algae, it was found in *Chlorella* by SOMMER and BOOTH (59) and by HOLZER (25). In *Euglena*, a considerable percentage of total P was found in the form of metaphosphate (ALBAUM *et al.*[2]). STICH (60) showed that metaphosphate occurs in cytologically demonstrable granules in *Acetabularia mediterranea*. In the blue alga *Phormidium ambiguum*, EBEL demonstrated the presence of polyphosphates (13).

WIAME (82) found that part of the metaphosphate from yeast is soluble in cold TCA. The larger, insoluble part could be removed from the residue with hot TCA. However, a certain degree of hydrolysis is to be expected when hot TCA is used, and has been observed by WIAME (82). As an alternative way to extract the insoluble metaphosphate, WIAME recommended alkaline extraction of the cells, after removal of lipid material with alcohol and alcohol-ether. This method was found to be very mild for yeast metaphosphate (82).

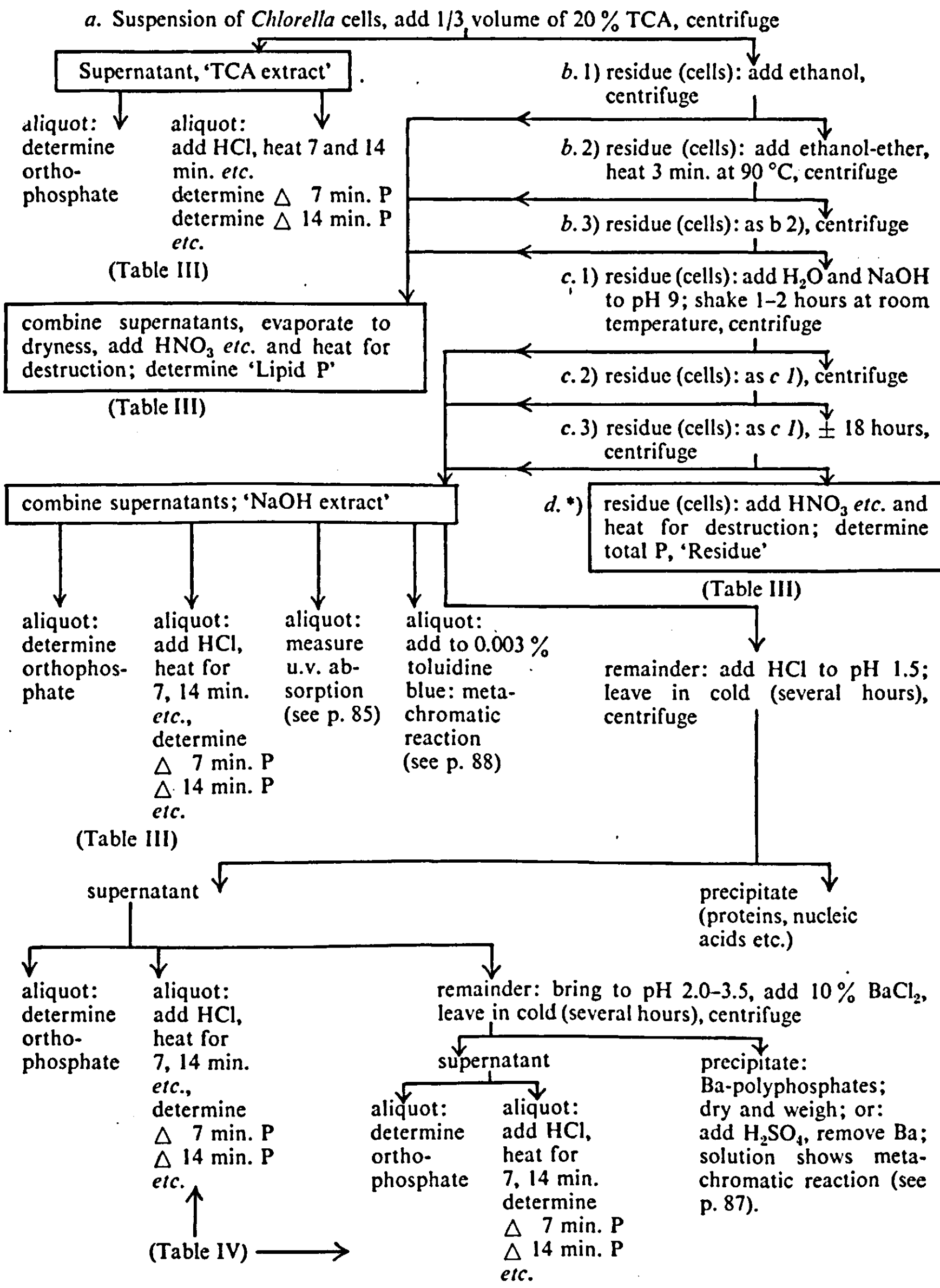
For that reason we have chosen it for the extraction of *Chlorella*.

b. Extraction of labile phosphate with dilute NaOH

Suspensions were prepared as usual (p. 73), but greater densities were taken sometimes, varying from 5–10 mm^3/ml. Samples of 100 or 150 ml were analyzed, immediately after preparation of the suspension, and also after 3–4 hours illumination at 25 °C in the absence of CO_2. The samples were first extracted with TCA at room temperature, final concentration 5 %, and centrifuged after a few minutes without neutralization. The supernatant was analyzed for orthophosphate, and samples were made up to 1 N with HCl and hydrolyzed for 7 and 30 minutes respectively, at 100 °C. The residue of the TCA extraction was treated successively with 10 ml ethanol at room temperature, and twice with an ethanol-ether mixture (3:1) at 90 °C for 3 minutes. The extracts, containing most of the pigments and the lipid material were combined and analyzed for total phosphate. According to WIAME's recommendations (82) the residue was suspended in distilled water and brought to pH 9 with NaOH. The duration of the alkaline treatment was longer than in WIAME's experiments (82); in our experiments the cells were extracted twice for 1–2 hours and a third time overnight. The combined alkaline extracts were analyzed for orthophosphate, and after addition of HCl to normality, samples were hydrolyzed respectively for 7 and 14 minutes, or longer, if desired. Finally, the residue of the NaOH extraction was submitted to wet destruction, and total phosphate was determined. For the sake of convenience, a schematical representation is added of the operations described here and in subsections *c* and *d*.

The results of some experiments of this type are contained in Table III and illustrated in fig. 6. Comparing the TCA extracts of the *Chlorella* samples before

Schema of extractions of *Chlorella* suspensions, before or after illumination in CO_2-free air. (Detailed description in Chapter III, § 3).



*) A fourth alkaline extraction was made between *c 3)* and *d)* in the expt. 1 of Table III.

TABLE III

Changes in the distribution of phosphate in a suspension of *Chlorella* as a result of illumination in CO_2-free air. Data in μg P/ml suspension. Sequence of extractions: TCA 5%, room temperature; alcohol and 2 × hot alcohol-ether (yielding 'lipid P'); dilute NaOH (pH 9, three extracts combined). △ 7 min. P etc.: phosphate released by hydrolysis in 1 N HCl, 100°C, for the time indicated.

Expt.	Fraction	Time 0	Light, CO_2 free air.	Difference
1.	TCA extract			
	orthophosphate	17.5	10.2	−7.3
	△ 7 min. phosphate	0.6	0.6	0.0
	△ 30 min. phosphate	0.0	0.0	0.0
	Lipid P	2.1	2.3	+0.2
	NaOH extract			
	orthophosphate	0.15	0.25	+0.1
	△ 7 min. phosphate	2.8	9.0	+5.2
	△ 14 min. phosphate	0.45	0.7	+0.25
	△ 30 min. phosphate	0.0	0.0	0.0
	NaOH, 4 th extraction			
	△ 7 min. phosphate	0.4	0.6	+0.2
	△ 20 min. phosphate	0.25	0.3	+0.05
	△ 30 min. phosphate	0.0	0.0	0.0
	Residue	5.05	6.15	+1.1
	Sum	29.35	29.1	−0.25
2.	TCA extract			
	orthophosphate	11.4	5.0	−6.4
	△ 7 min. phosphate	0.3	0.9	+0.6
	△ 30 min. phosphate	0.1	0.0	−0.1
	Lipid P	2.2	2.4	+0.2
	NaOH extract			
	orthophosphate	0.5	0.75	+0.25
	△ 7 min. phosphate	3.6	7.0	+3.4
	△ 14 min. phosphate	0.75	0.95	+0.2
	△ 210 min. phosphate . . .	0.5	0.55	+0.05
	Residue	4.9	6.2	+1.3
	Sum	24.25	23.75	−0.5
3.	TCA extract			
	orthophosphate	12.1	5.3	−6.8
	△ 7 min. phosphate	2.8	2.7	−0.1
	△ 15 min. phosphate	0.5	0.0	−0.5
	Lipid P	–	–	–
	NaOH extract (pH resp. 10; 9; 9)			
	orthophosphate	0.5	0.8	+0.3
	△ 7 min. phosphate	3.6	9.6	+6.0
	△ 14 min. phosphate	0.35	0.8	+0.45
	Residue	–	–	–

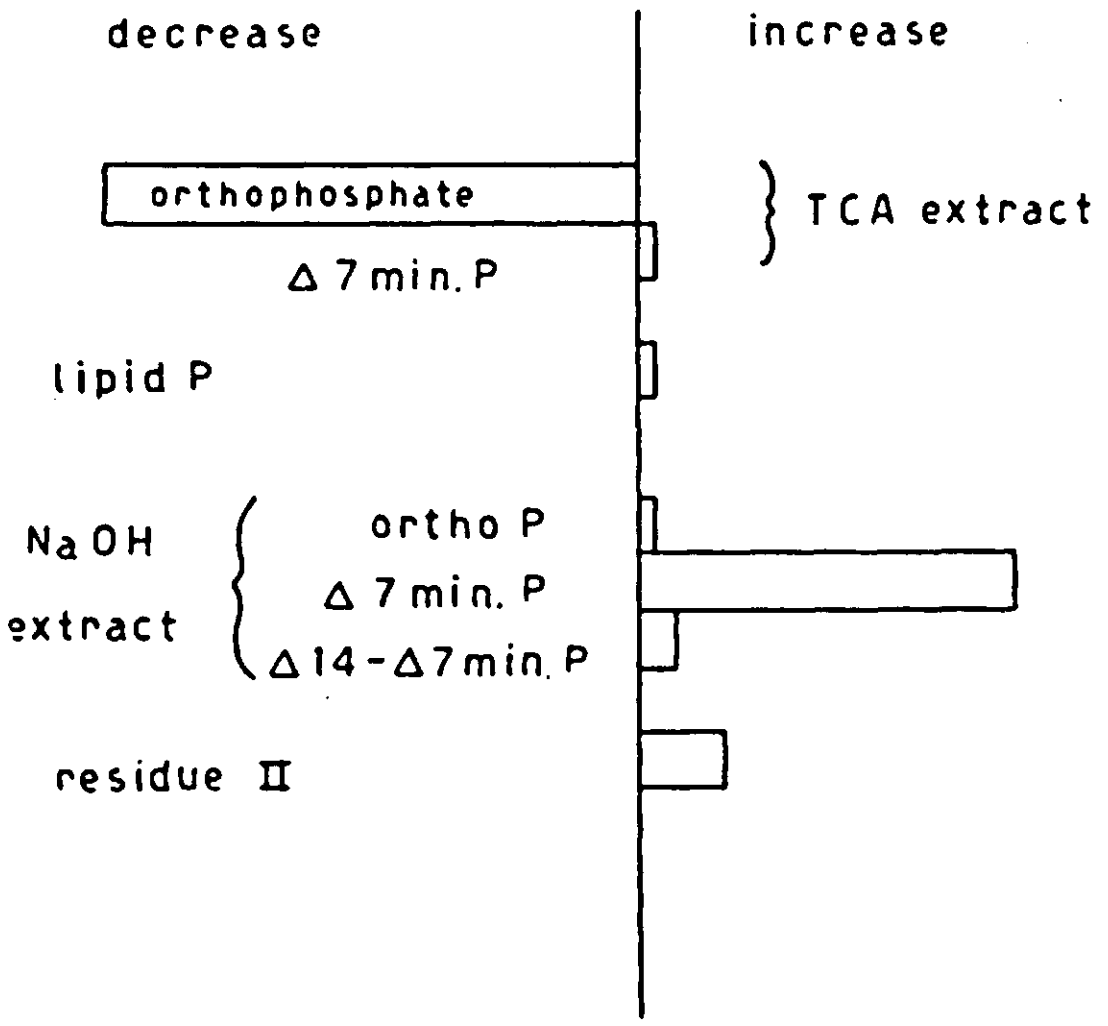


FIG. 6.
Changes in the distribution of phosphates over various fractions in a suspension of *Chlorella* after 3 hours illumination in CO_2-free air. Decrease in orthophosphate is accompanied by increase, especially in alkali-soluble, labile phosphate.

and after illumination, the latter show a considerable decrease in orthophosphate as expected, and a slight increase in △7 minutes labile phosphate. No important changes were found in the alcohol-ether extracts (lipid phosphate). The major part of the phosphate which has disappeared is recovered as △ 7 minutes phosphate in the NaOH extract. This is the fraction which should contain the polyphosphates (52, 82). There is also some increase upon illumination in orthophosphate and in more stable phosphates in the NaOH extract, but very little of the latter resists hydrolysis for more than 14 minutes. In the author's opinion, the presence of orthophosphate in the NaOH extract must be ascribed to a small degree of hydrolysis, even under these mild conditions of extraction. The observation of some phosphate of a more stable type may point to the presence of nucleic acid.

The u.v. absorption of the NaOH extract was considerable at 2570 Å, but there was neither correlation with the amount of △ 7 min. labile phosphate, nor with that of the more stable phosphate fraction. When the proteins were precipitated by acidification of the extract (see below, p. 86), the precipitate should contain the nucleic acids (52). However, it was found to contain only a small amount of phosphate, so that only small amounts of nucleic acids are present in the original NaOH extract. Moreover, according to EBEL (13) 60–80% of the phosphate of nucleic acid is resistent to 14 minutes hydrolysis in normal HCl. This resistent fraction, however, is absent or very small in our *Chlorella* extracts (Table III, expts. 1 and 2), and no increase is apparent upon illumination. We may therefore conclude that the fixation of phosphate does not result in formation of nucleic acids.

EBEL (13) has indicated that polyphosphate (GRAHAM's salt) is not completely hydrolyzed in 7 minutes, but that 15 minutes are required. It might, therefore, be that also the △ 14 min. phosphate represents polyphosphate. See, however, fig. 4 and Table II, which show complete hydrolysis in 7 minutes of the phosphate compounds formed in the light.

The residue of the alkaline extractions had a higher P content as a result of illumination, accounting for some of the phosphate fixed in the light. That some

alkali-soluble phosphate is still contained in this residue can be seen from Table III, expt. 1, where some of the phosphate which has disappeared was found in a fourth alkaline extract. A practically complete extraction of the fixed phosphate was obtained at pH 10 (see Table III, expt. 3).

c. Precipitation of labile phosphate with barium salts at low pH

The higher metaphosphates (polyphosphates) are precipitated by barium salts at low pH. With chemical preparations of polyphosphate complete precipitation is obtained at pH 2.5 (see [12]). Analyzing the phosphate compounds of *Euglena*, ALBAUM *et al.* (2) noticed precipitation of part of the polyphosphates at pH 1. According to EBEL (12) complete precipitation of the polyphosphates from yeast is obtained only at pH 4.5. Between pH 2.5 and pH 4.5, low-molecular weight polyphosphates (down to tripolyphosphate) are precipitated by barium salts, as was supported by the results of paper chromatography (12).

When, in our experiments, the alkaline extract of *Chlorella* was acidified, a precipitate containing very little phosphate was formed. According to EBEL, such precipitate consists of proteins (13). In order to remove these proteins, the extracts were brought to pH 1.5, and centrifuged. The supernatant was brought

TABLE IV

Precipitation with $BaCl_2$ at low pH of labile phosphate (in μg P), from the NaOH extract of *Chlorella*

	Before Ba-treatment	After Ba-treatment	Difference (precipitated)
a. Proteins removed at pH 1.5; barium precipitation at pH 3.5			
NaOH extract			
orthophosphate	260	290	−30
△ 7 min. phosphate	6340	1000	5340
△ 60 min. phosphate	500	230	270
Total P in precipitate			5580 [2]
Dry weight of precipitate			23,850 [2]
b. Proteins *not* removed; barium precipitation at pH 2.0 [1]			
NaOH extract, time zero			
orthophosphate	70	75	−5
△ 7 min. phosphate	545	75	470
△ 14 min. phosphate	55	60	− 5
Total P in precipitate			460
NaOH extract, after illumination of cells			
orthophosphate	120	140	−20
△ 7 min. phosphate	1435	140	1295
△ 14 min. phosphate	125	30	95
Total P in precipitate			1370

[1]) Same experiment as Table III, expt. 3.
[2]) % P in precipitate 23.3 %. Theoretical for $Ba(PO_3)_2$ 21.1 %

to pH values varying from 2 to 3.5, and a sample was taken for the estimation of orthophosphate, △ 7 min. and △ 14 or △ 60 min. phosphates. Excess 10% $BaCl_2$ was added to the remainder of the extract. A precipitate was formed which was removed by centrifuging. Determinations of ortho-, △ 7 and △ 14 or △ 60 min. phosphate were made again in the supernatant. No orthophosphate, about 85% of the △ 7 min. phosphate and variable amounts of the more stable phosphates, were precipitated by $BaCl_2$. Some results are shown in Table IV. Difficulties were encountered in trying to redissolve the barium precipitate by the addition of acid. Even at pH 1, not all of the precipitate goes into solution, and considerable hydrolysis takes place. Similar difficulties with yeast polyphosphates were reported by EBEL (11) and by DAMLE and KRISHNAN (9), and could be overcome by the use of cation exchanging resins. We have not yet applied this method in our experiments. After addition of 1 N H_2SO_4 to the above mentioned barium precipitate and removal of the $BaSO_4$, a solution was obtained which gave a metachromatic reaction with toluidine blue (see next section, p. 88), which supports the conclusion that indeed polyphosphates are present in the dissolved precipitate.

It appears from Table IV that not all of the 7 min. labile phosphate is precipitated as Ba-salt. It may be that the fraction which does not precipitate represents polyphosphates of low molecular weight, since the pH remained below 4.5, the limit indicated by EBEL (12) for complete precipitation of all polyphosphates.

d. The metachromatic reaction with toluidine blue

Metachromasy is a property of certain basophilic substances *i.e.* substances which are coloured by basic dyes. If suitable dyes of this type are applied to such substances, a complex is formed, the colour of which differs from that of the pure solution of the dye. The substances showing metachromasy are of high molecular weight, they are often colloids, and have free acid groups, *e.g.* carboxylic groups (arabic gum), sulfuric groups (cartillage, agar-agar), or phosphoric groups (polyphosphates, nucleic acids). The degree of metachromasy differs for various substances and can, moreover, be influenced by disturbing substances, as free acids ($H^{\cdot}$-ions) and salts. According to MASSART *et al.* (44), metachromasy as a rule is especially sensitive to low pH and salts when COOH groups are present, and less so when SO_4 groups are present. Among the substances with phosphoric groups there is much difference. Nucleic acids show metachromasy only when very little salt is present, and at neutral pH. MASSART *et al.* (44) attribute MICHAELIS' failure to obtain the metachromatic reaction with nucleic acids to the presence of acetate buffer in his experiments. WIAME found the metachromatic reaction with nucleic acids more sensitive to decrease in pH than with yeast polyphosphate (80). He even found nucleic acid to inhibit the metachromatic reaction of metaphosphate (81). Within the group of condensed phosphates, WIAME found the metachromatic reaction with toluidine blue specific for high-molecular phosphates (81). The absorption maximum of the dye at 630 mμ shifts to 530 mμ in the presence of polyphosphates. The ratio of the extinctions at these wavelengths ($\varepsilon_{530}/\varepsilon_{630}$) is taken as a measure of the metachromatic effect. Quantitative results have not been obtained by this method, since the effect depends on the proportion between the quantities of metaphosphate and toluidine blue. The effect decreases at both high and low values of the ratio metaphosphate/toluidine blue (81). DAMLE and KRISHNAN (9) tried to obtain quantitative results in estimating metaphosphate with the to-

luidine blue method, and stressed the necessity of control of acidity, salt concentration and temperature. They found that the intensity of the metachromatic reaction corresponds roughly to the molecular weight of the metaphosphate.

In our experiments, alkaline extracts of *Chlorella* as described on p. 82 showed a metachromatic reaction with a 0.003% solution of toluidine blue. This reaction is stronger with illuminated cells as compared with 'time zero' samples. After some time a purple precipitate develops. No quantitative results were obtained for the reasons given above.

The use of toluidine blue in the metachromatic reaction has developed from its use in cytological technique. Nucleic acids are coloured by this dye, as a rule without metachromasy. A stain containing formalin, acetic acid and toluidine blue was designed by LINDEGREN (38). This solution, if applied to yeast, colours volutin, but does not stain nucleic acids. LINDEGREN considers volutin as identical with metaphosphate (38). This identity is also pointed out by WINKLER (84).

When LINDEGREN's stain was applied to *Chlorella*, 1–5 coloured granules became visible in most cells. This seems to indicate that these granules contain polyphosphates. Similar structures became visible with another cytological stain for volutin, namely 1% methylene blue, followed by 1% H_2SO_4. No increase in the number or the size of these granules could be observed, however, after illumination in the absence of CO_2. Only a few observations were made, however, and it appears that reproducible results can only be obtained by strict control of conditions; variable percentages of coloured cells were obtained in samples from a single suspension. More specific cytological reactions for cellular polyphosphates have been developed recently by EBEL (14), and by EBEL and COLAS (17), with which we did not yet obtain a sufficient experience.

§ 4. CONCLUSION

It seems justified to conclude from the above, that polyphosphates are normally contained in *Chlorella* cells (*cf.* also [25, 59]), and are accumulated in light, especially in the absence of CO_2. Very probably, they also accumulate at a slow rate in the presence of CO_2, as is indicated by the occurrence of polyphosphates in freshly harvested cells, grown in the presence of 5% CO_2. STICH (60) observed the accumulation of polyphosphates during normal photosynthesis in *Acetabularia mediterranea*. Also in *Euglena*, polyphosphates are normal cell contents (2), evidently formed during normal growth in the presence of CO_2. The greatly accelerated rate of formation of polyphosphates as found in the experiments described in the present paper, is similar to the rapid rate of phosphate uptake and formation of polyphosphates in yeast, described by WIAME (82), SCHMIDT *et al.* (53, 54) and YOSHIDA (87, 88). The normally very slow rate of formation of polyphosphates in baker's yeast was enhanced by phosphate starvation and the presence of an energy source, such as glucose, pyruvate, acetate or ethanol (54).

In our experiments, the cells certainly were not phosphate-starved. The P content of the culture medium was 57 mg P/l, and the average P content of the cells was 5 μg P/mm³ packed wet cells. Assuming a dry weight of 25%, often found in our laboratory, this means 20 μg P/mg dry weight. KNAUSS and PORTER (34) have determined the uptake of phosphate by *Chlorella pyrenoidosa* as influenced by the composition of the culture medium. The P contents of their *Chlorella* cells and ours, when grown at comparable P contents of the medium,

are about equal. It seems justified to suppose that the maximum P content of our strain of *Chlorella* is not far from that found in *Chlorella pyrenoidosa* by the cited authors, *i.e.* 27 μg P/ml dry weight.

As to the energy source required for polyphosphate formation, in *Chlorella* light seems to replace the organic substrate necessary for yeast.

Certainly, the polyphosphates belong to the energy-rich phosphates, although few quantitative data have been published on their heat of hydrolysis (MEYERHOF *et al.*, [45]). Their formation in light represents a transformation of radiant energy into chemical energy. The amounts of energy fixed, however, are very small. The photosynthetic capacity of cells as used in our experiments is certainly not below 20–25 mm^3 CO_2/mm^3 cells/hour, *i.e.* about 5 μmoles CO_2/ml suspension/hour. The fixation of phosphate is at most 0.1 μmole P/ml suspension/hour. The amount of energy fixed per mole CO_2 assimilated is 112 kcal; the amount of energy per mole P converted will be of the order of 10 kcal. The ratio energy fixed in carbohydrate/energy fixed in polyphosphates then becomes 560. Such small quantities of energy could be derived from respiration, but the fact that light, and the absence of CO_2 are essential for the rapid formation of polyphosphates suggests that we are dealing here with a synthesis in which a photochemical step is involved.

Anticipating the general discussion, we can suppose that phosphate from energy-rich phosphate, formed in the light (see p. 72), is transferred to polyphosphates by way of enzymatic reactions. This would be especially noticeable when photosynthesis is limited by shortage of CO_2, in the fixation and reduction of which, probably ~ph is consumed. This implies that the production of ~ph in light is independent of CO_2 (*cf.* STREHLER, [62, 63]). From this point of view, the influence of several factors on phosphate accumulation was studied, and compared with their effects on photosynthesis. Comparison of the data thus obtained may aid in establishing the connection between photosynthesis and the formation of polyphosphates in light. The influence of several factors is described in the following Chapters.

CHAPTER IV

THE INFLUENCE OF SOME METABOLIC FACTORS ON THE FIXATION OF PHOSPHATE

§ 1. THE INFLUENCE OF LIGHT INTENSITY

Light intensity curves of photosynthesis and phosphate fixation were determined in the WARBURG apparatus.

Two 150 W sodium lamps below the glass bottom of the thermostat were used as a light source. Different light intensities were obtained by the use of incidentally available coloured glass filters which proved to be suitable for the purpose; these filters were mounted immediately below the vessels. The vessels were painted white except for the bottom, in order to prevent the entry of scattered light and, moreover to achieve a stronger and more uniform illumination of the algal suspension. Light intensities were measured with a thermopile. Because only the photosynthetically active light was of interest, the radiation with wavelength > 7000 Å was measured separately by inserting the SCHOTT RG 8 filter, and subtracted. Four ml of suspension, as described on p. 73, were introduced into each vessel. In some experiments the vessels were ventilated with air, containing 5% CO_2, in others 0.2 ml 2% KOH were placed in the side

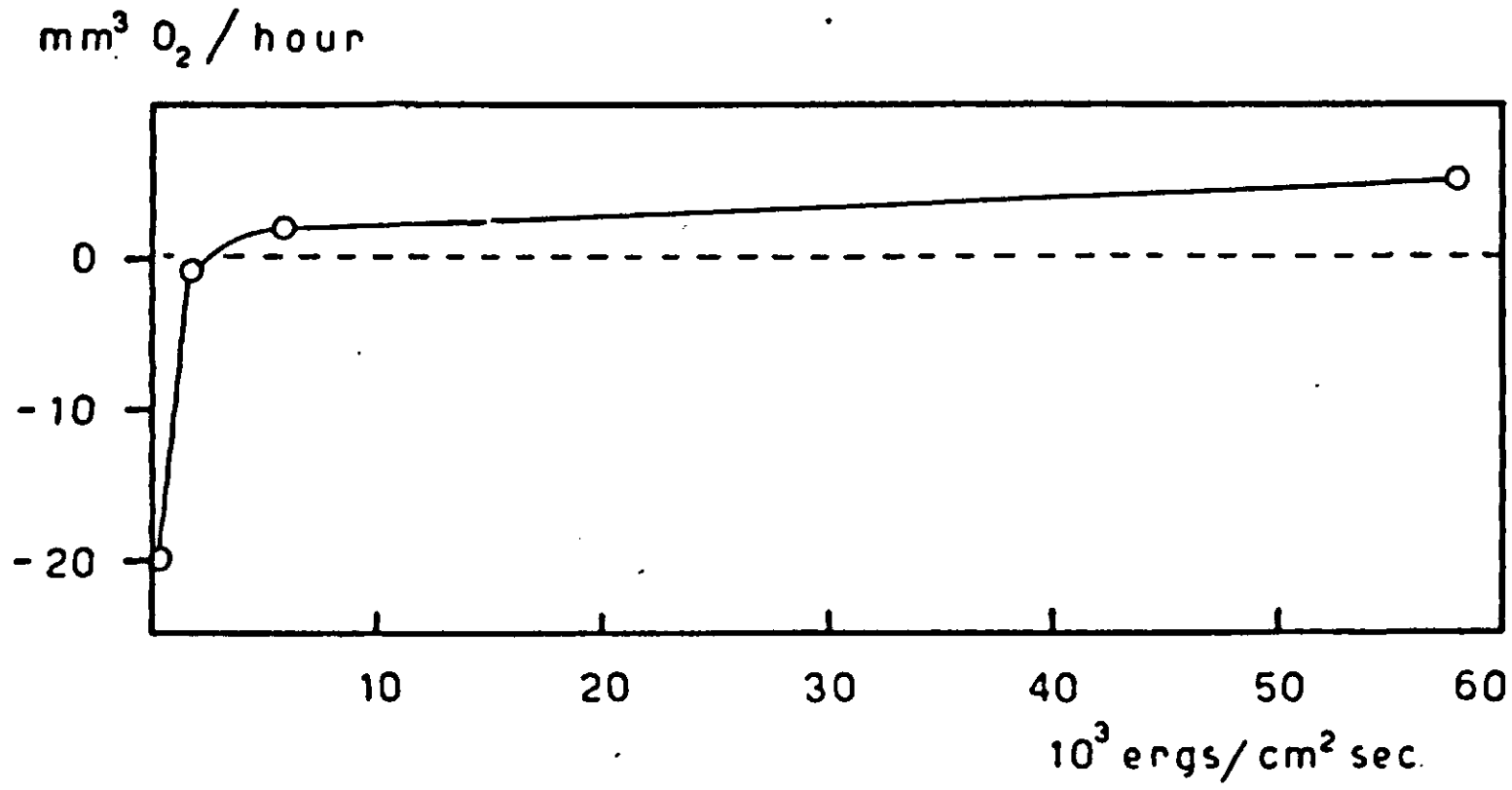


FIG. 7. Gas exchange of *Chlorella* in CO_2-free air, at different light intensities. ~ 20 mm³ cells/vessel; pH ± 4.0.

arm. Photosynthesis was measured at 25°C and calculated as CO_2 uptake, assuming the quotient $-O_2/CO_2 = 1.05$. — It may be remarked that omission of KOH did not perceptibly influence the results in experiments in which no CO_2 was added. The reason is that a vessel with a gas volume of about 16 ml, and 0.05% CO_2 contains less than 10 mm³ CO_2 at the start, which are removed by photosynthesis in a few minutes.

In a gas phase containing 5% CO_2, light saturation of photosynthesis was reached at intensities above 35,000 ergs/cm² sec (*cf. e.g.*, fig. 14). In air freed from CO_2, light saturation, measured as O_2 output, is already reached at about 5000 ergs/cm² sec (fig. 7).

Curves of phosphate fixation, determined under similar conditions in the absence of CO_2, show light saturation at about 13,000 ergs/cm² sec (fig. 8 and Table V). Since the rate of accumulation of polyphosphates declines after a relatively short time (1–3 hours), light saturation might depend on the duration of exposure. It could be supposed, namely, that a reservoir of restricted capacity

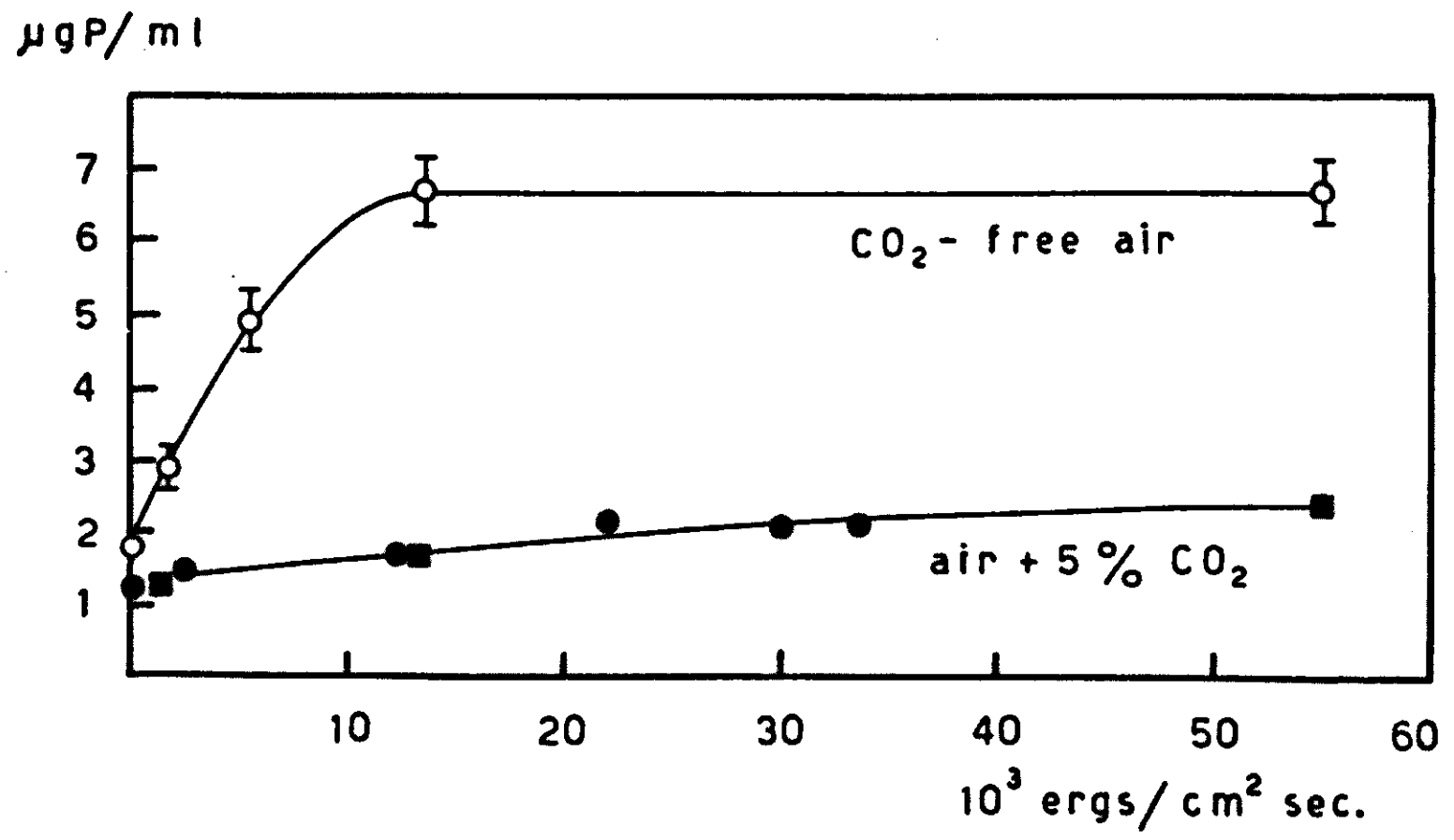


FIG. 8. Fixation of orthophosphate by *Chlorella* at different light intensities in the presence and in the absence of CO_2. pH ± 4.0; 25°C; ± 5 mm³ cells/ml; illumination for about 3 hours.

TABLE V

Decrease of orthophosphate in suspensions of *Chlorella* after about 3 hours in CO_2-free air at different light intensities. Data in μg P/ml; 25°C; pH 4.

Light intensity	μg P/ml converted	Number of observations	Differences between given and next lower light intensity	Number of paired observations
0 ergs/cm² sec . .	1.8 ± 0.1	12		
1,500 „ „ „ . .	2.9 ± 0.3	10	1.15 ± 0.25	10
5,000 „ „ „ . .	4.9 ± 0.45	12	2.3 ± 0.3	10
13,500 „ „ „ . .	6.7 ± 0.5	15	2.3 ± 0.45	9
55,000 „ „ „ . .	6.7 ± 0.45	18	0.03 ± 0.02	15

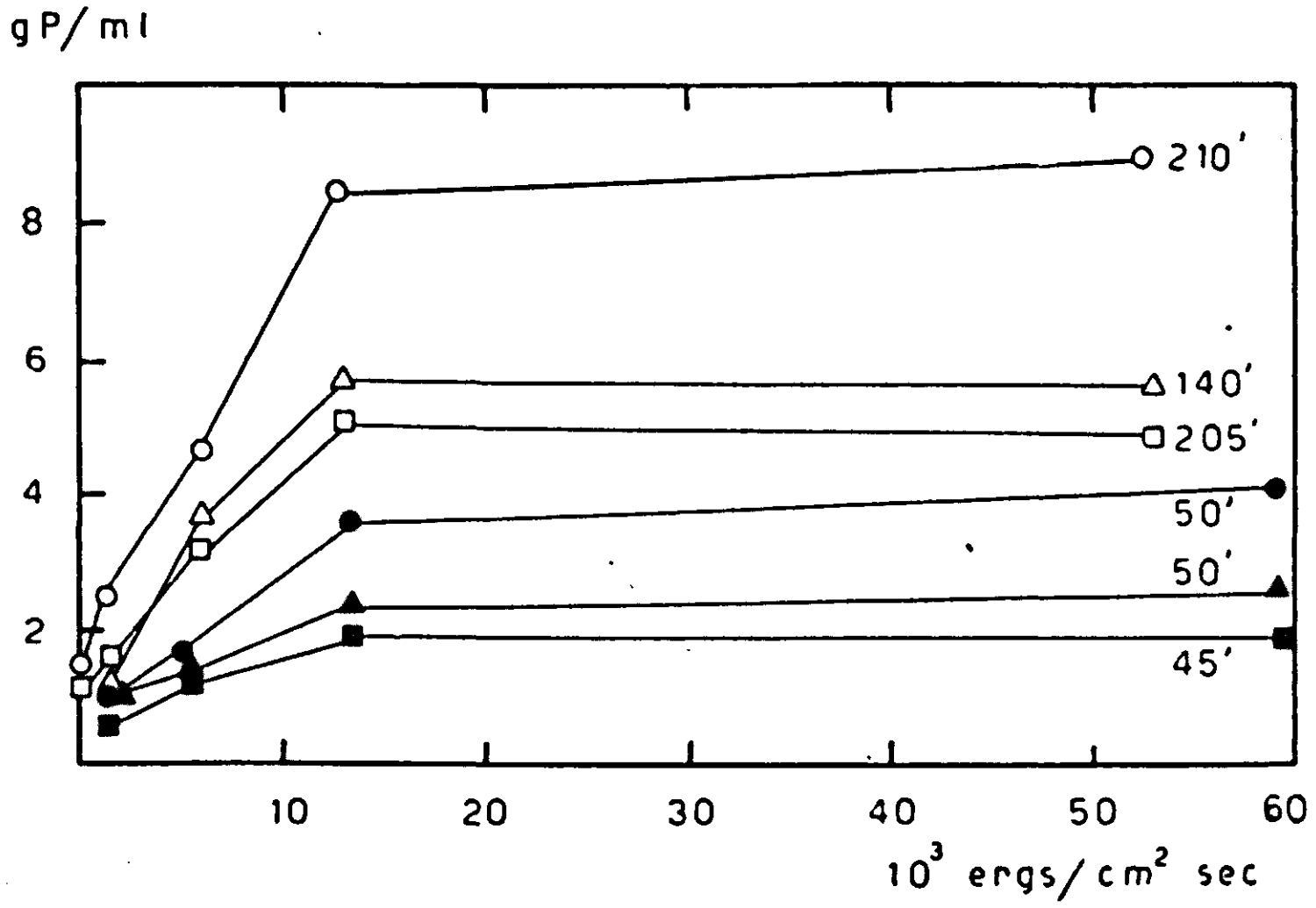


FIG. 9. Uptake of phosphate by *Chlorella* in CO_2-free air, as a function of light intensity, at various times of exposure. ~ 5 mm³ cells/ml; pH ± 4.0. Corresponding open and closed symbols belong to one experiment.

TABLE VI

Decrease of orthophosphate in suspensions of *Chlorella* after about 3 hours in air + 5% CO_2, at different light intensities. Data in μg P/ml; 25°C; pH 4.

Light intensity	μg P/ml converted	Number of observations	Experiment series nr.
0 ergs/cm² sec	1.2 ± 0.2	5	2
	1.2 ± 0.2	6	1
1,500 „ „ „	1.3 ± 0.25	5	2
2,500 „ „ „	1.5 ± 0.1	5	1
12,000 „ „ „	1.7 ± 0.2	6	1
13,500 „ „ „	1.7 ± 0.2	3	2
20,000 „ „ „	2.2 ± 0.15	6	1
30,000 „ „ „	2.1 ± 0.15	6	1
34,000 „ „ „	2.1 ± 0.15	6	1
55,000 „ „ „	2.4 ± 0.2	5	2

is filled during illumination, which would proceed more rapidly as the light intensity was increased, so that light saturation would shift towards lower light intensities with increasing duration of illumination. However, fig. 9 shows that this is not the case, light saturation being independent of the time of exposure.

With 5% CO_2 in air, the result is not very clear (fig. 8 and Table VI). Two series of experiments, made with a considerable interval of time, indicate either saturation at low light intensity (series 1) or at high intensity (series 2). However, the data from both series can be fitted into one graph without difficulty; the experimental errors weigh heavily on the small differences in this case, and no definite conclusion can be reached.

§ 2. THE EFFECT OF pH

Samples of a suspension prepared as usual were brought to the desired pH by dilute NaOH or H_2SO_4, then introduced into WARBURG vessels and illuminated in CO_2-free air. The results of a number of experiments are shown in Table VII and illustrated in fig. 10. It is evident that phosphate fixation depends

TABLE VII

The fixation of phosphate by *Chlorella* in light and CO_2-free air, at different pH values. Suspensions adjusted to desired pH with diluted NaOH or H_2SO_4.

Expt.	Duration, minutes	Initial P, µg/ml	Initial H_2PO_4', µg P/ml	Final P, µg/ml	Initial pH	Final pH	P converted, µg/ml
1	180	11.6	11.6	1.8	3.5	4.4	9.8
		12.9	12.9	3.2	4.3	–	9.7
		11.9	11.2	6.0	5.4	5.7	5.9
2	210	26.8	26.8	17.6	3.5	3.7	9.2
		30.2	30.2	20.8	4.4	4.4	9.4
		29.7	28.0	24.0	5.6	5.4	5.7
		26.4	8.0	25.5	7.4	6.4	0.9
3	180	50.5	50.5	45.7	3.3	4.0	4.8
		48.1	42.1	42.3	4.1	4.7	5.8
		50.8	47.0	47.3	5.5	5.4	3.5
		50.4	16.0	49.8	7.1	6.5	0.6
4	180	25.6	25.6	18.8	3.5	3.4	6.8
		25.6	10.2	26.0	7.0	7.4	–0.4
5	190	16.1	16.1	11.0	3.9	4.3	5.1
		16.1	7.0	15.3	6.9	7.1	0.8
6	180	12.0	12.0	6.8	3.6	–	5.2
		12.7	2.2	11.5	7.5	–	1.2

strongly on pH and is appreciable only in acid media. It is well known that photosynthesis shows no such pH dependence, and it seems somewhat puzzling that a photochemical production of ~ph should be so sensitive to the acidity of the external medium. However, WIAME (80) found a similar influence of pH on the accumulation of 'polyphosphorylated matter' in yeast. A maximum was found there at pH 5, whereas accumulation was zero at pH 8.3.

Some suggestions can be made in order to provide an explanation for this phenomenon:

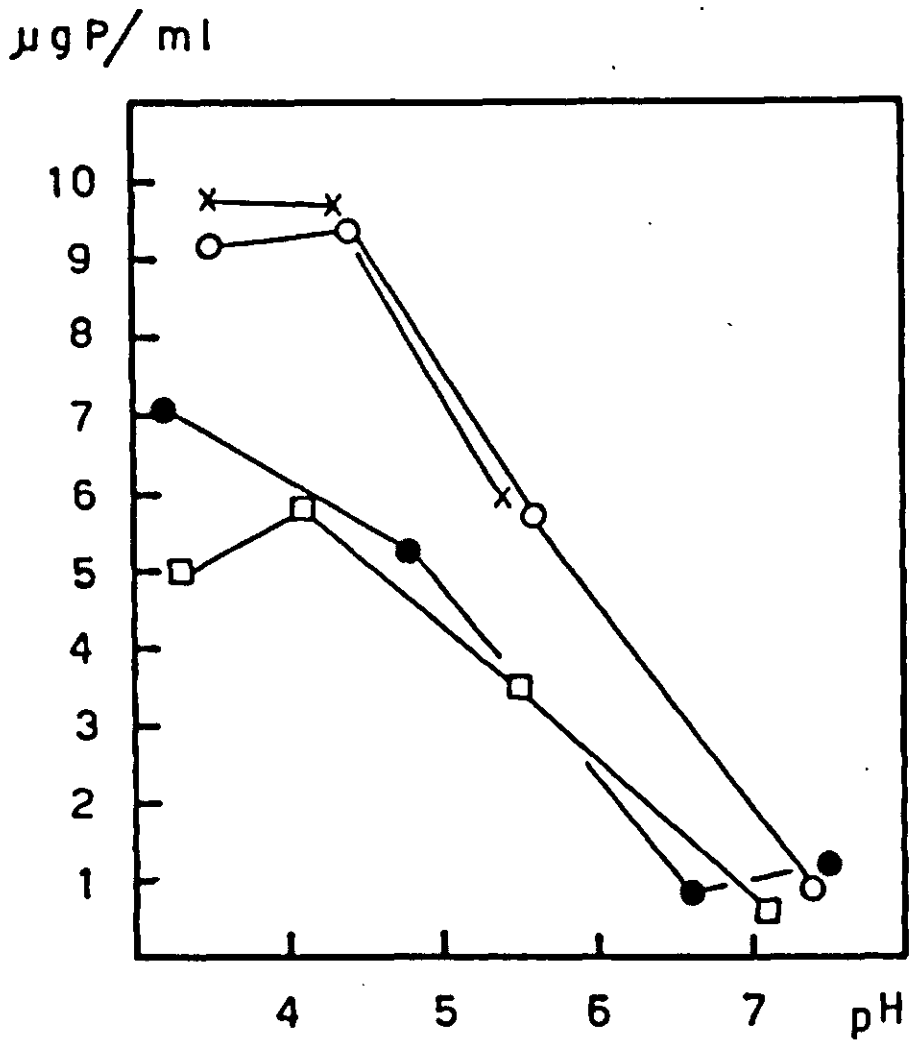


FIG. 10. Phosphate fixation in *Chlorella* in light, in CO_2-free air, as influenced by pH.

a. A preferent absorption of univalent over divalent ions (H_2PO_4' resp. HPO_4''). VAN DEN HONERT (27), working with *Saccharum* in water cultures, found the rate of phosphate absorption dependent on pH, increasing steadily from pH 7 to pH 4.5. He concluded that the rate of absorption is dependent on the concentration of H_2PO_4' ions, since for the ionization $H_2PO_4' \leftrightharpoons HPO_4''$, pK = 6.7, so that practically all phosphate will be present in the univalent form at pH 4.5, but only about 1/3 at pH 7. However, in our experiments, decreased phosphate fixation is already evident at pH 5.5, at which pH more than 90% of the phosphate still is present as H_2PO_4'. Moreover, an increase in the amount of phosphate so as to obtain a sufficient concentration of univalent ions even at higher pH values, did not remove the pH effect. So, *e.g.*, in expt. 3 of Table VII, at pH 7 from about 50 µg P/ml, 16 µg P/ml were present as H_2PO_4' at the start of the experiment. The uptake in 3 hours was only 0.6 µg P/ml, compared with 5.8 µg P/ml at pH 4.1. These experiments were repeated by J. ROMBACH in our laboratory with the same results. From this it appears that the ionic form of the phosphate cannot explain the pH effect.

The pH *vs.* phosphate uptake curves of fig. 10 remind of a curve recently published by SWANSON and WHITNEY (64). These authors established that the rate of absorption of labeled phosphate through the cuticle of bean leaves is

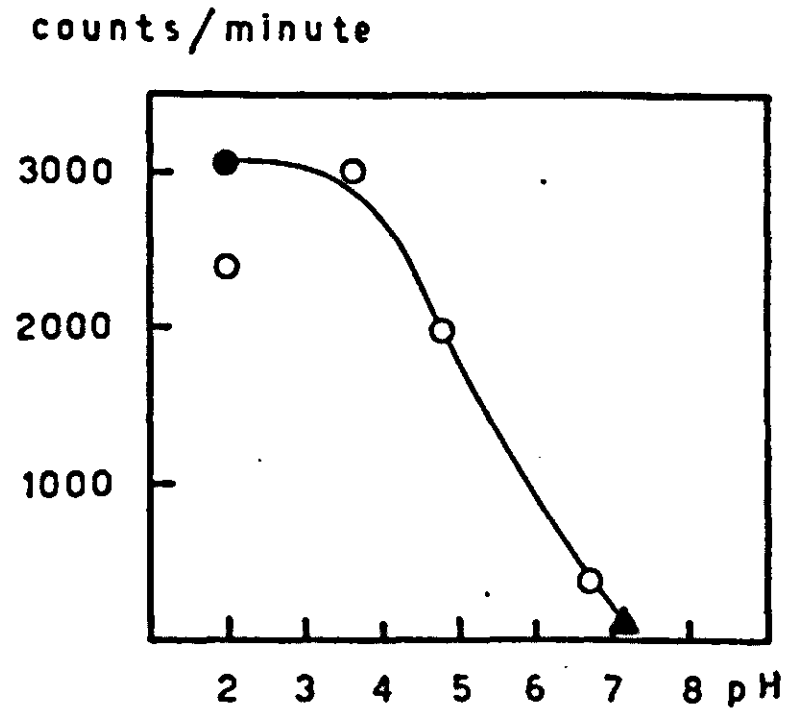


FIG. 11.
"Level of P^{32} activity found in the petiole" (of *Phaseolus vulgaris*) "following a 4-hr period of translocation from the blade as a function of pH of applied solution. Each point is the average of at least 3 plants. ○, ●, ▲; data taken from several experiments." From (64).

dependent on the pH of the applied drop. After absorption the tracer phosphate is transported downward. The rate of absorption and translocation was determined by measurement of radioactivity in the petiole after 4 hours. Fig. 11, reproduced from their publication, shows a pH dependence very similar to that of fig. 10; a marked decrease is visible already at pH 5, the absorption becomes zero at pH 7. As it may be assumed that the internal pH of the leaf is not influenced by the applied drop, any influence of pH on translocation is improbable, and only an effect on absorption is to be considered. Like in our case, the experimental curve and the dissociation curve of phosphate do not coincide. Also in the experiments of SWANSON and WHITNEY (64), therefore, a relation between uptake of phosphate and its ionic form, seems improbable.

b. A second possibility for explaining the effect of pH on phosphate accumulation is a direct effect on the permeability of the cell membrane. It is now generally assumed that the process of anion absorption requires respiratory energy. The necessary enzymes must be situated in the outer layer of protoplasm. An influence of pH on such frontier-layer enzymes does not seem impossible. In the mentioned article of SWANSON and WHITNEY (64), both possibilities as discussed sub *a* and sub *b* are considered in connection with the effect of pH on phosphate absorption.

Two other suggestions to explain the decreased formation of polyphosphates at higher pH may be mentioned.

c. Influence of pH on the transformation of a primary phosphorylation product to polyphosphate.

d. Increased activity of internal polyphosphatase at higher pH, resulting in breakdown of the formed compounds.

These explanations, however, imply that pH inside the cell is influenced by pH of the medium.

§ 3. THE EFFECT OF ANAEROBIOSIS

Effects of oxygen on the formation of energy-rich phosphates in the light have been reported. HOLZER (25) proposed a scheme for the participation of ~ph in photosynthesis, in which ATP was considered important. This compound was supposed to be generated by way of oxidative phosphorylation in connection with the oxidation of photochemically reduced TPN. Reduction of DPN and TPN by chloroplasts in the light has been actually demonstrated by TOLMAČH (66) and by VISHNIAC and OCHOA (68, 69). The last mentioned authors also observed the formation of ATP in illuminated suspensions containing chloroplasts, mitochondria and DPN, for which formation oxygen was necessary (69). ARNON *et al.* (3) demonstrated formation of ATP in illuminated chloroplasts, which was inhibited in the absence of oxygen. On the other hand, formation of ATP in the light by intact *Chlorella* cells as measured by STREHLER (62, 63) was independent of oxygen.

In connection with these observations it appears of interest to discuss some experiments in which we measured the uptake of phosphate upon illumination in normal air, and in nitrogen, both with and without CO_2. Suspensions of *Chlorella* were illuminated in glass cylinders, and flushed with the required gas mixture.

Nitrogen ('extra pure', from a steel flask) was freed from traces of oxygen by passing it over electrically heated copper gauze, followed by a bottle containing an alkaline solution of pyrogallol as a check, which showed only very slight darkening, even after 3 hours. In the presence

TABLE VIII

Phosphate fixation by *Chlorella* in light, in the presence or in the absence of oxygen; pH 4; 25° C; 2 hours light. Data in μg P/ml.

Expt. nr.	CO_2-free air	Nitrogen	Difference
1	3.0	3.4	0.4
2	3.7	4.0	0.3
3	2.8	3.3	0.5
4	5.5	6.3	0.8
5	4.0	4.5	0.5
6	5.8	6.4	0.6
Average	4.1 ± 0.5	4.65 ± 0.55	0.5 ± 0.07
Expt. nr.	Air + 5% CO_2	Nitrogen + 5% CO_2	Difference
7	1.3	1.3	0.0
8	1.6	1.8	0.2
9	1.7	1.7	0.0
10	2.6	2.6	0.0
Average	1.8 ± 0.3	1.85 ± 0.25	0.05 ± 0.05

of CO_2 this check, of course, had to be omitted, but we felt confident that the copper gauze acted properly also under these conditions.

Table VIII presents the results of some of these experiments. Variation between cultures on different days is rather great, as was often encountered. Phosphate fixation was found to be slightly greater in nitrogen than in CO_2-free air, whereas no difference appears between nitrogen + 5% CO_2, and air + 5% CO_2.

In another set of experiments phosphate fixation in the dark was especially studied. A difference was found between suspensions flushed with CO_2-free air and those flushed with nitrogen.

These experiments differed in some respects from the standard procedure. Cultures were grown in phosphate-free medium for the last 18–24 hours before the experiment. The harvested cells were resuspended in fresh, phosphate-free medium, and adjusted at various pH values, as indicated in Table IX. 'Extra pure' nitrogen was freed from traces of O_2 and CO_2 by passing it through a bottle containing: 15% KOH, 10% $Na_2S_2O_4$ (hydrosulphite) and 0.5% anthraquinone sulphonic acid ('silver salt'), followed by a sodium lime tube and a bottle containing an alkaline solution of pyrogallol as a check. Air was freed from CO_2 as usually (p. 74). Somewhat longer exposures were given in the hope of getting larger changes in phosphate levels; the results, however, were rather negative. The cells may have suffered from the phosphate starvation, since also fixation of phosphate in light, in CO_2-free air, was rather low.

Aerobically, phosphate was taken up at a slow rate in all experiments. In N_2, phosphate uptake was low, while at higher pH, the uptake was even replaced by a release of phosphate into the medium (Table IX). In one case pH was brought from 3.8 to 6.7 after 4 hours; in the aerobic sample, phosphate uptake then became very small, while in the anaerobic sample the uptake was replaced by a release. Phosphate conversion in dark thus seems to be inhibited at higher pH, just as was found in the light, as discussed in § 2 of this Chapter. However, the uptake of phosphate in dark requires oxygen, contrary to that in light. The fact that respiratory phosphorylation accounts for part of the phosphate fixed in air

TABLE IX

Phosphate fixation by *Chlorella* in darkness, in the absence of CO_2, in air or nitrogen. Data in μg P/ml.

Expt. nr.	Time in hours	Air	Nitrogen	pH
1	3	0.8	–0.8 [1])	6.2
	7	0.6	–1.7	
2	2	0.8	0.1	8.0
	5	1.1	–0.7	
3	2	0.2	–0.3	6.2
	5½	0.9	0.0	
4a	2	0.5	0.2	3.8
	4	1.7	1.0	
4b [2])	17½	0.6	–1.4	6.7

[1]) The negative sign indicates release of phosphate.
[2]) Experiment 4b was a continuation of 4a after pH had been brought to 6.7. Phosphate fixation in 4b is relative to the beginning of the 17½ hour period at pH 6.7.

strengthens the results of Table VIII, in which light-induced phosphate fixation appears to be favoured in the absence of oxygen.

In some further experiments cells were analyzed to see whether polyphosphates could act as phosphate storage. Suspensions were made as usually and illuminated for 3 hours in CO_2-free air, to bring polyphosphates at a high level. Thereafter, half of the cells were analyzed, the remainder of the suspension was brought to a pH of 6–7 and flushed overnight with nitrogen in darkness. For analysis TCA extraction was applied, followed by removal of the lipids and alkaline extraction, as described on p. 82. As could be expected, the changes in the phosphate fractions were small. After the cells had been in dark for 18 hours under a gas phase of nitrogen, the TCA extract showed an increase in orthophosphate of 1.1 ± 0.3 μg P/ml ($n = 5$), while the $\triangle$ 7 min. labile phosphate in the NaOH extract had decreased by about 1.0 ± 0.3 μg P/ml ($n = 3$). It thus seems that the increase in orthophosphate originates from polyphosphates which constitute the greatest part of the labile, NaOH soluble phosphate.

These observations are comparable with the finding of WIAME (82) that in yeast, the (acid-insoluble) polyphosphates disappear during growth in the absence of phosphate. Moreover, STICH, by microscopical observation, found that polyphosphates in *Acetabularia* disappear when the cells are kept in darkness for long periods (60).

§ 4. THE INFLUENCE OF NITRATE

It has been variously claimed that nitrate can be reduced in a photochemical process. BURSTRÖM, *e.g.* found that in *Triticum* leaves nitrate assimilation is dependent on light and on the presence of CO_2 (7). The energy necessary for this assimilation cannot be supplied by respiration. BURSTRÖM supposes that also in *Chlorella* CO_2 and nitrate are assimilated simultaneously to proteins or their precursors. However, *Chlorella* can assimilate nitrate also in darkness, in which

case, obviously, the energy is supplied by respiration (8). KOK (36) found that a release of oxygen by *Chlorella* in the light could be observed in the presence of nitrate, even when no CO_2 was present. When nitrogen was given only in the ammonium form, no net oxygen production ever occurred in the absence of CO_2. In this he saw an argument for the occurrence of a photosynthetic assimilation of nitrate, parallel to the assimilation of CO_2. KANDLER (30) proposed a scheme in which ~ph is generated in the light, and is available equally for the reduction of CO_2 and of nitrate. Also VAN NIEL *et al.* (49) conclude that nitrate is a hydrogen acceptor in the light, alternative to CO_2. DAVIS (10) however, found relations more complicated in *Chlorella*. In CO_2-free air, oxygen was produced in the light when KNO_3, glucose and phosphate were present. About 2 moles of O_2 were produced per mole of assimilated nitrate. The participation of respiratory energy in the reduction of nitrate, and subsequent assimilation of respiratory CO_2 were proposed as an explanation.

TABLE X

Phosphate fixation by *Chlorella* in light, as influenced by nitrate. pH 4; 25°C. Data in μg P/ml, fixed in 3 hours.

Expt.	No nitrate	1 m Mole KNO_3/l	Difference
1. CO_2 absent . . .	7.5	6.6	0.9
2. ,, ,, . . .	5.3	7.8	–2.5
3. ,, ,, . . .	5.9	6.3	–0.4
4. ,, ,, . . .	4.1	3.8	0.3
Average	5.7	6.1	–0.4 ± 0.75
5. CO_2 present . . .	2.1	2.3	–0.2
6. ,, ,, . . .	2.6	1.5	1.1
7. ,, ,, . . .	3.2	3.2	0.0
8. ,, ,, . . .	2.0	1.5	0.5
9. ,, ,, . . .	3.3	1.8	1.5
10. ,, ,, . . .	2.9	2.9	0.0
11. ,, ,, . . .	2.8	2.0	0.8
12. ,, ,, . . .	2.0	2.7	–0.7
Average	2.6	2.25	0.4 ± 0.25

With respect to these findings, nitrate could be supposed to interfere with the accumulation of polyphosphates in the light in the same way as CO_2. In order to check this possibility, some experiments were made in which KNO_3 was added to the suspension medium. Nitrate, in concentrations up to 10^{-3} molar had no influence on the rates of respiration or photosynthesis in air + 5% CO_2. In the absence of CO_2, small positive readings (gas evolution) were observed in the light, both in the presence and in the absence of nitrate (*cf.* fig. 7). Phosphate fixation was measured in light, with and without CO_2. No significant effect of nitrate was found, as can be seen in Table X. In another set of experiments, 10^{-2} molar KNO_3 was given. In this case, respiration, and oxygen production in the light were somewhat stimulated by this nitrate supply. In some cases, cellular nitrogen was determined with a modified KJELDAHL method. In the nitrate-free controls, the cells lost 10–15% of their nitrogen in about 3 hours. In the pre-

sence of 10^{-2} molar KNO_3, no such loss occurred, or some rise in nitrogen content of the cells was observed. Differences in nitrogen metabolism were also indicated by a tendency of pH to rise strongly in the presence of nitrate. However, there was no influence of nitrate on the accumulation of polyphosphates in the light, provided pH was properly controlled. These results, therefore, fail to show an influence of nitrate on the studied phosphate conversions.

§ 5. THE INFLUENCE OF GLUCOSE

Some results concerning the influence of glucose on the fixation of phosphate by *Chlorella* have already been published from our laboratory (79). The results are briefly repeated in Table XI, and illustrated in figs. 12 and 13. Glucose diminishes the amount of phosphate fixed in the light, both in the presence and in the absence of CO_2. This shows that the observed effect is not due to the extra respiratory carbon dioxide, produced during the conversion of the glucose. No

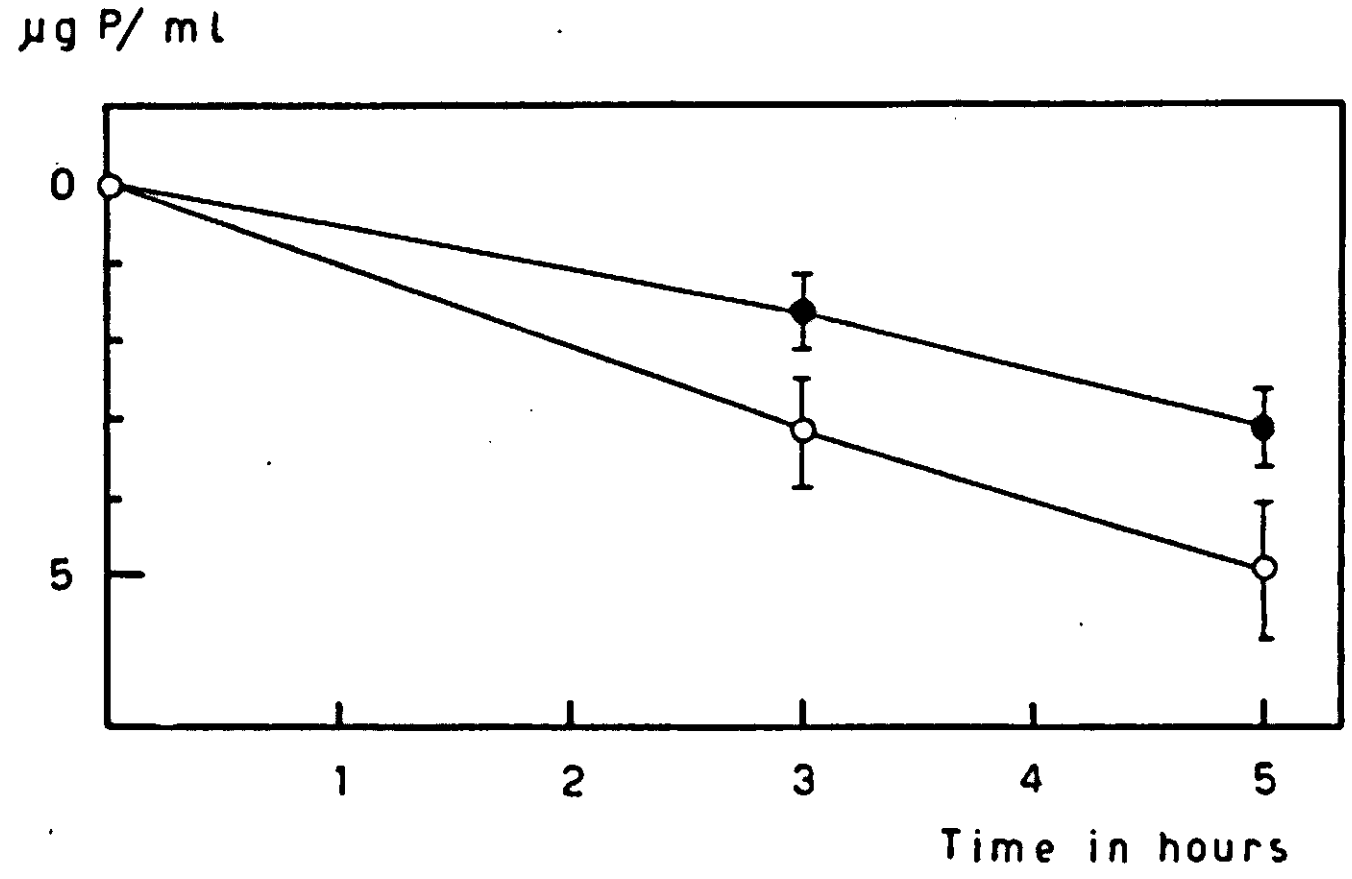


FIG. 12. Decrease in orthophosphate of suspensions of *Chlorella* in CO_2-free air, in the presence (●) and in the absence (○) of 0.2% glucose. ~ 4 mm³ cells/ml; pH ± 4.0. Average of 5 experiments. From (79).

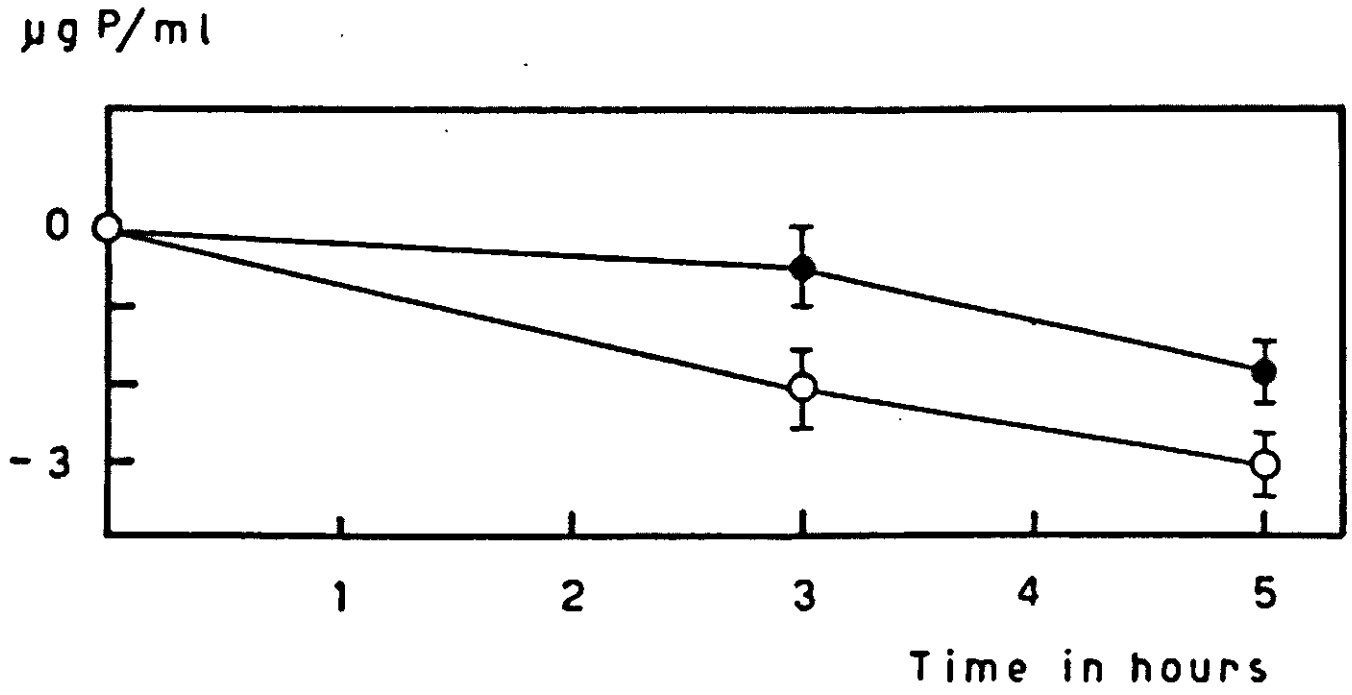


FIG. 13. Decrease in orthophosphate of suspensions of *Chlorella*, in air + 5% CO_2, in the presence (●) and in the absence (○) of 0.2% glucose. ~ 4 mm³ cells/ml; pH ± 4.0. Average of 5 experiments. From (79).

TABLE XI

Phosphate fixation in *Chlorella* as influenced by 0.2% glucose, in light and in darkness, in the presence and absence of CO_2. pH 4; 25° C. From (79).

Treatment	P converted, μg P/ml in:		Number of experiments
	3 hours	5 hours	
1. Light; CO_2 free-air . .	3.2 ± 0.7	5.0 ± 0.9	5
2. As 1; + glucose . . .	1.7 ± 0.5	3.2 ± 0.5	5
3. Difference; row 1–2 .	1.55 ± 0.3	1.8 ± 0.4	
4. Light; air + 5% CO_2 .	2.1 ± 0.5	3.1 ± 0.4	5
5. As 4; + glucose . . .	0.5 ± 0.5	1.9 ± 0.4	5
6. Difference; row 4–5 .	1.6 ± 0.4	1.2 ± 0.3	
7. Dark; CO_2 free-air . .	0.0 ± 0.4	0.4 ± 0.4	3
8. As 7; + glucose . . .	–0.2 ± 0.4	0.0 ± 0.6	3
9. Dark; air + 5% CO_2 .	0.5 ± 0.1	0.7 ± 0.2	3
10. As 9; + glucose . . .	1.0 ± 0.4	1.2 ± 0.4	3

influence of glucose on the phosphate fixation in darkness was observed.

The major products of phosphate fixation in light, in the absence of CO_2, were identified as polyphosphates only after the experiments on the effects of glucose had been made. Its influence appears to be quite opposite to what has been found with polyphosphate formation in yeast, for which the necessity of a respirable substrate, *e.g.* glucose, providing the energy for the phosphate bonds, is stressed by all authors (54, 82, 87). Apparently, in *Chlorella* the respiration of glucose does not build up a sufficient concentration of ~ph to allow an appreciable synthesis of polyphosphates. It may be supposed, instead, that glucose competes with the polyphosphate forming system for ~ph generated in the light, just as has been suggested for carbon dioxide. In this connection it is of importance that glucose is not completely respired by *Chlorella*, but that it is largely transformed into cellular material. This conversion is very efficient; according to MYERS, 5/6 of the available glucose is assimilated, the rest is respired (47). Also KANDLER (31) observed high efficiencies for glucose assimilation by *Chlorella*. These observations support the above conclusion that the respiration of glucose does not result in an important concentration of ~ph available for polyphosphate synthesis. If glucose acts as an acceptor of photochemically produced ~ph, there might be an influence of light on the assimilation of glucose by *Chlorella*. MYERS found that the time, required for the assimilation of a limited quantity of glucose was independent of the light (47). KANDLER, however, has observed that assimilation of glucose by starved *Chlorella* cells was accelerated in the light (31). He supposes that in starved cells the rate of glucose assimilation was limited by lack of ATP, which is required for the hexokinase reaction. To a certain degree, this shortage could be overcome by light. Furthermore, the conversion of glucose was slightly depressed in the presence of CO_2, indicating a competition for light energy, or ATP, between glucose conversion and CO_2 assimilation. Whereas dark assimilation of glucose was strongly depressed in the absence of oxygen, in the light this depression was largely overcome. This can only mean that no oxygen is required for the formation of ATP in the light. These observations are well in agreement

with our findings concerning the effects of glucose, CO_2 and O_2 on the formation of polyphosphates in the light. We may, therefore, accept that glucose is phosphorylated, *e.g.* by ATP, formed in the light. Probably polysaccharides or other phosphate-free products are formed from the glucosephosphates. Hence the assimilation of glucose is competitive of the fixation of phosphate since it tends to keep phosphate in 'circulation'.

CHAPTER V

THE INFLUENCE OF INHIBITORS

§ 1. PHENYLURETHANE

The inhibitory effect of the urethanes on photosynthesis is known since long (WARBURG, [72]). The most active of these compounds is phenylurethane. It was assumed that the urethanes act by virtue of their surface activity, covering the surface of enzymes, and thus blocking the access of substrates or reaction intermediates, an action called narcotization.

In general the narcotics are not very specific enzyme inhibitors. Photosynthesis is inhibited by phenylurethane to about the same degree at all light intensities (72). This indicates inhibition of a photochemical reaction, and of a dark reaction responsible for rate limitation at high light intensities (50, 72). Respiration of *Chlorella* is less sensitive to phenylurethane than photosynthesis, and is even stimulated by concentrations of the inhibitor which already strongly depress photosynthesis. This shows that some degree of specificity must be ascribed to phenylurethane. Very little was known about the influence of phenylurethane on the phosphate metabolism. In the experiments to be described, we investigated the influence of phenylurethane upon the fixation of phosphate in darkness and light, and upon the gas exchange, in the presence and in the absence of CO_2.

In view of the slight solubility of phenylurethane in water, it was found convenient first to dissolve the phenylurethane in a small quantity of ethanol, and to use this solution for making the adequate dilutions in aqueous media. In the experiments to be described, the inhibitor solution consisted of: 33 mg phenylurethane, 1 ml ethanol + 99 ml suspension medium (p. 73). The control solution contained a similar amount of ethanol. Equal amounts of inhibitor and control solution were added to comparable samples of a dense suspension of *Chlorella* so as to obtain comparable conditions, and the desired concentration of inhibitor. In most experiments the concentration of ethanol was 0.6%. The effect of ethanol on metabolism was not studied specifically, since no experiments were made in which ethanol was applied as the variable factor for comparison with an ethanol-free control. The measurements of photosynthesis and phosphate fixation which have been compared were made, however, within a short time, using cells cultivated and treated in the same way. It is felt justified, therefore, to compare the results, and the following conclusions can be drawn from the data obtained: 1) Ethanol considerably stimulates respiration, which is contrary to the finding of MYERS (47) who reported no influence. 2) No significant differences were found between the rates of photosynthesis in suspensions with and without ethanol, measured at various light intensities. 3) The fixation of phosphate in CO_2-free air, measured at a series of light intensities, was not significantly different in suspensions with and without ethanol (*cf.* the control lines of figs. 15 and 27, and the corresponding data in Tables XII and XIV). It thus seems justified to neglect the influence of ethanol on the results of the experiments concerning the influence of phenylurethane.

Photosynthesis, measured at pH 4, with air + 5% CO_2 as a gas phase, was found to be inhibited by phenylurethane in concentrations between 10^{-4} and 10^{-3} molar, in accordance with WARBURG's findings (71). The fixation of phos-

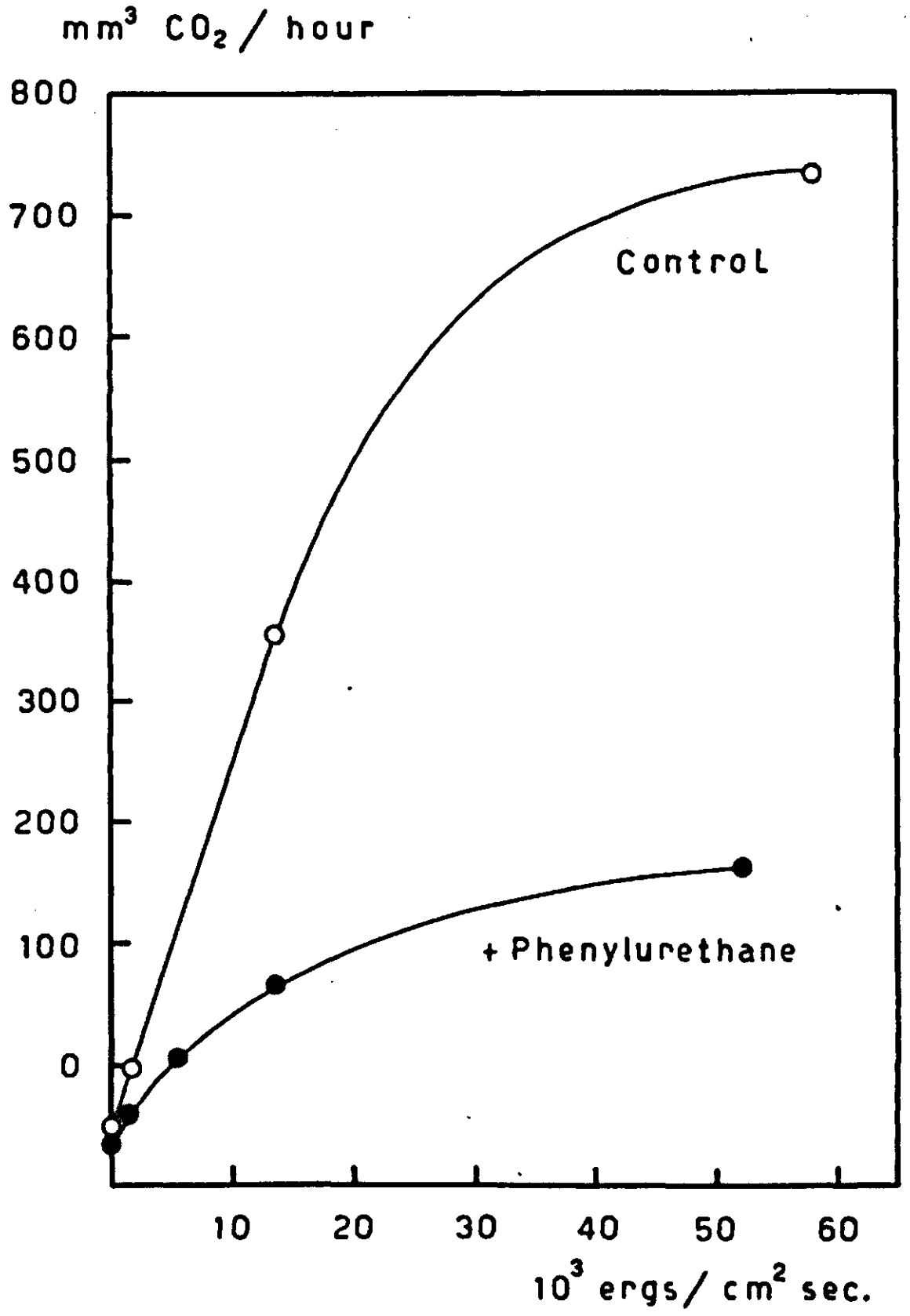


FIG. 14. Photosynthesis of *Chlorella* at various light intensities, in the presence (1.2×10^{-3} molar) and in the absence of phenylurethane. 20 mm³ cells/ vessel. pH 4.0; 25°C; air + 5% CO_2. Rate of photosynthesis calculated, assuming the ratio $-O_2/CO_2 = 1.05$.

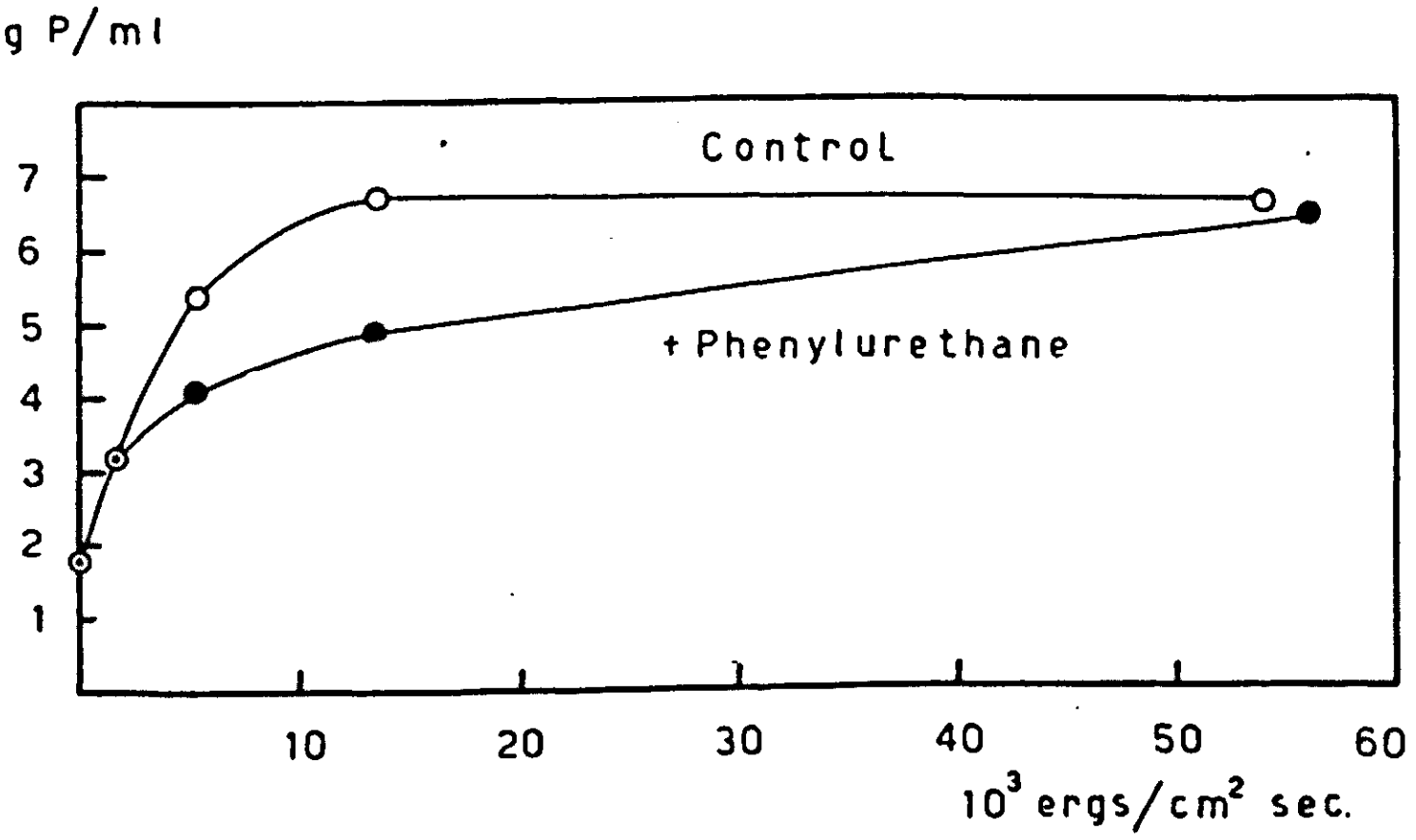


FIG. 15. Phosphate fixation by *Chlorella* in CO_2-free air, at various light intensities, as influenced by phenylurethane (1.2×10^{-3} molar). 5 mm³ cells ml; pH ± 4.0.

phate in CO_2-free air was much less sensitive to phenylurethane than photosynthesis. Moreover, phosphate fixation and photosynthesis differed in their dependence on light intensity. Fig. 14 shows the influence of 1.2×10^{-3} molar phenylurethane on respiration and photosynthesis at various light intensities. The inhibition of photosynthesis is about 75% at all light intensities. Fig. 15 shows phosphate fixation in CO_2-free air at the same light intensities and with the same concentrations of phenylurethane as used for the measurements of photosynthesis of fig. 14. A moderate degree of inhibition is apparent at intermediate light intensities only. In dark, and at low light intensities no inhibition was found. Light saturation was shifted to higher light intensities; at about 55,000 ergs/cm² sec the same rate of phosphate fixation was reached as in the control. From Table XII the degree of significance of the observed differences may be seen.

TABLE XII

The fixation of phosphate by *Chlorella* in CO_2-free air, at different light intensities, in the presence and absence of phenylurethane. pH 4; concentration of inhibitor: 1.2×10^{-3} molar. All suspensions contained 0.6% ethanol. Data in µg P/ml, fixed in 3 hours.

a	b	c	d	e	f
Light, ergs/cm² sec.	Control, µg P/ml fixed	Phenylurethane, µg P/ml fixed	Difference (columns b–c)	Number of observations	% of Control
0	1.8 ± 0.1	1.8 ± 0.1	0.0 ± 0.15	6	100
1,500	3.2 ± 0.35	3.2 ± 0.2	–0.05 ± 0.2	6	100
5,000	5.4 ± 0.6	4.1 ± 0.35	1.3 ± 0.35	6	76
13,500	6.7 ± 0.6	4.9 ± 0.35	1.75 ± 0.5	12	75
55,000	6.6 ± 0.6	6.5 ± 0.4	0.1 ± 0.4	12	98

In a few experiments the influence of phenylurethane on phosphate fixation was determined in the presence of 5% CO_2. Under these conditions only very small changes in phosphate level were observed (fig. 16). In dark, and at low light intensities the accuracy of the determination of phosphate is of the same order of magnitude as a possible effect of the inhibitor. Only at the highest light intensity phosphate fixation is definitely inhibited. Therefore, the sensitivity to phenylurethane of phosphate fixation in relation to light intensity seems to be different in the presence and in the absence of CO_2. It has been remarked that the phosphate compounds formed in the presence of CO_2 differ, at least partly, from those formed in its absence, as was concluded from their difference in

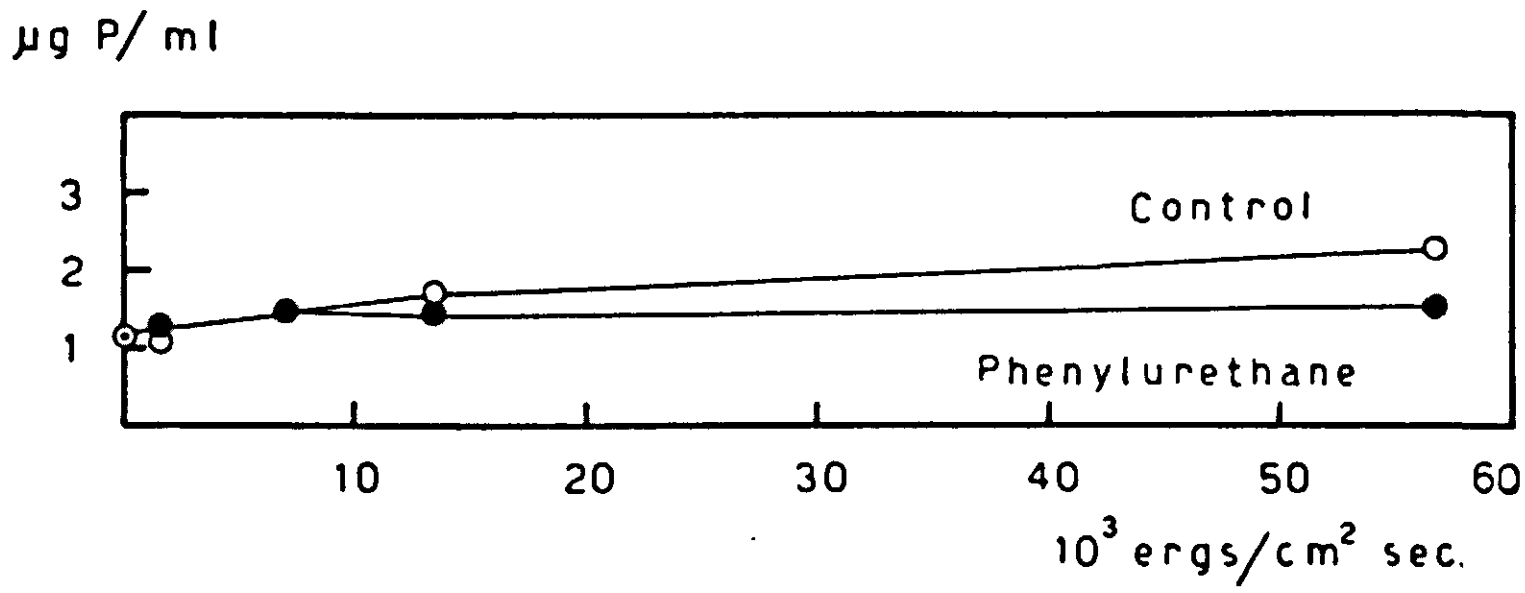


FIG. 16. Phosphate fixation by *Chlorella* in air + 5% CO_2, at different light intensities, as influenced by phenylurethane (1.2×10^{-3} molar). 5 mm³ cells/ml; pH ± 4.0.

stability during extraction with hot, 1 N HCl (Chapter III, § 1, and [86]). The observations on the effect of phenylurethane also point to a difference in the processes of phosphate fixation in the presence and absence of CO_2. For further discussion, see Chapter VI, § 1.

§ 2. 2–4 DINITROPHENOL

Dinitrophenol (DNP) is of interest in the scope of the present investigation, because it is known to inhibit phosphorylations, notably the formation of ATP, accompanying respiration. It is remarkable that the uptake of oxygen, and the release of carbon dioxide are not affected to the same degree. The gas exchange can be stimulated even at concentrations of DNP which strongly inhibit phosphorylation. This 'uncoupling' action was first described by LOOMIS and LIPMANN (40). Moreover, DNP is known to affect photosynthesis. GAFFRON (21) found that concentrations of DNP inhibiting respiration, also inhibit photosynthesis in *Chlorella*. Small concentrations which stimulated respiration, however, had no influence on photosynthesis. GAFFRON, therefore, concluded that the photosynthetic and respiratory systems are independent. HOLZER (25) observed that photosynthesis in *Chlorella* was already inhibited by concentrations of DNP which stimulate respiration. He suggests that, primarily, uncoupling of phosphorylation from oxidation occurs, so that the reduction of CO_2 is limited by lack of ATP. HOLZER also made observations on the products of phosphate metabolism in *Chlorella* in light. Metaphosphate, partly soluble in cold, partly in hot TCA, was found. The uptake of phosphate in light was very small, and no influence of light on the amount of TCA-soluble metaphosphate in the cells appeared (25). This is not in contradiction to our results, since in HOLZER'S experiments, at pH 8.3, in the presence of CO_2, conditions were not suitable for the accumulation of metaphosphate. HOLZER made no observations on phosphate metabolism in the presence of DNP.

Concerning the influence of DNP on metaphosphate metabolism the following reports are available. YOSHIDA (87) found that in yeast, formation of metaphosphate was inhibited by concentrations of DNP which did not inhibit respiration. STICH and GRELL (61) found that the formation of metaphosphate in *Acetabularia* in the light, is inhibited by DNP.

The experiments to be reported concerning the influence of DNP may be divided into four parts:

a. The effects of various concentrations of DNP on photosynthesis, and the influence of pH.
b. The effects of DNP on phosphate fixation in the light, and the influence of pH.
c. The effect of DNP on photosynthesis at various light intensities.
d. The effect of DNP on phosphate fixation at various light intensities.

a. The effects of various concentrations of DNP on photosynthesis, and the influence of pH

Photosynthesis was measured with the WARBURG technique, at 25 °C, with air + 5 % CO_2 as a gas phase, at pH about 4. Photosynthesis appeared very sensitive to DNP at low pH, so that concentrations between 10^{-6} and 10^{-5} molar had to be applied. It had been our intention to measure photosynthesis, and the photosynthetic quotient, with the two-vessel method, but very deviating results were obtained when WARBURG's formula was applied, as will be discussed below.

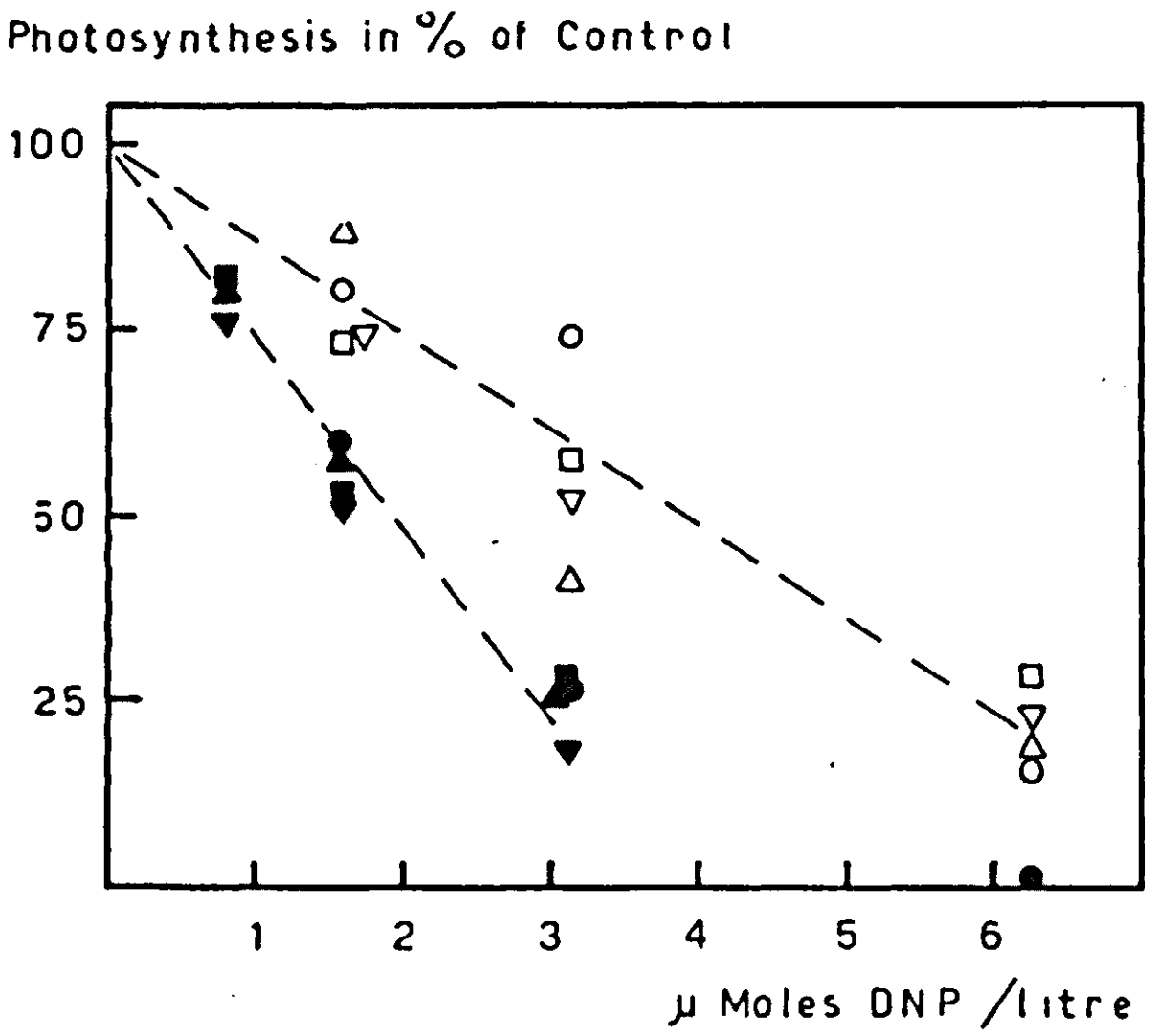


FIG. 17. The effect of DNP in different concentrations (μ Moles/litre) on photosynthesis of *Chlorella*. pH 3.8–4.0; air + 5% CO_2. Photosynthesis calculated, assuming the ratio $-O_2/CO_2 = 1.05$, and plotted in % of control. 20 mm³ cells in 3 ml (open symbols) or 6 ml fluid respectively (black symbols).

Therefore, the gas exchange has been calculated for each vessel under the assumption that the ratio $-O_2/CO_2 = 1.05$. Data obtained in this way are given in fig. 17 for two parallel sets of observations, differing only in the volumes of suspension, which were 3 and 6 ml, respectively. It will be noted that inhibition at a given concentration of DNP is stronger with the larger v_F than with the smaller. With a concentration of DNP = 3.1×10^{-6} molar, pH 4.0, 20 mm³ cells/vessel, at 25 °C we observed for:

vessel 3 (v_F = 3.035 ml; v_G = 18.86 ml): h = 53 mm/hour
vessel 2 (v_F = 6.07 ml; v_G = 15.58 ml): H = 31 mm/hour

Calculating photosynthesis from these data with the two-vessel formula, we find: O_2 = –250 mm³/hour (uptake!) and CO_2 = + 385 mm³/hour (release!). In the same experiment, concentration DNP = 6.2×10^{-6} molar, gave:

vessel 9 (v_F = 3.07 ml; v_G = 18.78 ml): h = 14 mm/hour
vessel 8 (v_F = 6.14 ml; v_G = 13.76 ml): H = –6 mm/hour.

Vessel 8 gave negative readings, and at the end of the experiment the cells were brownish, apparently dead, in contrast with those in vessel 9 which had remained normally green in the presence of the same concentration of DNP as in vessel 8. An explanation may be found in the difference in total amount of DNP in the two vessels. When, namely, the cells would absorb all or most of the DNP present, the total amount of DNP per vessel would determine the activity of the inhibitor instead of its concentration. In the given case this would mean that a 'valid' vessel pair would be nr. 9 and nr. 2, both containing 3.5 μg DNP/vessel. Calculating photosynthesis for this pair of vessels, we obtain: O_2 = 177 mm³/hour, and CO_2 = –173 mm³/hour. These are acceptable figures in relation to those found in the pair of control vessels, *viz.* O_2 = 490 mm³/ hour, and CO_2 =

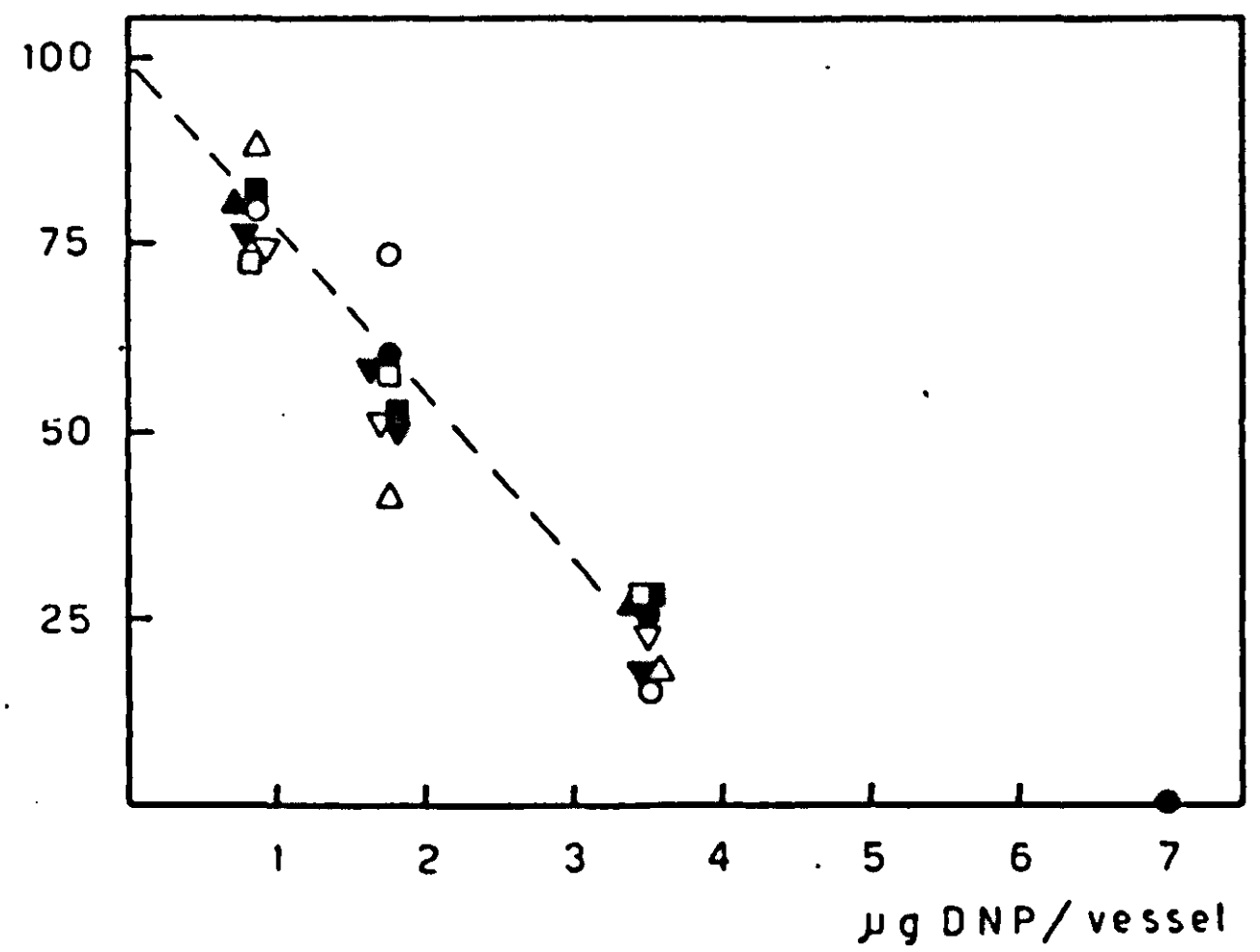


FIG. 18. The effect of DNP, in µg/*vessel* on photosynthesis of *Chlorella*. Same data and symbols as fig. 17.

–415 mm³/hour In fig. 18, the data of fig. 17 are given again, plotted against the amount of DNP/vessel. These data support the suggestion that in these experiments DNP was quantitatively absorbed by the cells.

An important factor in experiments on the biological activities of weak electrolytes, such as DNP, is the pH of the medium. Both GAFFRON (20) and HOLZER (25) draw attention to this point in relation to the effect of DNP on photosynthesis. Generally, it has been found that weak acids are more active in acid media than in neutral or alkaline ones. A general discussion of the phenomena is given by SIMON and BEEVERS (55).

Several experiments were made, in which photosynthesis was measured as influenced by DNP at different $H^{\cdot}$ concentrations. As expected, the percentage of inhibition decreased as pH increased. The amount of DNP was kept constant at 1.75 µg/*vessel*. Results are shown in fig. 19. Especially at pH below 4, it is again apparent that, with this way of dosage the volume of the suspension, v_F, has little influence on the degree of inhibition. At higher pH, however, there seems to be some separation between the open circles and the dots (representing $v_F = 3$ and $v_F = 6$ ml respectively), which indicates that here the concentration of the inhibitor is important. So at pH 4.5, where inhibition is still appreciable, the groups of points ('A' and 'B') do not coincide. At pH above 5 inhibition is slight, if any, so only small differences are to be expected.

In the mentioned article of SIMON and BEEVERS (55) several explanations for the effect of pH on the biological activity of weak electrolytes are discussed. HOLZER (25) *e.g.*, has supposed that activity would be due to the undissociated molecules only, because only these would be able to penetrate into the cells. With the formula $\log \frac{\alpha}{1-\alpha} = \text{pH} - \text{pK}$ (α = degree of dissociation), and assuming pK for DNP to be 4.0 (29), we can calculate the quantity of molecular DNP at any pH. Plotting photosynthesis against the amount of molecular DNP/vessel we obtain fig. 20, which contains the data of figs. 18 and 19. The divergence

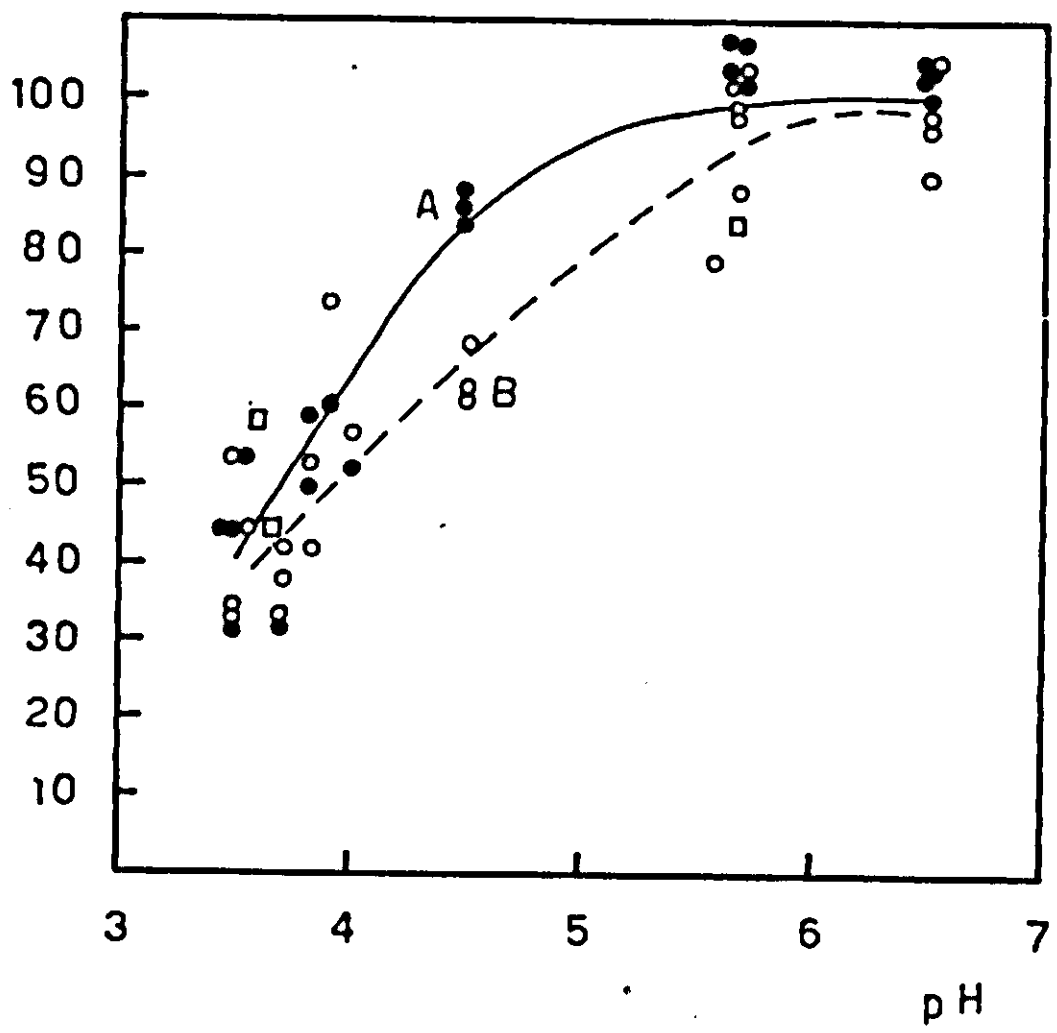


FIG. 19. Photosynthesis of *Chlorella* in the presence of 1.75 µg DNP/vessel containing 20 mm³ cells at various pH values. Photosynthesis in % of control. ○: v_F = 3 ml; □: v_F = 4 ml; ●: v_F = 6 ml.

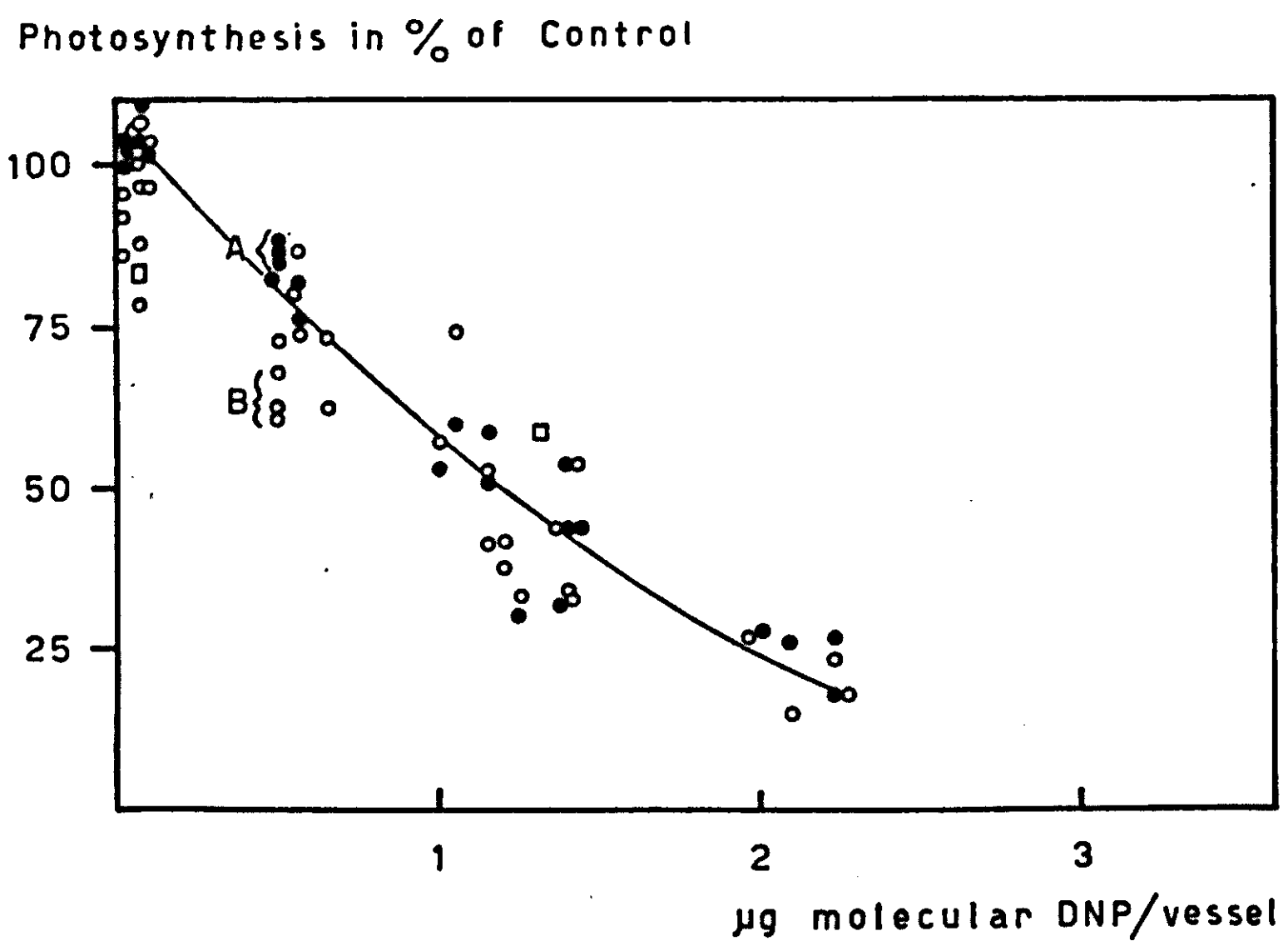


FIG. 20. Photosynthesis as influenced by DNP; 20 mm³ cells/vessel. Abscissa: amount of undissociated (molecular) DNP/vessel (pK = 4.0). Experiments with various doses of DNP (fig. 18), and those at various pH values (fig. 19) put together. Symbols as in fig. 19.

between the groups 'A' and 'B' remains (*cf.* fig. 19). The data given by HOLZER (25) do not fit into this figure. In his experiments 7×10^{-3} molar DNP at pH 8.3 caused 90% inhibition with about 20 mm³ cells in 2 ml suspension; at pH 9.2 inhibition was 62%. Total DNP = 2600 µg/vessel. pH – pK = 4.3; the ratio

ions/molecules is 20,000, so there is 0.13 μg molecular DNP/vessel at pH 8.3. For pH 9.2 we calculate: ions/molecules = antilog 5.2 = 160.000, and 0.016 μg molecular DNP/vessel. This points to a greater efficiency of free molecules at higher pH, or to an additional effect of the ions.

SIMON and BEEVERS (55), who made observations with several inhibitors over a wide range of pH, regularly noticed such an apparent higher efficiency of the molecular species at pH values far above pK. Discussing this, they point out the importance to distinguish the conditions in the suspension medium from those inside the cells. If the cell membrane is permeable to both molecules and ions, the presence of non-permeating electrolytes, *e.g.* proteins must be taken into account. In such a case the internal concentration of the ions of the added electrolyte would be determined by a DONNAN equilibrium. At present, however, no data are available which would permit a computation on this basis. Another possibility is that only the free molecules enter the cells. Very probably, the internal pH is largely independent of the external pH, resulting in different degrees of ionization inside and outside the cell. Fig. 21, taken from (55)

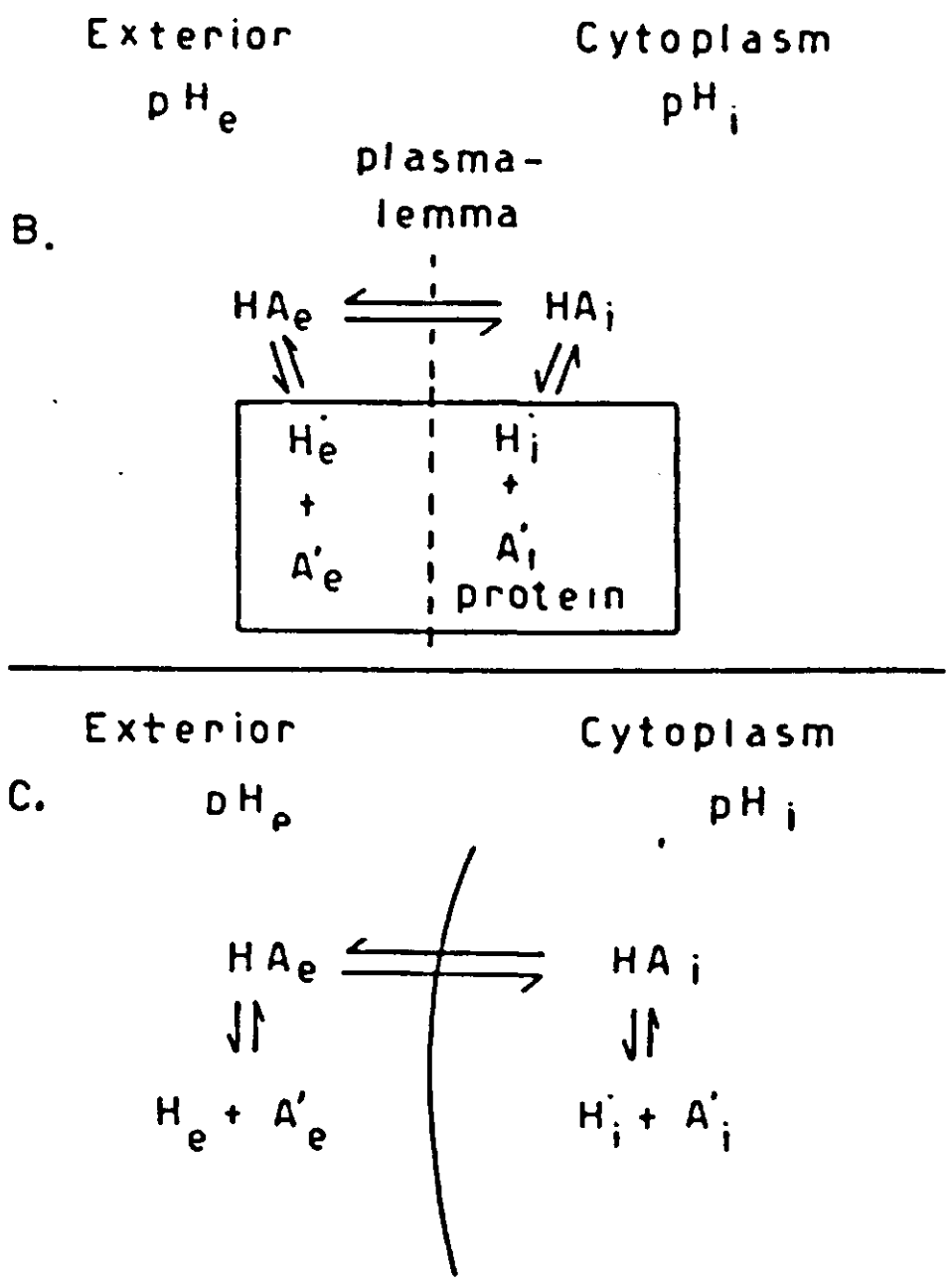


FIG. 21.
"Tentative scheme of distributions, envisaged at steady state... B. The system in a vacuolated cell. The external and internal molecules concentrations are equal. The internal ion concentration is determined by a DONNAN equilibrium. – C. The system in a cell with an outer semi-permeable membrane. The internal concentration is probably determined by the molecule concentration, the pK of the compound and the internal pH". From (55).

illustrates the mentioned possibilities. When arbitrary assumptions on the value of the internal pH are made, the second scheme (C) of fig. 21 permits to make some computations We will consider the case in which a specific amount of inhibitor, *viz.* 1.75 μg per vessel is administered. Omitting brackets, concentrations are indicated by HA and A′, meaning free acid and anion respectively. Index i means internal, index e external. At $pH_e = 3.5$, dissociation of DNP is 20%, hence $A_e' = 0.25\ HA_e$. With $pH_i > pK$, we find $A_i' \gg HA_i$. When we assume $pH_i = 7$, we obtain $A_i' = 1000\ HA_i$. For total DNP (index t) we thus find:

$$DNP_t = DNP_e + DNP_i = (HA_e + A_e') v_e + (HA_i + A_i') v_i \qquad (1)$$

Cell volume (v_i) is 20 mm³ in all experiments.

With v_e = 3000 mm³, and substituting in (1) we find:

$$DNP_t = 1.25 \times 3000\ HA_e + 1000 \times 20\ HA_i$$

With free permeability of the molecules $HA_e = HA_i$. Thus, DNP_t = 23,750 HA_e and DNP_i = 20,000 HA_e, so that about 85% of the DNP_t is inside the cells; 85% of 1.75 µg = 1.5 µg.

When v_e = 6000 mm³, substitution in (1) gives:

$$DNP_t = 1.25 \times 6000\ HA_e + 20{,}000\ HA_e = 27{,}500\ HA_e$$

so that DNP_i = 20,000/27,500 DNP_t, or 73%; 73% of 1.75 µg = 1.25 µg.

This is in agreement with the finding that in experiments at low pH little influence on inhibition is found when v_e is increased (figs. 18 and 19). When, however, $pH_e > pK$, we will find $A_e' \gg HA_e$, and the first term of (1) dominates over the second term. At pH_e = 5.5, A_e' = 31 HA_e.

For v_e = 3000 mm³, substitution in (1) gives:

$$DNP_t = 32 \times 3000\ HA_e + 20{,}000\ HA_e = 116{,}000\ HA_e$$

so that DNP_i = 20,000/116,000 = 19% of DNP_t; 19% of 1.75 µg = 0.33 µg.

For v_e = 6000 mm³ we get:

$$DNP_t = 32 \times 6000\ HA_e + 20{,}000\ HA_e = 216{,}000\ HA_e$$

so that DNP_i = 20,000/216,000 = 9,5% of DNP_t; 9,5% of 1.75 µg = 0.16 µg.

We may expect that in this case the activity of the inhibitor is proportional to its concentration, as is in reasonable agreement with the findings shown in fig. 19. Fig. 22 shows data on photosynthesis from all available experiments, plotted against DNP_i, calculated on the assumptions outlined. Note the better fit of the groups 'A' and 'B'.

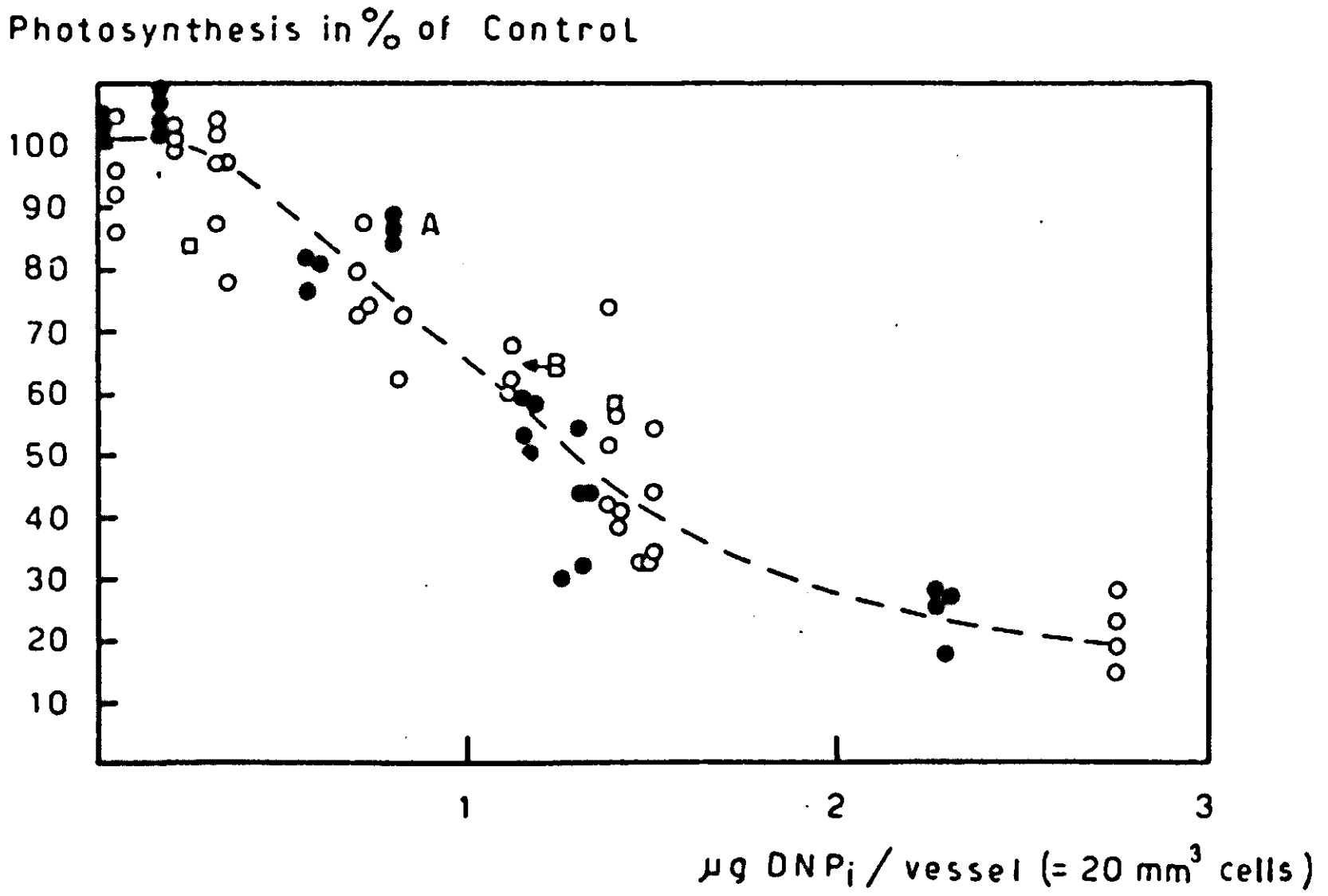


FIG. 22. Photosynthesis of *Chlorella* as influenced by DNP. The same experiments as in fig. 20, plotted here against the amount of DNP, computed to be inside the cells. See text p. 108.

Applying these computations to the data given by HOLZER (25), we arrive at the following. Total volume = 2000 mm^3, containing 2.3 mg dry weight/ml, or about 10 mm^3 fresh cells/ml; v_i = 20 mm^3. DNP was 7×10^{-3} molar = 1300 µg/ml. DNP_t = 2600 µg. At pH_e = 8.3, the ratio ions/free molecules of DNP = 20,000, or $A_e' = 20{,}000\ HA_e$. Substituting in (1) we get:

$$DNP_t = 20{,}000 \times 2000\ HA_e + 20{,}000\ HA_e = 4.10^7\ HA_e = 2600\ \mu g$$

so that $DNP_i = 2.10^4/4.10^7$ = 0.05 % of 2600 µg = 1.3 µg (90 % inhibition was found).

At pH_e = 9.2, we find A_e'/HA_e = 160,000. Equation (1) then becomes:

$$DNP_t = 160{,}000 \times 2000\ HA_e + 20{,}000\ HA_e = 32.10^7\ HA_e = 2600\ \mu g$$

so that $DNP_i = 2.10^4/32.10^7 \times 2600$ = 0.16 µg (62 % inhibition was found).

Probably the strain of *Chlorella* used, and also its cultivation and further treatment were different in HOLZER's experiments and in ours. Therefore, the fact that the 'active' quantities of DNP were found to be of the same order of magnitude is already satisfactory. Nothing is known, moreover, concerning the relation between pH_i and pH_e when the latter is changed. When $pH_e \gg pK$, a rise of pH_i of 0.3 units would double the amount of DNP_i, as can easily be computed.

b. The effects of various concentrations of DNP on the fixation of phosphate in light, and the influence of pH

The fixation of phosphate in light, at pH 4, in the absence of CO_2 was affected by DNP in concentrations comparable to those inhibiting photosynthesis (fig. 23). When total DNP was kept constant, and pH increased, a decrease in inhibition was found, as in the case of photosynthesis (see section *a*). Ad-

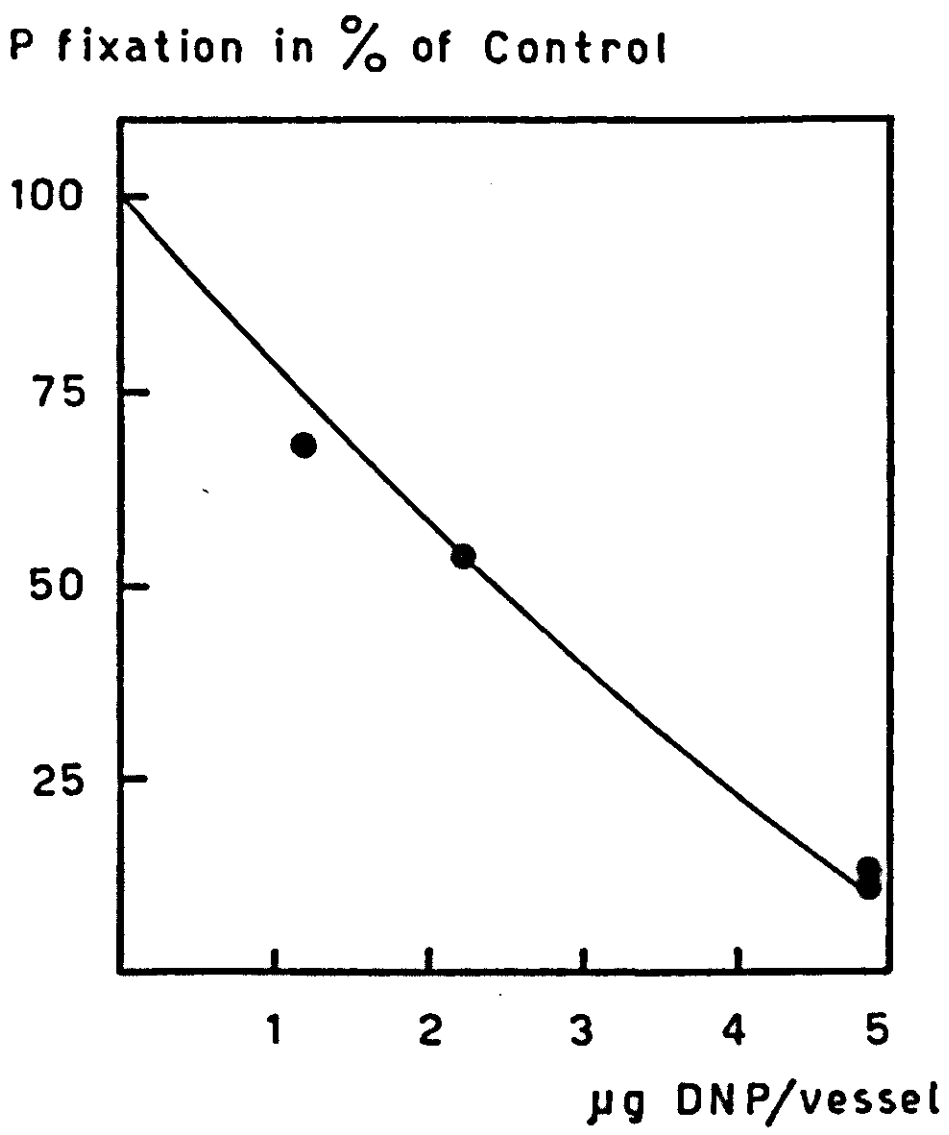


FIG. 23. Phosphate fixation in *Chlorella* by CO_2- free air, in % of control, as influenced by various concentrations of DNP. ~ 4 mm^3 cells/ml; pH 4.0.

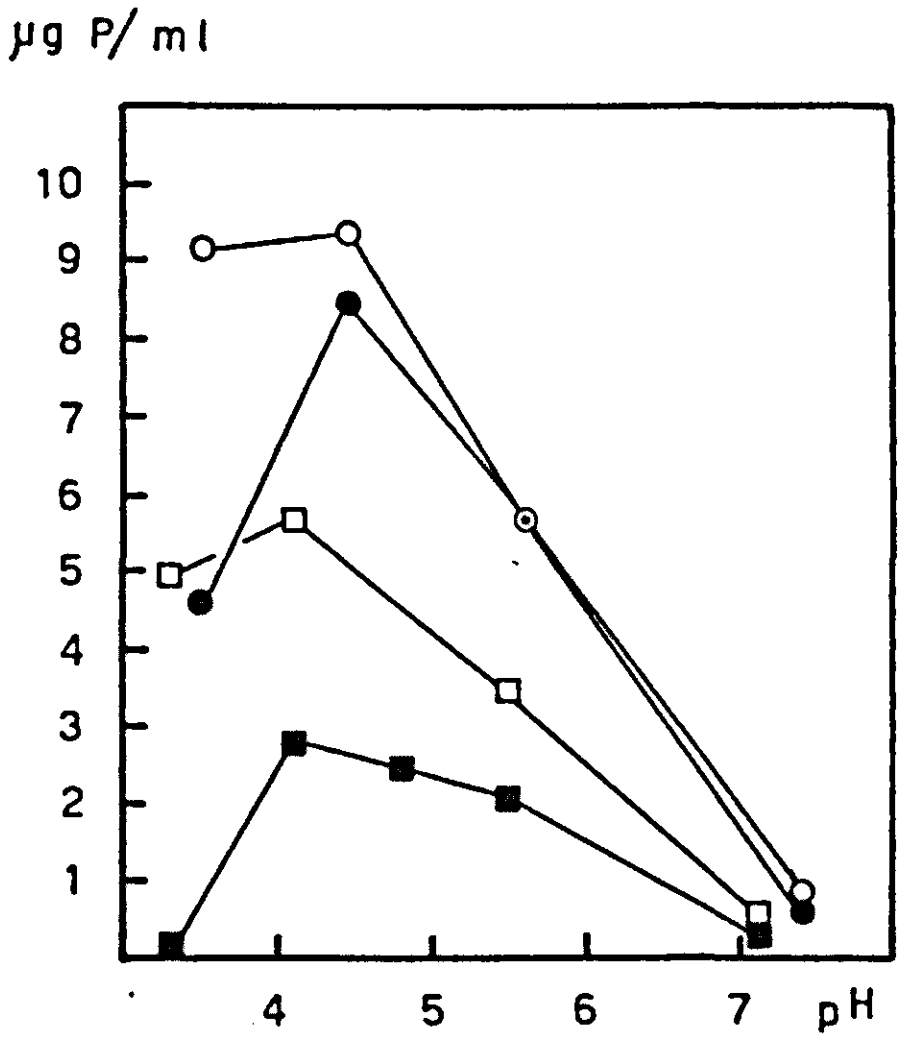


FIG. 24. Phosphate fixation by *Chlorella* in CO_2-free air. Influence of DNP at various pH values. Open symbols: control; black symbols: + DNP, 1.75 µg/vessel, containing 20 mm^3 cells/4 ml.

ditionally, we must take into consideration the strong effect of pH on the uptake of phosphate in this case, as was discussed in Chapter IV, § 2 (p. 92). Fig. 24 shows the results of two representative experiments. It is apparent that the percentage of inhibition decreases at higher pH.

c. The effect of DNP on photosynthesis at various light intensities

Very few data are available on the inhibition of photosynthesis by DNP in relation to light intensity. GAFFRON (21) has measured photosynthesis of *Chlorella* in the presence of DNP, at two rather low light intensities, *viz.* 900 and 1700 lux. Since inhibition was similar at both light intensities, it seems that the inhibitor is active already in the range of light limitation.

In experiments with the WARBURG technique (pH about 4.0, 4 ml suspension per vessel, with 20 mm^3 cells and 1.6 µg DNP), we found respiration always stimulated. Under these conditions the light intensity curve of photosynthesis had

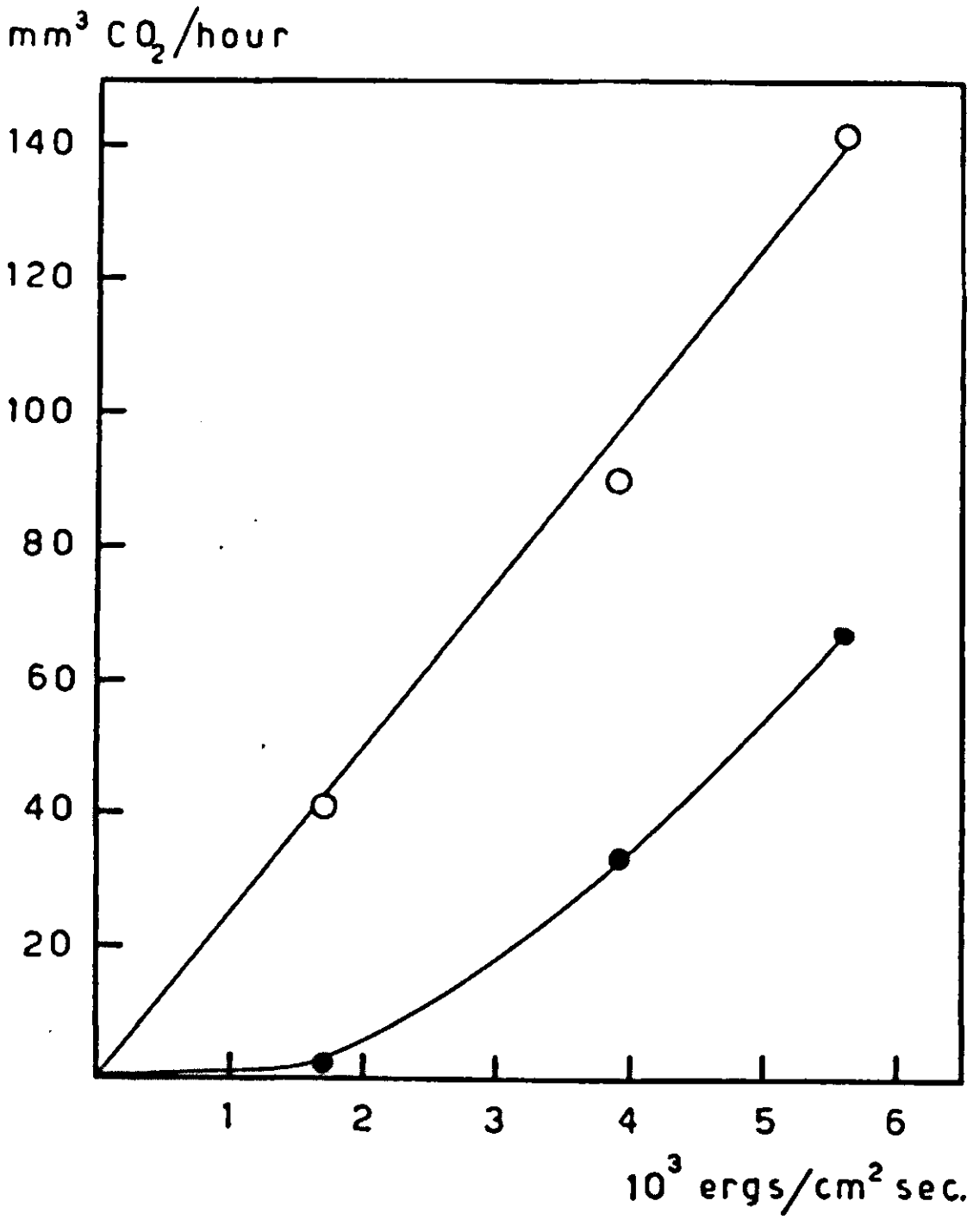


FIG. 25.
Photosynthesis of *Chlorella* at different light intensities, in the absence (○) and in the presence (●) of DNP (0.4 µg/ml); pH 4.0; air + 5% CO_2; 20 mm^3 cells/4 ml. Photosynthesis calculated, assuming the quotient $-O_2/CO_2 = 1.05$. Average of several experiments.

TABLE XIII

Photosynthesis of *Chlorella* at different light intensities, in the presence or absence of DNP. Air with 5% CO_2; 25° C; DNP: 0.4 µg/ml; 20 mm^3 cells in 4 ml; pH 4. Data in mm^3 CO_2/vessel hour, computed on the assumption that $-O_2/CO_2 = 1.05$, and corrected for respiration.

Light, ergs/cm² sec.	Control, mm^3 CO_2	Number of observations	+ DNP, mm^3 CO_2	Number of observations	% of Control
1700	42 ± 3	10	2^5 ± 4^5	7	6
3900	90 ± 7	4	33^5 ± 7	7	37
5600	142 ± 8	10	67 ± 10	7	47

a sigmoid shape; up to about 1500 ergs/cm² sec the slope was distinctly smoother than above this point, so that the percentage inhibition decreased with increasing light intensity (*cf.* fig. 25 and Table XIII in which inhibition is over 90 % up to 1700 ergs/cm² sec, but only about 35 % between 3900 and 5600 ergs/cm² sec). Fig. 26 represents the result of an experiment made with a new volumeter, developed by KOK (37), which combines high sensitivity and rapidity of reaction. Measurements were made over 2 minutes periods. Because of the very small size of the ves-

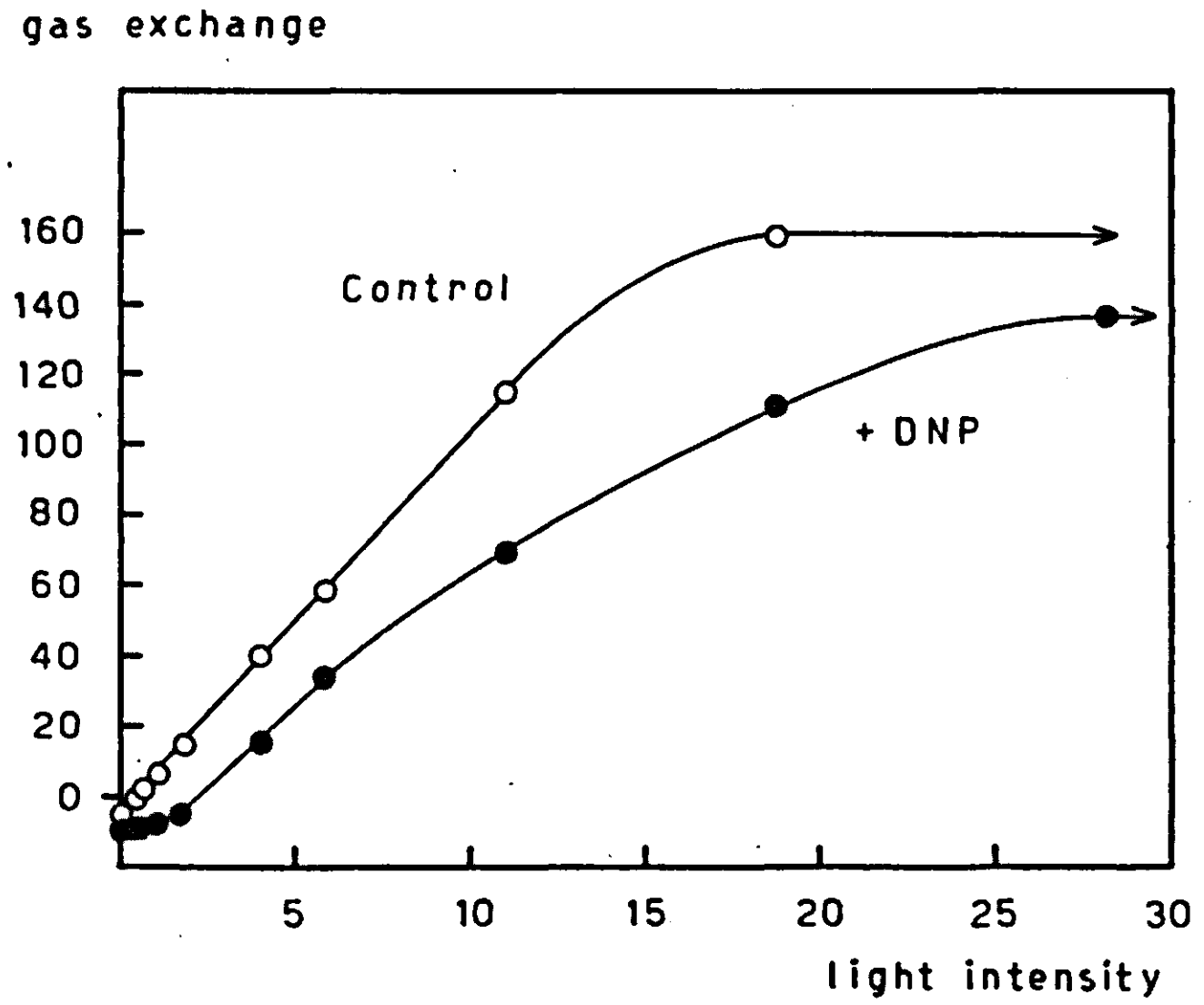


FIG. 26. Photosynthesis of *Chlorella* at different light intensities, in the presence and in the absence of DNP (0.3 μg/ml suspension); pH 4.0; air + 5% CO_2; 5 mm³ cells/ml. Arbitrary units of light intensity and gas exchange.

sel, very high light intensities are obtainable by using suitable optical systems. It is, however, rather difficult to measure light intensities in absolute units, so that they are given here in relative units. At the highest intensity shown in fig. 26 (28 units), photosynthesis in the control vessel soon decreased owing to photo-oxidation. In the poisoned suspension, however, photosynthesis remained constant at light saturation, up to a relative intensity of 59 (not shown in the figure). The percentage of inhibition may become very small at these very high light intensities. The fact that a similar type of curve is found when photosynthesis is measured over periods of 2 minutes, as in the volumeter, or over 45–60 minutes as in the WARBURG vessel, indicates that decomposition of the inhibitor in the light cannot be considered as an explanation for the decrease of inhibition with increasing light intensity. For further discussion, see Chapter VI, § 1.

d. The effect of DNP on phosphate fixation at various light intensities

As in the case of phenylurethane, the effect of DNP on phosphate fixation in the absence of CO_2 was studied at various light intensities. Applying 0.4 μg DNP/ml, at pH 4.0, we found the fixation of phosphate in dark strongly inhibited (whereas oxygen uptake was stimulated; *cf.* p. 110). In the presence of

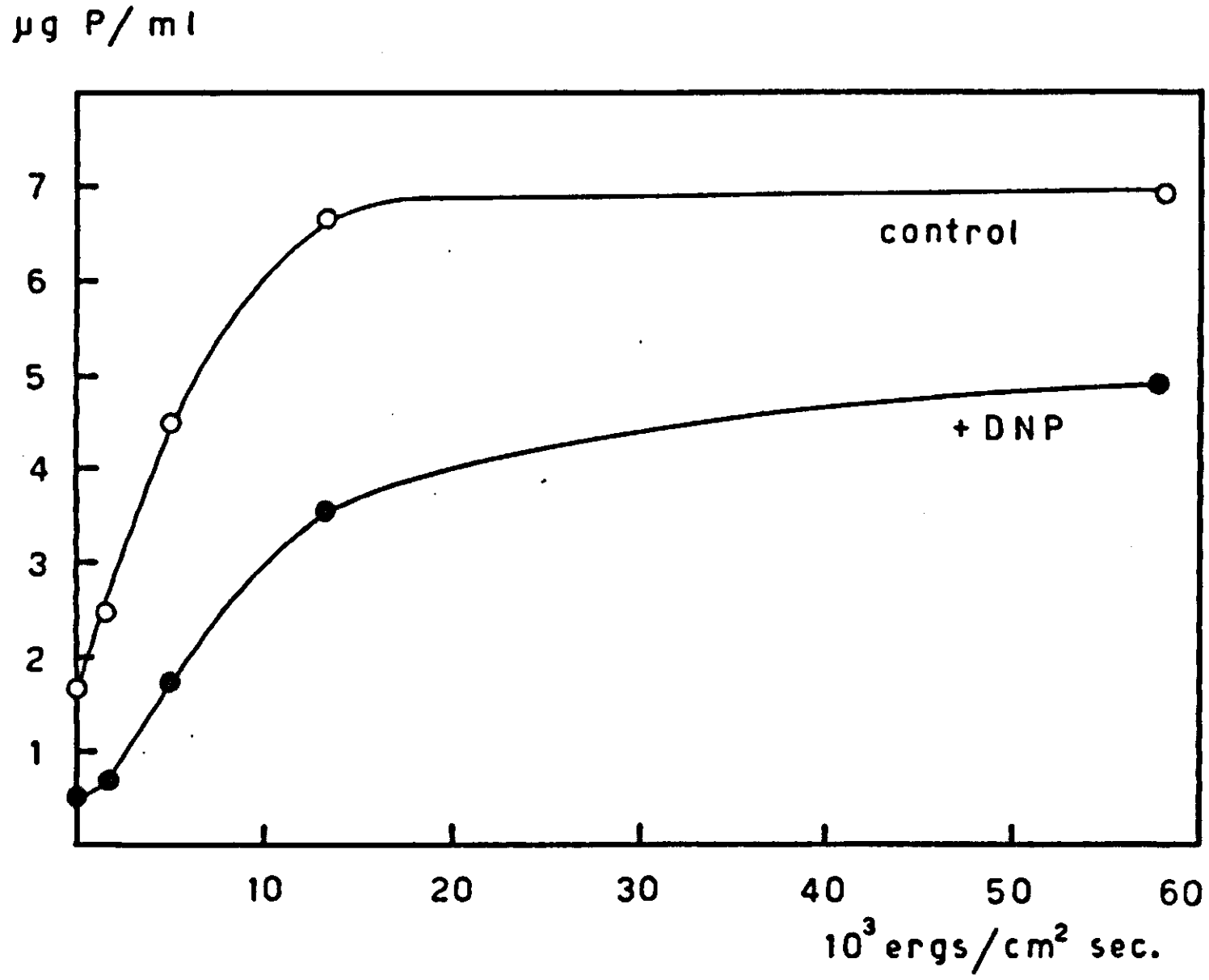


FIG. 27. Phosphate fixation by *Chlorella* in CO_2-free air at various light intensities, as influenced by DNP (0.4 µg/ml, pH 4.0). Suspensions containing 5 mm³ cells/ml. Averaged from several experiments.

TABLE XIV

The fixation of orthophosphate by *Chlorella* in CO_2-free air, at different light intensities, in the presence and absence of DNP. Data in µg P/ml fixed in 3 hours. 25° C; pH 4; concentration of DNP: 0.4 µg/ml.

a	b	c	d	e	f
Light, ergs/cm² sec.	Control, µg P/ml fixed	DNP, µg P/ml fixed	Difference (columns b–c)	Number of observations	% of Control
0	1.55 ± 0.2	0.45 ± 0.2	1.1 ± 0.15	7	29
1,500	2.4 ± 0.35	0.6 ± 0.25	1.8 ± 0.3	6	25
5,000	4.4 ± 0.6	1.8 ± 0.45	2.6 ± 0.35	7	41
13,500	6.2 ± 0.45	3.5 ± 0.35	2.7 ± 0.75	4	56
55,000	6.9 ± 0.4	4.8 ± 0.65	2.1 ± 0.35	6	70

TABLE XV

As Table XIV. Net light effects (light minus dark).

Light, ergs/cm² sec.	Control, µg P/ml fixed	DNP, µg P/ml fixed	Difference (columns b–c)	Number of observations	% of Control
1,500	0.9 ± 0.25	0.1 ± 0.17	0.8 ± 0.27	6	11
5,000	2.8 ± 0.5	1.3 ± 0.35	1.5 ± 0.35	7	46
13,500	4.75 ± 0.55	3.2 ± 0.5	1.5 ± 0.6	4	67
55,000	5.2 ± 0.3	4.3 ± 0.55	0.9 ± 0.35	6	83

DNP the light intensity curve of phosphate fixation has a somewhat sigmoid shape, as observed also in the case of photosynthesis (see p. 111). Fig. 27 and Tables XIV and XV illustrate this. At very low light intensities (about 1500

ergs/cm² sec) the fixation of phosphate in the presence of DNP was hardly increased over that in darkness, whereas in the control the efficiency of the light with respect to phosphate fixation is highest at these light intensities. With increasing light intensity the percentage inhibition of phosphate uptake decreases, as indicated by the steeper part of the curve in fig. 27. Comparing the steepest parts of both curves, between 1500 and 5000 ergs/cm² sec, we see that the inhibition owing to DNP is less than 50%, whereas in dark and up to 1500 ergs/cm² sec phosphorylation is more strongly inhibited. For a discussion see Ch. VI, § 1. Light saturation is shifted to higher light intensities in the presence of DNP, hence inhibition is minimal at the highest intensity studied (± 55,000 ergs/cm² sec). The data in Table XV represent the net light action, and demonstrate more clearly the strong inhibition at low light intensity. These data are useful for comparison with those on photosynthesis, presented in section *c* of this § (Table XIII).

§ 3. SODIUM AZIDE

Sodium azide is known as an inhibitor of photosynthesis. WASSINK *et al.* studied its influence on photosynthesis and fluorescence of *Chromatium* (73). They found photosynthesis inhibited at all light intensities, but especially so at low light intensities. Normally, the light intensity curves of photosynthesis of purple bacteria are slightly sigmoid. This feature was strongly increased in the presence of sodium azide, while a decrease of fluorescence indicated that azide is closely associated with the light sensitive system (73, see also 51). On the other hand, it is known that sodium azide influences respiration in much the same way as DNP, uncoupling respiration and phosphorylation (41). It was of interest, therefore, to study the effect of azide on phosphate accumulation in the light, as compared with photosynthesis.

First, it was found that concentrations many times smaller than those used by WASSINK *et al.* (73), were sufficient to obtain suitable inhibitions. This might be explained by a pH effect, as observed also for DNP, since in the case of sodium azide, the corresponding hydrazoic acid is a weak acid (pK = 4.7, *cf.* 29, 55). We found that photosynthesis at pH 4 was inhibited at NaN_3 concentrations of 10^{-6} to 10^{-5} molar, while inhibition of photoreduction in *Chromatium* at pH 6.3 required concentrations of 10^{-2} to 10^{-3} molar (73). Fig. 28 shows photosynthesis of *Chlorella* as influenced by various concentrations of azide. Respiration was stimulated by concentrations up to 10^{-5} molar in our experiments and dark fixation of phosphate was inhibited (*cf.* the results obtained with DNP, § 2, *c* and *d* of this Chapter).

Phosphate fixation in light was studied at pH 4, in CO_2-free air, using the same concentrations of azide as applied in the measurements of photosynthesis. Table XVI and fig. 29 show the inhibition of phosphate fixation in CO_2-free air measured after about 3 hours of illumination as a function of the applied concentration of azide.

Phosphate fixation in light, in air with 5% CO_2 was found less sensitive to azide (Table XVI). There seems to be even a slight stimulation although experimental errors weigh heavily upon small differences as found in this case.

It has been suggested by RABINOWITCH (51) that azide decomposes in the light owing to a close connection with the chlorophyll (*l. c.* p. 959). Some results were obtained which seem to support this hypothesis. When phosphate uptake in

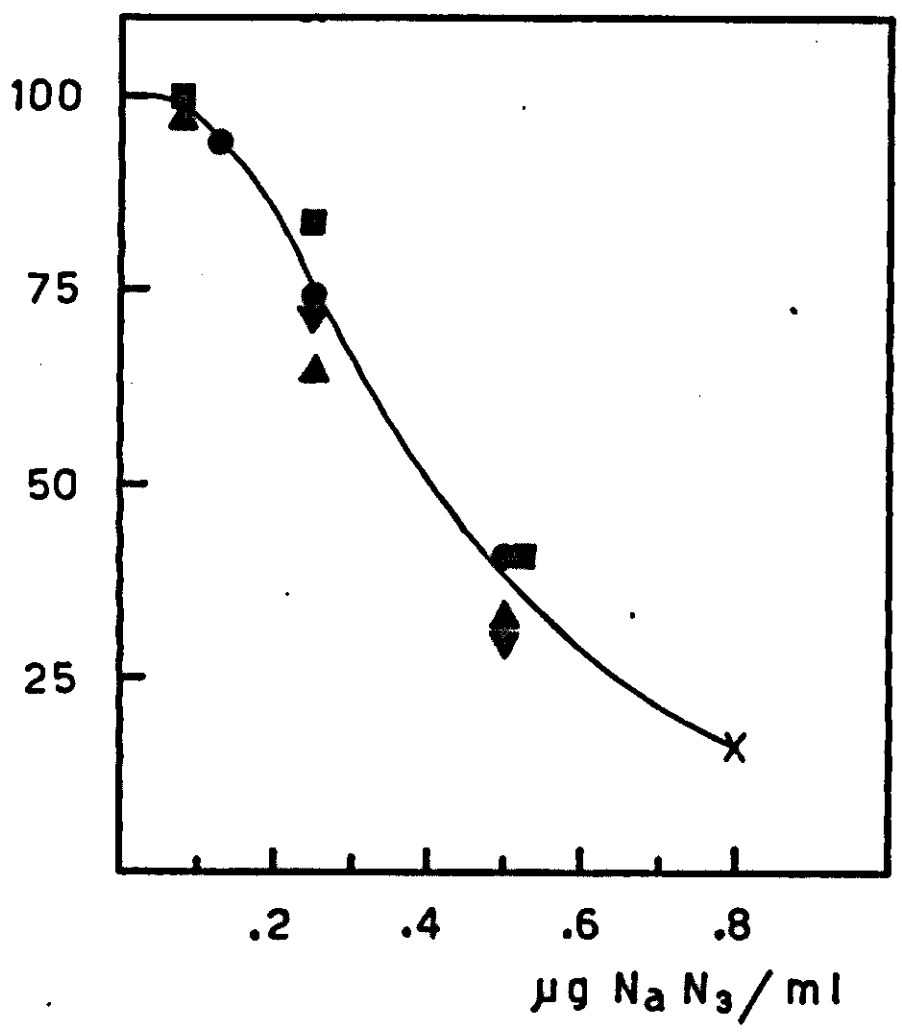


FIG. 28. Photosynthesis of *Chlorella* as influenced by sodium azide, in % of control. pH 4.0; air + 5% CO_2; 5 mm³ cells/ml. Measurements over 45–60 minutes, during the first hour of illumination.

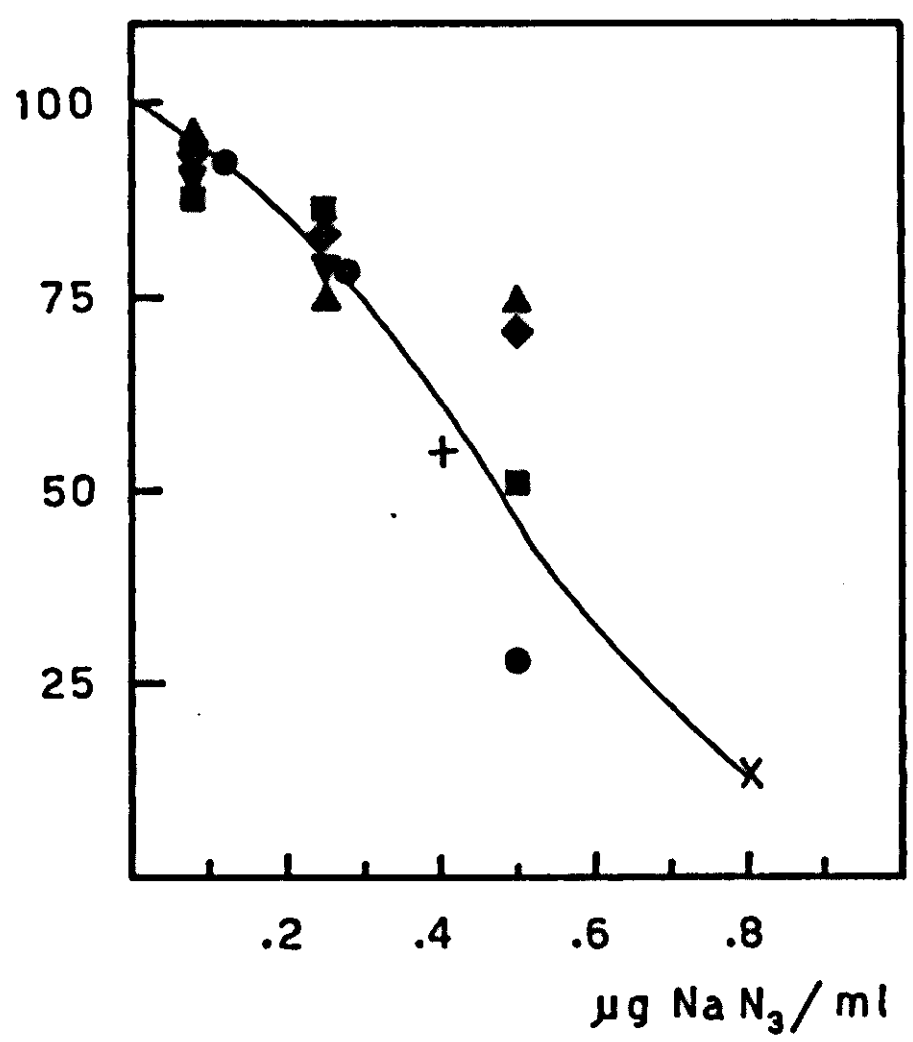


FIG. 29. Phosphate fixation by *Chlorella* in light in CO_2-free air, in the presence of sodium azide; in % of control. Uptake measured after 3 hours of light. ~ 4 mm³ cells/ml; pH 4.0.

TABLE XVI

Fixation of phosphate by *Chlorella* in light, as influenced by sodium azide. Data in µg P/ml, fixed in 3 hours. Temperature 25°C; pH 4; 4mm³ wet cells/ml.

Concentration	0	1.2×10^{-6}M	3.7×10^{-6}M	7.5×10^{-6}M	1.2×10^{-5}M
CO_2-free air . . .	5.7 ± 0.25	5.1 ± 0.25	4.5 ± 0.25	3.2 ± 0.7	0.9
Number of observations . . .	6	5	5	4	1
% of control . . .	100	89	79	56	(16)
Air + 5% CO_2 .	1.35 ± 0.2	1.7 ± 0.2	1.7 ± 0.25	1.05 ± 0.27	0.9
Number of observations . . .	5	3	5	6	1
% of control . . .	100	125	125	78	(67)

TABLE XVII

Changes in the rate of phosphate fixation by *Chlorella* in the light in CO_2-free air, in the presence and absence of sodium azide. pH 4.0; 25° C. Data in µg P/ml.

a	b	c	d	e
	Control	+ NaN_3	Difference (columns b–c)	Number of observations
1st hour	2.4 ± 0.37	1.3 ± 0.4	1.1 ± 0.25	7
2nd hour	2.35 ± 0.3	1.65 ± 0.25	0.7 ± 0.15	6
3rd hour	2.1 ± 0.2	1.4 ± 0.1	0.7 ± 0.2	6

light, in CO_2-free air was followed a decrease in inhibition with time was often observed (Table XVII).

In the experiments of Table XVII, the results were not influenced by the presence or absence of alkali in the side arm of the WARBURG vessels. Therefore, distillation of free hydrazoic acid (boiling point 37° C) cannot explain these results. Also the decrease in inhibition with time was not correlated with changes in pH during the course of the experiments.

It is improbable that free nitrogen was formed from the azide in our experiments. Up to 500 μg/ml NaN_3 was given in some experiments. This high concentration gave rise to a small uptake of gas in the absence of CO_2, whereas the corresponding control showed a slight gas evolution. Theoretically about 1 ml of free nitrogen could arise from the azide given. Suspensions with this amount of azide were bleached at the end of the illumination period.

The curves of fig. 28 for photosynthesis and fig. 29 for phosphorylation are very similar, but taking into account the differences in exposition time, phosphate fixation in CO_2-free air seems to be somewhat more sensitive to NaN_3 than photosynthesis.

§ 4. SODIUM FLUORIDE

Sodium fluoride in concentrations between 10^{-2} and 10^{-1} molar has been reported to inhibit photosynthesis of *Mnium undulatum* (56) which inhibition was stronger at high than at low light intensities. The best known effect of fluoride is the inhibition of the step converting phosphoglyceric acid into pyruvic acid in glycolysis. This prevents the formation of ~ph normally accompanying that step. Also, generally, the transfer of ~ph to the adenytic acid system is prevented by fluoride (56). HOLZER and HOLZER (26) have made probable that respiration of *Chlorella* mainly uses the pathway of glycolysis. Concerning the metaphosphate metabolism, YOSHIDA (88) found that the formation of metaphosphate in yeast was inhibited by NaF.

We have measured photosynthesis of *Chlorella* in the presence of NaF in acid media, pH 4, with 5% CO_2 in air. Photosynthesis was inhibited by concentrations from 1 to 5 × 10^{-3} molar. Respiration, however, was unaffected, or even slightly stimulated at these concentrations (*cf.* 56).

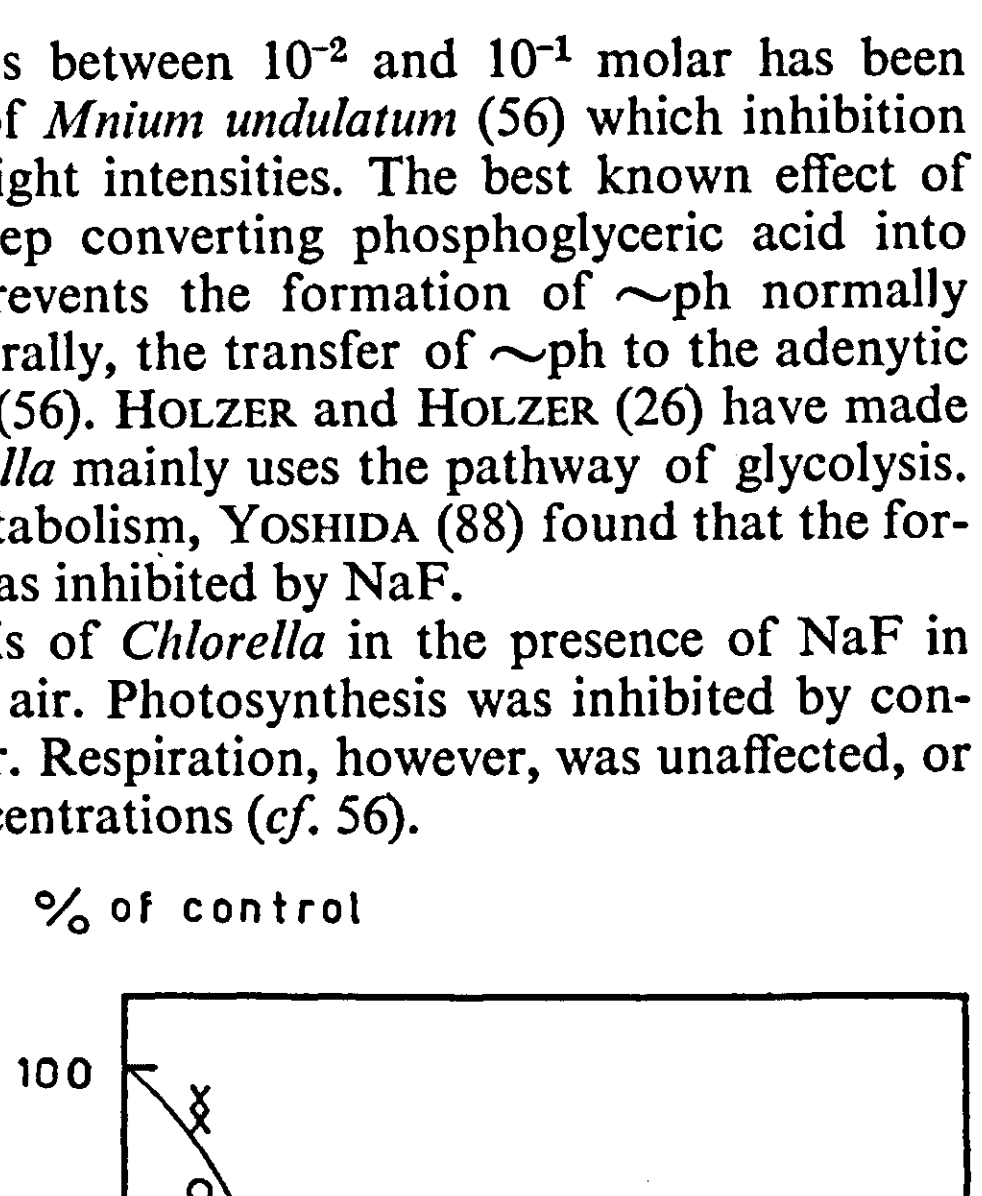


FIG. 30.
Photosynthesis (×) and formation of polyphosphates in CO_2-free air (O) by *Chlorella*, as influenced by various concentrations of sodium fluoride, in % of control.
~ 5 mm³ cells/ml; pH 4.0.

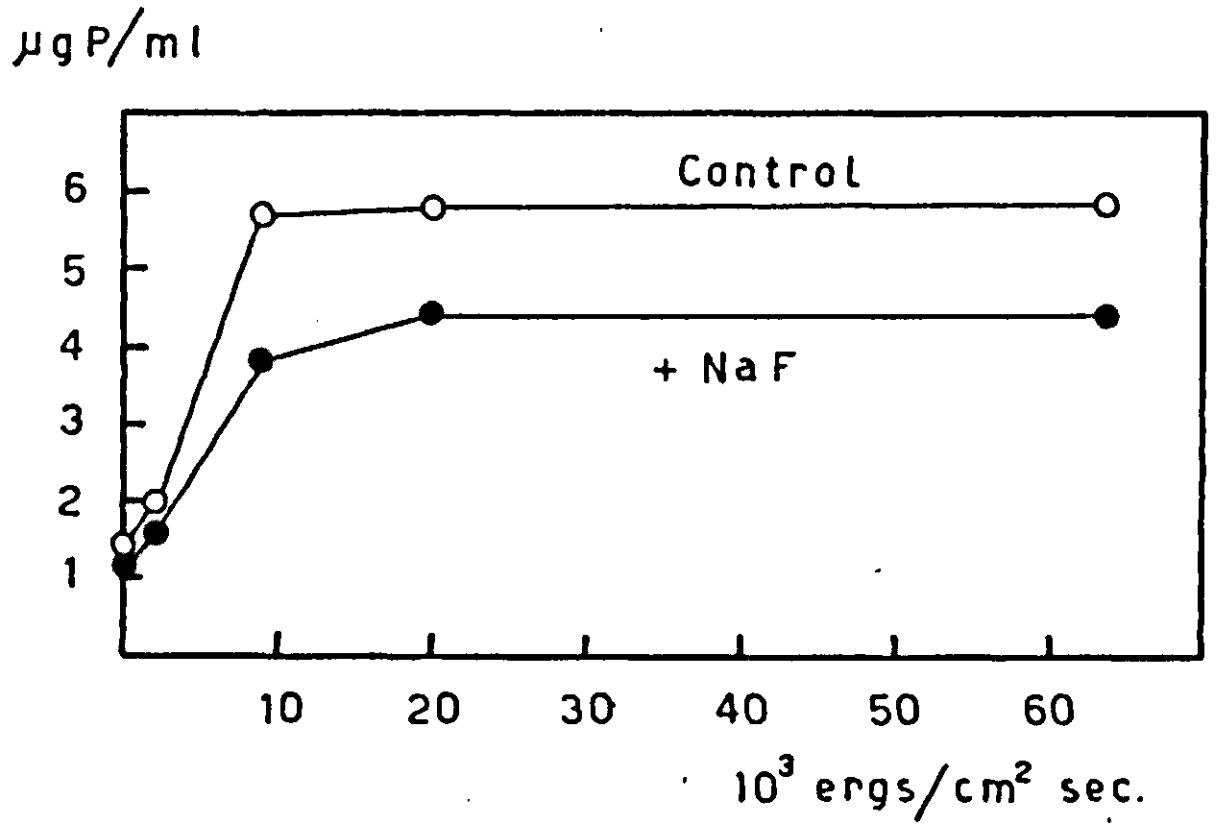


FIG. 31.
Fixation of phosphate by *Chlorella* in CO_2-free air at various light intensities, as influenced by sodium fluoride (10^{-3} molar) at pH 4.0; ~ 4 mm³ cells/ml.

Accumulation of polyphosphates was inhibited to about the same degree as photosynthesis. Fig. 30 shows the inhibition of both photosynthesis and polyphosphate accumulation in the presence of various concentrations of NaF. Fig. 31 shows an experiment on phosphate fixation at different light intensities. Inhibition is about equal at all light intensities, whereas dark fixation is not inhibited by the concentrations used.

CHAPTER VI

DISCUSSION AND SUMMARY

§ 1. GENERAL DISCUSSION

Chlorella, as used in our experiments, generally takes up orthophosphate and converts it into other phosphate compounds. This tendency is apparent in light and in darkness; the uptake of phosphate in darkness cannot be due to phosphate deficiency of the cells (*cf.* p. 88). It seems to require oxygen (see p. 95), and can thus be related to respiration. No observations were made concerning the nature of the products of dark fixation. An influence of pH on the rate of phosphate fixation was found, both in light and in darkness; fixation was maximal when the cells were suspended in an acid medium. Some suggestions for an explanation were made on pp. 93–94.

The fixation of phosphate in light in the presence of CO_2, and that in the absence of CO_2 show both quantitative and qualitative differences. The latter are expressed by the greater chemical stability of the phosphate compounds formed during photosynthesis as compared with those formed during illumination in CO_2-free air ([86], *cf.* p. 76) and also by the following observations:

1° In the presence of phenylurethane, at high light intensities phosphate fixation is inhibited in the presence of CO_2, but not in its absence (figs. 15, 16).

2° The inhibition of phosphorylation by azide is stronger in the absence of CO_2 than in its presence (cf. Table XVI). There are no data on the nature of the phosphorylated compounds, formed in the light in the presence of CO_2. Part of them may well be polyphosphates, since these are normal cell constituents, as pointed out on p. 88. The phosphate uptake in CO_2-free air in light seems to represent a special situation resulting in the accumulation of polyphosphates. This rapid rise in polyphosphate content under somewhat abnormal conditions

is not without parallel. In *Neurospora*, metaphosphate is normally present in small amounts (28). HOULAHAN and MITCHELL found four genetically different mutants, in which metaphosphate could accumulate up to many times the normal content, especially when a required special factor was supplied in growth limiting quantities. The mutant genes in the phosphate accumulating strains were not related, and genetic analysis showed that the presence of a special 'P-mutant gene' was improbable. These authors therefore concluded that the phosphate accumulation probably "results from the abnormal functioning of a general phosphorylating agent" (28). In some *Mycobacteria* species, a rapid increase in metaphosphate was observed, following the addition of special substrates and other agents (83).

Also in our case it is plausible that the polyphosphates are derived from a normal intermediate, the utilization of which is blocked when photosynthesis is limited by lack of CO_2. These derived products are rather stable, also in darkness, and cannot be considered as immediately available stored 'reducing power' (*cf.* p. 72). It is tempting to consider ATP as a precursor of these polyphosphates. Some arguments herefor can be given and are listed under *a*) and *b*) below.

a) The *photochemical formation of ATP* has been reported by several investigators. VISHNIAC and OCHOA (68, 69) have reported the reduction of di- and triphosphopyridine nucleotides (DPN and TPN) by chloroplasts in the light. Reoxidation of the coenzyme could be mediated by several enzymatic reactions. So, in the presence of mitrochondria and oxygen, oxidation of photochemically reduced DPN was accompanied by the formation of ATP. The authors suggest that reduced coenzymes are general reducing agents, and that the biochemical diversity among organisms is founded upon the diversity of their mechanisms to produce hydrogen (69). According to the mentioned authors, in photosynthesizing organisms, the production of 'hydrogen' from water in light and its transfer to coenzyme would be such a specific mechanism.

A recent paper by ARNON *et al.* (3) indicates that ATP may be formed by illuminated chloroplasts without addition of coenzyme or mitochondria. The authors suppose that the generation of ~ph is coupled to the reoxidation of a reductant, produced by photolysis of water:

$$H_2O \xrightarrow[\text{light}]{\text{chloroplasts}} 2\,e + 2\,H^{\cdot} + \{O\}$$

$$2\,e + 2\,H^{\cdot} + \{O\} + n\,AMP + 2n\,PO_4 \longrightarrow H_2O + n\,ATP$$

$$n\,AMP + 2n\,PO_4 \xrightarrow{\text{light}} n\,ATP$$

in which AMP means adenosine monophosphate.

It is not known whether {O} represents free oxygen or its precursor. No gas exchange could be observed manometrically, but aerobic conditions were necessary for continued operation of this process of photosynthetic phosphorylation (3). Furthermore, chloroplasts as prepared by ARNON *et al.* (3) are capable of fixation and reduction of CO_2 in the light. The latter properties are soon lost; moreover they are inhibited in the presence of oxygen and phosphate. Phosphorylation in the light, however, proceeds both in the presence and absence of CO_2. These experiments show, even more clearly than those of VISHNIAC and OCHOA (68), that phosphorylation is intimately connected to the photochemical reaction.

The experiments of STREHLER (62, 63) show that in intact *Chlorella* cells, the formation of ATP is accelerated in the light, and that its utilization is more rapid in light than in darkness.

Its formation was observed when the cells were illuminated after anaerobic incubation in darkness, so that obviously oxygen is not required herefor. Formation of ATP in darkness was observed only when oxygen was admitted. The dark phosphorylation was strongly dependent on temperature, contrary to the light induced formation of ATP. The steady state level of ATP in light was not influenced by CO_2. This indicates, according to STREHLER, that "carboxylations are not the major drain on ATP".

b) A *transfer of ~ph from ATP to polyphosphates* seems quite feasible. HOFFMANN-OSTENHOF and WEIGERT (23) have suggested that metaphosphates in lower organisms, play a rôle in storing phosphate bond energy, interchangeable with the adenosinephosphoric acids. This might be represented by the following equation:

$$ATP + (NaPO_3)_n \rightleftarrows ADP + (NaPO_3)_{n+1}.$$

In a recent note, HOFFMANN-OSTENHOF *et al.* (24) report that they have found a yeast enzyme catalyzing at least the reaction from right to left. In the presence of this yeast enzyme and ADP, tracer from labeled metaphosphate was incorporated into the nucleotide fraction. In view of the similarity in energy-content of phosphate bonds of polyphosphates and the terminal bonds of ATP (phosphoric anhydride bonds), a reversibility of this reaction is very probable, but not yet demonstrated (24).

Assuming that ATP is formed in close connection with the primary photochemical reaction, it is entirely possible that transfer of ~ph to polyphosphates constitutes a drain on ATP alternative to carboxylation or carbon dioxide reduction. Light saturation of photosynthesis and of phosphate accumulation then will depend on the capacity of the respective dark systems, and need not coincide. The low light intensity at which saturation for phosphate accumulation occurs, points to a restricted capacity of the ~ph transferring system. STREHLER calculated that the minimum rate of formation of the terminal ~ph of ATP, at light saturation was 3.6×10^{-11} moles ~ph/mm^3 cells/sec in his experiments (62). The maximum rate of phosphate fixation in our experiments was 0.1×10^{-6} moles P/ml suspension/hr or $0.5–0.6 \times 10^{-11}$ moles P/mm^3 cells/sec which means a ratio between both values of at least 6–7. That the capacity for phosphate fixation is much smaller than the capacity for photosynthetic fixation of CO_2 was pointed out on p. 89. It is of course to be expected that the yield of polyphosphate formation is smaller than that of ATP generation, but it is interesting that these values agree within one order of magnitude. In our experiments oxygen was required for fixation of phosphate in the dark, but not in the light. In these respects our findings are comparable with STREHLER's observation on formation of ATP in dark and light.

Our data so far do not indicate that nitrate is an alternative acceptor for photochemically produced ~ph in competition with CO_2 or the polyphosphates.

The observations of a decreased fixation of phosphate in light in the presence of glucose are best explained by assuming that glucose acts as an acceptor of photochemically formed ~ph, along with CO_2 and the polyphosphates (see p. 99–100). This conception is supported by results of KANDLER (31) who found an increased rate of glucose assimilation in the light by starved *Chlorella* cells,

which he explained by an increased supply of ATP, generated in the light. The increased utilization of glucose in the light is therefore considered as an indicator for a light dependent phosphorylation (31). As already remarked on p. 100, the effects of CO_2 and O_2 in the experiments of KANDLER are in a line with the results of our experiments on polyphosphate formation.

Phenylurethane was found to inhibit photosynthesis much more than polyphosphate formation (*cf.* figs. 14 and 15 respectively, and Table XII). The light intensity curve of phosphate fixation indicates a preferent inhibition of the photochemical reaction. Apparently the involved dark systems are less sensitive, since at sufficiently high light intensities, the rate of phosphate fixation equals the maximum rate of the control. However, it must be assumed that also some dark system of the photosynthetic chain is inhibited, because inhibition of gas exchange is found at all light intensities. The photochemical reaction which is sensitive to phenylurethane may be the production of a photoreductant, the partial reoxidation of which – yielding ATP – would be insensitive as well as the transfer of ~ph to polyphosphates. In a study on photosynthesis with $C^{14}O_2$ in the presence of phenylurethane, NEWBURGH and BURRIS (48) observed that the total fixation of CO_2 was decreased and that the distribution of tracer over different compounds was changed. Phosphate containing compounds, such as phosphoglyceric acid and hexose diphosphate contained relatively less C^{14} than phosphate-free compounds such as alanine. The authors, therefore, supposed that phenylurethane interferes primarily with a light reaction leading to the formation of high energy phosphate.

In dark and at a light intensity of 1500 ergs/cm² sec, no inhibition of phosphate fixation was observed in our experiments. Some remarks on this will be given below.

With dinitrophenol as an inhibitor, the strong inhibition of dark phosphorylation together with a stimulation of respiration, was not unexpected, and in accordance with other observations. Photosynthesis was already inhibited by concentrations of DNP which stimulated respiration. Regarding this inhibition of photosynthesis by DNP it was especially remarkable that at low pH the *amount* of inhibitor present (per vessel containing a given amount of cells), rather than its *concentration* determined the degree of inhibition. This can be understood if, following suggestions outlined by SIMON and BEEVERS (55), we assume that the cells are permeable to the undissociated molecules of DNP only, while their internal pH does not deviate appreciably from neutrality, irrespective of the external pH. At low pH this situation would lead to a conspicuous accumulation of the poison inside the cells (see p. 104–108).

The formation of polyphosphates and photosynthesis were found to be about equally sensitive to DNP. The similarity in shape of the light intensity curves for both processes (*cf.* figs. 27 and 25, 26), suggests that DNP inhibits a step which both processes have in common, and all that is known about the activity of DNP suggests that this is a phosphorylation. HOLZER had already observed that photosynthesis is more strongly inhibited by DNP than respiration. He supposed that the generation and partial oxidation of a photoreductant continues in the presence of DNP, but that the generation of ~ ph, normally coupled to this phenomenon, is inhibited. Consequently, photosynthesis would be limited by lack of ATP (25). In a similar way, in our experiments, the formation of polyphosphates might be curtailed. The inhibition is visible already in the light limiting part of the curves, showing that the inhibited step is closely

connected to the photochemical reaction. NEWBURGH and BURRIS (48) observed that in the presence of DNP, apart from an overall inhibition of $C^{14}O_2$ fixation, a relatively smaller amount of the tracer was incorporated into P-containing compounds than into P-free ones, just as had been found with phenylurethane (see above). The authors concluded that also DNP acts upon a light reaction which leads to the formation of ~ph.

It was noted above (p. 113, see fig. 27 and Table XV) that the inhibition of phosphorylation was somewhat smaller in light than in darkness. Recent data of KANDLER (32) [1]) point to a similar difference. KANDLER who considered the rate of glucose assimilation by starved *Chlorella* as an index for ATP formation (*cf.* p. 99, 118), found glucose assimilation in darkness depressed in the presence of DNP. The extra-assimilation in the light, however, was less sensitive to the inhibitor. KANDLER concludes that the generation of ATP *via* oxidative phosphorylation, coupled to reoxidation of a photoreductant, is not the basic mechanism for production of ~ph in the light, although this process may well occur normally (32). In discussing the results, obtained with this and several other inhibitors, KANDLER concludes that a still closer connection exists between light absorption and formation of ~ph than is implied in the 'recombination theory' assumed by HOLZER (25), STREHLER (62, 63), ARNON *et al.* (3) VISHNIAC and OCHOA (68, 69). I cite:

"Grundsätzlich muss auch daran gedacht werden, dass die unmittelbare Folge der Quantenabsorption nicht die Wasserspaltung zu sein braucht, sondern dass auch primär eine energiereiche Phosphatbindung, bzw. eine Verbinding hohen Gruppenpotentials, die mit dem Adenylsäuresystem in Gleichgewicht steht, aufgerichtet werden könnte" (32).

It may be remarked that the different oxygen requirements for the formation of ~ph by intact *Chlorella* cells in light and darkness (31, 62, 63) are also in a line with the view that different mechanisms are operative in both cases.

In this context, attention may also be drawn to the observations of KANDLER (30) and of WASSINK and ROMBACH (74) on changes in orthophosphate levels in *Chlorella* at the start of an illumination. In the first minute in the light, two minima in orthophosphate were observed. WASSINK and SPRUIT (75), comparing various induction effects related to photosynthesis, have suggested that the maximum in ATP after about 40 sec light (STREHLER, [62, 63]) corresponds to the second minimum in orthophosphate. The first minimum then might indicate the formation of another, unknown phosphate compound "which is built at the energy transfer system in close connection with the act of energy transfer, and that it is closely related to the primary energy acceptor in photosynthesis" (75). The compound, recently suggested by KANDLER (32) may well be identical with the one to the existence of which WASSINK and SPRUIT have concluded previously (75).

In view of the fact that polyphosphates are found normally in separate cell particles (14, 60) it still seems most plausible that the immediate donor of ~ph for the polyphosphates is a generally distributed compound like ATP, *cf.* p. 117.

The sigmoid shape of the light intensity curves of both phosphate fixation and photosynthesis in the range of light limitation in the presence of DNP, asks for a special comment. At light intensities below the compensation point, the 'dark' type of inhibition of phosphorylation seems to prevail over the 'light'

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[1]) I wish to thank Dr O. KANDLER sincerely for sending me this paper in manuscript.

type. One may think that the first amounts of ~ph arising in the light, are preferently used for the basic metabolism, which is deficient in ATP as a result of the 'uncoupling' owing to DNP. Further amounts of ~ph would be available for phosphate fixation, or photosynthesis, in proportion as they arise.

Another approach would be possible if we assume that a special mechanism is operating at low light intensities, different from the system, operating in photosynthesis at higher light intensities. KOK (35) and VAN DER VEEN (67), *e.g.* have assumed that, below the compensation point, a special, more efficient mechanism operates, which also is connected with the production of high energy phosphates. Our observations would then lead to the supposition that this special mechanism is very sensitive to DNP, whereas the mechanism working at higher light intensities is less sensitive. Gas exchange reflects the state of phosphorylation in the same way as the formation of polyphosphates does. In the experiments with phenylurethane the data indicated that phosphorylation in dark and at low light intensities were somewhat less sensitive to the inhibitor than the generation of ~ph at higher light intensities. Also this observation could fit in the scheme of different mechanisms below and above compensation. The compensatory mechanism would be insensitive in this case, causing a steeper slope of the curve at low light intensities. It must be remarked, however, that in no instance a higher efficiency of phosphate fixation, or of gas exchange, in the way suggested by KOK (35), was observed in our controls. For a review of the pertinent literature, see *e.g.* (6).

STREHLER's observations (62, 63) may be quoted here. As mentioned on p. 118, he found an accelerated rate of generation and utilization of ATP by *Chlorella* in light. The stationary level, after 10 minutes illumination was maximal at a low light intensity. For an explanation, STREHLER suggests:

> "If ATP were both generated and utilized by photochemically driven reactions, whose rate constants and saturation values are different, the level should first rise, then fall off somewhat as the second process gains ascendancy" (63).

This could mean, that extra-ATP is available at low light intensities, when respiration is compensated by photosynthesis, with a higher efficiency than that obtaining for photosynthesis at higher light intensities.

The data available on inhibition by sodium azide seem to indicate that the sensitivity to this inhibitor decreases in the following order: polyphosphate formation, photosynthesis, phosphate fixation in the presence of CO_2, (*cf.* p. 113) and respiration. The action of azide resembles that of DNP in several respects.

No experiments were made with NaN_3 at various light intensities, because the activity of this inhibitor seemed to decrease with time. This seemed to be due to the illumination, (see p. 113) so that the rate of decomposition would be higher at high light intensities. This would cause a decrease in inhibition at the high light intensities, especially with the long exposures necessary for the study of formation of polyphosphates.

The formation of polyphosphates was about equally sensitive as photosynthesis to NaF, whereas at the concentrations used, the endogenous respiration was still unaffected, or even slightly stimulated. The active concentrations were about 10 times smaller than those in the photosynthesis experiments of SIMONIS (56), and in the experiments on metaphosphate formation in yeast by YOSHIDA (88). This again must be ascribed to the low pH used in our experiments. The formation of polyphosphates was inhibited at all light intensities. This indicates

that various processes are affected by fluoride, as has also been reported by SIMONIS (56) and KANDLER (32).

Concerning the role of polyphosphates in metabolism, the suggestions and observations made by HOFFMANN-OSTENHOF and his collaborators (23, 24) on the transfer of ~ph between polyphosphates and the adenylic acid systems are valuable. In *Chlorella*, anaerobic incubation in darkness led to a slow disappearance of polyphosphates (this paper). In yeast, metaphosphate disappeared when cells were grown in phosphate-free medium (WIAME, [82]). It thus seems that the polyphosphates constitute a reserve of energy and of phosphate. WIAME has found that the metaphosphate fraction which is insoluble in cold TCA, was more active physiologically than the soluble fraction (82). Apparently the TCA soluble fraction was mobilized only after depletion of the TCA-insoluble one. A recent article by KATCHMAN and VAN WAZER (33) is important in this respect. The polyanionic polyphosphates build complexes with polycationic proteins. The degree of complex formation depends on the dimensions of proteins and polyphosphate chains, and is stronger with long-chain polyphosphates than with short-chain ones. On treatment with TCA, polyphosphates will be precipitated along with the proteins. KATCHMAN and VAN WAZER showed experimentally that precipitation is more complete, the greater the average chain length of the polyphosphates is (33). The reason for the correlation between chain length and physiological activity is unknown so far.

§ 2. SUMMARY

The relations between phosphate metabolism and photosynthesis were studied in the green alga *Chlorella*.

In suspensions of this organism orthophosphate is converted into cellular phosphates at low pH in the light. In the presence of CO_2 this conversion is much smaller than in its absence (Ch. II, § 2; see [76]).

In the absence of CO_2 the greater part of the phosphate is transformed to polyphosphates, which are mostly insoluble in cold TCA; they are soluble in hot HCl, or in dilute NaOH at room temperature (Chapter III). This formation of polyphosphates in the light continues for several hours, at a slowly decreasing rate. It is light saturated at much lower light intensities than is photosynthesis of similar suspensions in the presence of 5% CO_2 (Ch. IV, § 1). It is maximal about pH 4, and decreases towards zero at pH 7–8 (Ch IV, § 2). It does not require oxygen (Ch. IV, § 3), and is not affected by nitrate (Ch. IV, § 4). In the presence of glucose the formation of polyphosphates decreases (Ch. IV, § 5). The formation of polyphosphate in light is much less sensitive to phenylurethane than photosynthesis, and its inhibition is due to an effect on the photochemical reaction only. Light saturation is therefore shifted to higher light intensities (Ch. V, § 1).

2–4 Dinitrophenol affects photosynthesis and the formation of polyphosphates in a similar way at all light intensities. For both processes inhibition is particularly strong at low light intensities, and decreases steadily with increasing light intensities (Ch V, § 2). At low pH, the effect of DNP was influenced also by the density of the suspension. The observations could be reasonably explained by assuming that the cell membrane is impermeable to the ionic form of the inhibitor and that pH inside the cells was several units higher than in the medium (Chapter V, § 2, *b*). Sodium azide inhibits photosynthesis and formation of

polyphosphates to about the same degree, the latter process being somewhat more sensitive (Ch. V, § 3). Sodium fluoride inhibits photosynthesis and formation of polyphosphates to about the same degree (Ch. V, § 4). The reported experiments can be understood by assuming that energy-rich phosphates are formed in close connection with the photochemical reaction of photosynthesis, and that ~ph is transferred to polyphosphates when photosynthesis is curtailed by lack of CO_2. The experiments indicate that also glucose is an acceptor for ~ph in light, independent of CO_2.

Phosphate fixation in light in the presence of CO_2, differs not only quantitatively, but also qualitatively from that in the absence of CO_2, as is indicated by the greater chemical stability of the compounds formed (see [86]), the greater sensitivity of this accumulation to phenylurethane (Ch. V, § 1) and its smaller sensitivity to azide (Ch. V, § 3).

Phosphate fixation in darkness is distinguished from the formation of polyphosphates in the light in that it requires oxygen. The dark fixation is less sensitive to glucose, phenylurethane and NaF, and more sensitive to DNP than fixation in light.

A slow decomposition of polyphosphates accompanied by release of orthophosphate was observed in the dark, in suspensions at pH 7 under anaerobic conditions (Ch. IV, § 3).

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STELLINGEN

I

Lichtenergie kan door *Chlorella* photosynthetisch worden vastgelegd in polyphosphaten wanneer het normale photosynthetische ketenproces is geblokkeerd door afwezigheid van koolzuur.

Dit proefschrift

II

Voor de vorming van energierijke phosphaten door *Chlorella* in het photosynthese proces is geen zuurstof vereist.

Dit proefschrift

III

De waarnemingen betreffende de invloed van dinitrophenol op het metabolisme van *Chlorella* pleiten ervoor dat slechts ongedissociëerde moleculen in de cel binnendringen.

Dit proefschrift

IV

Er is geen afdoende bewijs dat het auxine metabolisme een causale rol speelt bij de bloeiinductie.

V

Het begrip 'endogeen rhytme' is nuttig ter verklaring van photoperiodiciteitsverschijnselen, maar mag daarbij niet worden opgevat als autonoom rhytme.

P. F. WAREING, Phys. Plant. 7, 157–172 (1954)
E. BÜNNING, Phys. Plant. 7, 538–547 (1954)

VI

Ten onrechte meent SCHAFFALITZKY DE MUCKADELL, dat bij een meerjarige beuk een deel van de boom in een jeugdstadium verkeert.

Phys. Plant. 7, 782–796 (1954)

VII

De overeenkomst tussen viren en genen ligt in het feit, dat beide structuren, specifieke enzymatische processen vanuit centrale punten dirigeren.

VIII

Het voorkomen van ballistosporen bij de *Tilletiaceae* moet in aanmerking worden genomen bij de systematische plaatsbepaling van deze familie.

A. H. R. BULLER en T. C. VANTERPOOL,
Researches on fungi, V, 1933

IX

Het verdient aanbeveling, de mogelijkheid het ingenieursexamen aan de Landbouwhogeschool te beperken tot 2 of 3 vakken, ook open te stellen voor candidaten die tijdens het candidaatsexamen geen blijk hebben gegeven van buitengewone bekwaamheid.

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Wageningen 1955