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STUDIES ON THE CYCLE OF ELEMENTS IN FRESH WATER

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CHAPTER I

INTRODUCTION

In this paper a report is given of an investigation into the chemical cycle in dutch fresh water lakes. In the biocoenosis in these lakes two cycles occur, that of the organisms and that of the chemical elements. Both cycles are very closely interwoven.

In the cycle of organisms the algae occupy a primary position. The existence of all subsequent heterotrophic organisms in the cycle depends fundamentally on the consumption of the autotrophic ones. We here make a differentiation between consumers – organisms, that live exclusively on phytoplankton – and predators, organisms, that feed on consumers and predators.

The cycle of elements is determined chiefly by the uptake from and release into the milieu of chemical components by the living organisms, together with the abiotic processes in the surrounding medium which are indispensable to convert the excreted compounds into substances, that can be absorbed again. The cycle consists of a great number of continuously interlocking conversions. It is, however, useful to divide the cycle artificially into a number of discontinuous steps; an element is considered to have changed from one step to the next, when it has been transferred to a compound, classed differently in chemistry. The requirements for this classification can be different for various cases. Ecological equivalence is more important here than chemical equivalence. When a thus defined transfer has resulted from a change in the cycle of organisms, we speak of a link between both cycles.

A link between both cycles is met in photosynthesis. Another link occurs, when an alga dies – by whichever cause – and returns to the water a part of the assimilated elements. In general the various elements are given off from dead algae into the water at different rates, some compounds having such a low rate of dissolution, that we are inclined to speak of slags. The type of the biocoenosis determines the nature and the amount of the slags. Small modifications in the

composition of the biocoenosis can involve the conversion of a slag into an absorbable compound; this can also be effected by abiotic processes.

Phenomena, in principle analogous to those happening at the death of an alga, occur at its consumption by a heterotrophic organism, or, more generally, at the transfer of matter from one organism to another. For, when a consumer contains two elements in a ratio differing from that in the consumed organism, the quantity of the element, present in the latter in excess, will be excreted by the former and therefore liberated. The other element will be liberated later, viz. when the consumer in turn dies off. Here also the originally simultaneously bound elements reappear in the water at different moments.

Caused by these processes shiftings in the mutual ratios of the concentrations occur. These are often of greater importance than the concentrations of certain chemical substances as such for the selection of the organisms, which, under certain outward circumstances, get a chance to develop.

Thus, for instance, we may observe that the consumption of organism A by B opens up the way for C to develop, either because A feeds on C, or because A through its composition disturbs the concentration ratio, necessary for the growth of C, and this ratio is restored after the consumption of A by B. In the latter case the influence comes about via the cycle of elements. This cycle is as much dependent on as it is necessary for the cycle of organisms, as least as long as we can exclude external influences. For, the concentrations of the chemical elements, necessary for growth, and the forms in which those elements occur, are dependent immediately on the passage of these elements through the various organisms. Nitrogen, which may occur in the water in various organically bound forms, as three different ions and finally even as dissolved N_2, is an excellent example of this. It is forthwith clear, that we have a very complex situation here, about which very little is known quantitatively.

The possibilities here mentioned are only a few out of many, but they serve to illustrate that the starting-point for any ecological-hydrobiological research must be the knowledge – both analytical-chemical and physiological – of the individual needs and composition of the various organisms involved. Composition and structure are important because the nature of the involved material determines whether in a change of circumstances the elements will find their way through the cycle easily or with difficulty.

Understanding of the whole of processes enacted in a lake can only be acquired by approximation of the separate facets, by dividing the biocoenosis and through it the cycles in small parts and subjecting these isolated parts to a quantitative investigation in a laboratory. After that it must be tried to fit the acquired data into a larger ecological unit, where every change in a concentration results from the difference between increase and decrease, out of which at least one process has to be known quantitatively to acquire an idea of what

really happens. The fitting of data from a smaller into a larger ecological scale will have to be done by degrees, by changing from the laboratory to big tanks of several cubic meters, filled with known starting-material, and subsequently to an investigation in a natural lake.

The first requirement to be made for quantitative laboratory investigation is the determination of the distribution of a chosen element over the various ecologically determined fractions, while the sum of these fractions always has to be equal to what was present at the start of the experiment. The greatest difficulty is here that it has to be possible to draw a sample at random from the total amount of material present in the experiment. It must be feasible to make the studied object homogeneous.

To obtain a square balance of the material we have set ourselves the restriction for the present to involve no elements in our investigation that can take a part in the processes in gaseous state. Otherwise the work would have to be done in a fully closed system, which entails experimental difficulties.

The facet dealt with in this paper – which is to be considered, not only as a survey of the results obtained, but even more as a working-programme – concerns the passage through algae of certain elements, which are essential for the growth of these algae. We have restricted ourselves for the present to nitrogen, phosphorus, silicium and iron.

More than to the uptake of these elements by the algae – to which moreover rather much attention has been paid in literature – we have directed our attention to the question what the fate of these elements is, when an alga dies. Does the dead cell withdraw the absorbed elements from the cycle of elements for a prolonged time, thus rendering impossible a subsequent bloom of algae, or do these elements leave the cells speedily by whatever mechanism? It is of great importance here to be acquainted with the differences in dissolution rates of the various elements and the ratio between the amount of incorporated material and the amount of still present reserve material.

The solution of this problem in ecology is of great importance for the question, what the influence is on the cycle of the in nature often occurring bloom of algae – the curious phenomenon that, usually in spring and autumn, a special alga develops suddenly in quantity in a certain lake and often dies off at an even quicker rate.

We have been able to answer this question in part, by which it has become possible in principle to foretell, what changes will occur in a lake, when a once appeared bloom of algae dies off again. When moreover the most favorable conditions for the growth of the more commonly occurring algae are known, it must be possible to foretell more or less, what will happen in the next period.

When this work will be extended to other organisms or to an investigation in lakes, it will become more and more difficult to meet the above made experimental requirements, especially when the

biocoenosis deals with organisms of a larger size and also when the organisms can cause an inhomogeneous distribution over the milieu. To a lesser extent we encounter this difficulty also in nature when working with algae, because they accumulate in the upper, more illuminated, layers and are deposited in the lower layers after dying off. In the laboratory it was always possible to make homogeneous suspensions, by stirring through aeration or by shaking.

It is outside the scope of this work to go extensively into the function of the mud in a lake and the experimental difficulties which may arise there. The determination only of the concentration of an element in water in contact with mud is quite senseless, when one can not at the same time determine the possibly important reserve stock of that element in the mud. Notwithstanding repeated efforts – partly with the use of radio-active isotopes – there is as yet no method known to measure satisfactorily the buffering effect of mud on the cycle of elements.

In laboratory experiments we can make an instantaneous picture at any moment of the distribution of an element over its compounds and of its localisations; in investigations in natural water this will hardly ever be the case. But it may prove to be of significance, when we can give meaning to a certain phenomenon in a lake on account of similar observations obtained from laboratory experiments.

CHAPTER II

THE ORGANISMS

Choice of object

As has been mentioned in the introduction, we wanted for our investigation an alga that is a common inhabitant of the dutch lake water, and that can be easily cultivated under conditions similar to those in a lake, especially as far as the concentrations of the required nutrients are concerned, while its chief physiological properties must be rather accurately known.

Of the algae obtained by us – vid. page 6 – *Scenedesmus quadricauda* and *Stephanodiscus Hantzschii* meet these requirements very well and were used in our experiments.

Technique of isolation

Bacteria-free, unialgal cultures were obtained in different ways.

Lake water was placed in the laboratory in the light till a growth of algae became distinctly visible. When necessary 70 mg of KNO_3 and 5 mg of KH_2PO_4 were added to 1 liter of water, the concentrations being nearly equal to Rhode's culture medium No. 8 (Rhode 1948). The algae were isolated from this solution, either with a micropipette or by inoculating in Petri dishes on an agar-medium, consisting of enriched lake water solidified by 2.5 % agar (Pringsheim 1946). The first method is not suitable for diatoms, because these may become attached to the glass.

Sterility was tested by means of peptone-glucose plates or of liquid media. Not always the obtained cultures proved to be sterile. Repeated subculturing of the non-sterile cultures on Petri dishes usually led to sterile cultures. Occasionally penicillin was added.

The following species were obtained:

Scenedesmus quadricauda (Turpin) Kützing
Ankistrodesmus falcatus (Corda) Ralfs
Nitzschia communis Rabenhorst
Chlamydomonas, not further identified
Ankistrodesmus convolutus Corda
Gonium pectorale O.F.M.
Stephanodiscus Hantzschii Grun.
Chlorella vulgaris Beyerinck
Stichococcus bacillaris Naegeli

The bacteria-free cultures were kept, partly in liquid media, partly in agar media in tubes. The stock cultures were subcultured every two or three months.

Culturing

As a culture medium for the green algae we used Rodhe's culture solution No. 8 with a few minor modifications. The amount of $Ca(NO_3)_2$ used was only 10 % of that indicated by Rodhe. As a source of nitrogen we used NH_4NO_3, the pH of the solution remaining more constant then during the growth of the algae. $NaHCO_3$ was added, when a rapid growth was required. Fe-citrate was substituted by Fe-EDTA (ethylenediaminetetraacetic acid), because this stock-solution can be kept longer. In a few special experiments, in which no nitrate was allowed to be present, NH_4Cl was used, whereas $Ca(NO_3)_2$ was substituted by $CaCO_3$, which counteracts a possible acidification of the medium. More EDTA is then added, because otherwise Ca binds the EDTA of the Fe-EDTA solution.

Modified Rodhe's culture medium for green algae:

$Ca(NO_3)_2.4\ H_2O$ 10 mg or $CaCO_3$ 50 mg + EDTA 185 mg; $MgSO_4.7\ H_2O$ 10 mg; KH_2PO_4 5 mg; NH_4NO_3 30 mg; $NaHCO_3$ 75–100 mg; Fe-EDTA sol.[1]) 5 ml; H_2O 1000 ml.

The green algae could be cultured on this medium for long periods. Some of the species we have been subculturing for six years now.

The diatoms could not be cultured on this medium, when only Na_2SiO_3 was added. They grew fairly rapidly after adding purified yeast-autolysate, or according to Pringsheim (1946), soil-extract. In high concentrations non-purified yeast-autolysate proved to act as an inhibitor.

Modified Rodhe's culture medium for diatoms:

$Ca(NO_3)_2.4\ H_2O$ 10 mg; $MgSO_4.7\ H_2O$ 10 mg; $Na_2SiO_3.9\ H_2O$ 50 mg; KH_2PO_4 5 mg; NH_4NO_3 30 mg; Fe–EDTA–sol.[1]) 5 ml; H_2O 1000 ml, Soil extract 20–50 ml; purified yeast-autolysate 5–10 ml.

The purified yeast-autolysate was prepared as follows:

1 Kg of yeast in 1 liter of H_2O is kept at 60° C during one night. This suspension

[1]) In a solution of 0.8 g of Na_2–EDTA per liter 1.0 g of $(NH_4)_2SO_4.Fe_2(SO_4)_3.24\ H_2O$ is dissolved without heating.

is boiled and after cooling centrifuged. To the supernatant three times its volume of acetone is added, followed after one hour by filtration. The filtrate is evaporated to 200 ml and kept in a refrigerator for one night. After filtration a clear pale yellow solution is obtained, which, after sterilisation, can be stored for many months. Higher concentrations than the above mentioned had neither a more stimulating nor an inhibiting effect on the growth of the diatoms.

Instead of by yeast-autolysate or soil-extract, modified Rodhe's No. 8 solution could be improved also by using p.a. chemicals (Merck or BDH) and adding $NaHCO_3$ and $CaCO_3$. As no Na_2SiO_3 p.a. is obtainable, this was purified by precipitation of the SiO_2 with H_2SO_4, washing with water and fusing afterwards with $NaHCO_3$ p.a. at 1000° C.

With p.a. chemicals and purified Na_2SiO_3 the growth of the diatoms was as good as in modified Rodhe's solution with purified yeast-autolysate or soil-extract; the only difference being, that they deteriorated quickly in the light after the growth had stopped by lack of nutrients.

The cells were cultured in Fernbach-flasks or in flat-bottomed 10 liter flasks.

The Fernbach flasks are filled with one liter of culture medium and closed with rubber stoppers provided with an inlet and outlet of air; the latter is also used for inoculating. Seven of these flasks are placed in an aquarium tank of 3 × 15 dm with a glass bottom, under which two fluorescent lamps (Philips 65 Watt) have been mounted, so that the distance to the flasks is about 15 cm. Through the tank – filled with distilled water – run two metal tubes, through which tap-water can be led, via a waterseal operated by means of a thermostate. As a rule we cultured our material at 20° C.

For special purposes the cells are also cultured in 10 liter flat-bottomed flasks. Through the rubber stoppers are now fitted three tubes, the inlet and outlet of air and a siphon. The inlet ends 5 cm above the bottom of the flask in a horizontally placed quarter circle with a radius slightly smaller than that of the flask at that level. In this glass loop holes have been made of about 1 mm diameter, spaced at a distance of 1 cm, causing sufficient bubbling of air to keep the green algae in a homogeneous suspension, which is not the case in the aeration of the Fernbach flasks. The inlet is, via a rubber tube lined inside with talcum-powder, connected with a small bell-jar closed with cotton-wool. The rubber tube is closed during sterilisation. Inoculation is performed through the air outlet. Samples of the culture medium can be siphoned out of the flasks without contamination, as the outside leg terminates in a little bell-jar with a downwards facing aperture. During sterilisation the siphon is drawn out of the liquid and closed with cotton-wool and afterwards pushed down and closed with a rubber stopper.

This apparatus enables us to obtain about 8 liters of culture, from which at different moments about 5 liters in total can be drawn and, if required, replaced by fresh culture medium.

Most experiments were carried out with *Scenedesmus quadricauda*. We also cultured *Stephanodiscus Hantzschii* and *Nitschia palea*, both of which

grew better without aeration. A disadvantage was, that the diatoms clot together very closely. A homogeneous suspension was best obtained by carefully shaking the diatoms with glass pearls of 5 mm and removing the remaining clots by filtering over glass wool.

At two to thee weeks' intervals so much nitrogen, phosphorus, silicium, iron and magnesium was added to the remaining cultures, that the original concentrations were attained once more. We noticed, that after a few days the culture solution contained no traceable amounts of silicium and phosphorus any more. For nitrogen the same applies after a slightly longer period. After depletion of nitrogen and phosphorus the dry weight of the cells can still increase for a prolonged time.

Filtration and centrifugation

The quantitative separation of *Scenedesmus* cells and their liquid medium can be obtained in principle by filtration or centrifugation. Both methods have their specific difficulties.

For filtrations very hard filters have to be used, entailing very low filtration-rates. The filters are soon blocked, thus rendering subsequent washing of the cells impossible. It is also very difficult – usually even impossible – to remove the cells from the paper. This method is therefore only applicable when the filtrate is wanted. When the cells have to be isolated, the suspension has to be centrifuged. The best method is to concentrate the suspension by leaving the cells to settle, and to centrifuge afterwards, as this decreases the disturbing whirling of the cells. In this connection it is important to remark that the centrifugation tubes with round bottoms give the best results.

Repeated washing of an amount of *Scenedesmus* cells entails increasingly worse results.

Recently we obtained good results by using Schott G 5 M glass filters. This method proved to be successful for obtaining cells (even washed ones) as well as fluid.

In the course of our experiments we had to centrifuge the *Scenedesmus* cells from solutions of 20 % and 60 % ethanol and 5 % trichloroacetic acid. No difficulties were met with, even after repeated washings. This did not apply to *Ankistrodesmus* and the diatoms used by us. The separation between fluid and these cells was effected by filtration through a Schott G 5 M glass filter.

CHAPTER III

METHODS

In this chapter a survey is given of the various analytical methods used. In general the common procedures were applied; the modifications that were necessary are discussed in detail. Each procedure used was tested thoroughly for recovery of known quantities added.

It has to be stressed that any given procedure yielding satisfactory results for our extracts and the lake water used by us is not necessarily suited to any other type of water.

All determinations were carried out colorimetrically by means of the Zeiss photometer Elko III.

DETERMINATIONS

Ammonia

The amount of ammonia, present in the solution (lake water or culture medium) was determined by distillation of the alkaline solution and nesslerization of the distillate.

An aliquot of the solution, containing less than 1 mg of $N(NH_3)$, is made alkaline with a saturated borax-solution with phenolphtalein as an indicator, to pink colour, and distilled with a microdistillation apparatus after Parnas–Wagner into a 30 ml volumetric flask, containing 2.5 ml of 0.03 N H_2SO_4.

It is imperative to have the aperture of the condenser end under the surface of the H_2SO_4. If this is omitted – as happens rather frequently – losses up to 30 % may occur.

1 ml of Nessler's reagent is added to 20 ml of distillate. The extinction is measured, after one hour at room temperature, by means of filter 42. If necessary, the distillate is diluted 4 times beforehand. The extinction is proportional to the quantity of $N(NH_3)$ up to 200 μg. The smallest quantity that can be determined with an experimental error of 1.5 % is 3 μg when a 5 cm cuvette is used.

Nessler's reagent is prepared by dissolving 100 g of HgI_2 (red) with 70 g of KI in 400 ml of H_2O. After adding 100 g of NaOH in 500 ml of H_2O, the solution is diluted to 1 liter (SNELL and SNELL 1954). This reagent can be stored in a refrigerator for several months.

This method of preparing Nessler's reagent is more satisfactory than that, generally used, in which $HgCl_2$ is dissolved in an excess of KI, in which case the calibration curve is often not a straight line and the molecular extinction varies greatly.

Nitrite

An aliquot of the solution to be tested on NO_2', containing less than 35 μg of $N(NO_2')$, is diluted to 100 ml in a 100/110 ml volumetric flask. At room temperature are added 5 ml of a 0.2 % sulphanilamide solution in H_2O, immediately followed (or simultaneously) by 2 ml of a 20 % HCl solution, after 3 minutes by 1 ml of a 0.5 % ammonium-sulphamate solution in H_2O, and after another 3 minutes by 1 ml of an 0.1 % solution of N-(1-naphtyl)ethylene diamine.di-HCl in H_2O. (This reagent has to be kept in a refrigerator).

Finally the solution is diluted to 110 ml.

The extinction can be measured after 15 minutes by means of filter 53 and is proportional to the quantity of $N(NO_2')$ up to 35 μg. The smallest quantity that can be determined with an experimental error of about 1.5 % is 1 μg of $N(NO_2')$ per 100 ml of the solution in a 5 cm cuvette. The blank is prepared by addition first of the ammoniumsulphamate solution and then of the other reagents.

This method is similar to that described by SHINN (1941) and

modified by SIJDERIUS (1954). Sijderius, however, adds first the HCl and then the sulphanilamide. In the lake water in question we recovered by this procedure only 90 % of the nitrite added. This deficit increased, when the interval between the addition of the HCl and the sulphanilamide is prolonged. It is obvious, that the HNO_2 disappears from the acidified lake water.

Nitrate

Nitrate was determined by means of the xylenol method as published elsewhere (GOLTERMAN 1955).

The extinction is proportional to the quantity of $N(NO_3')$ up to 500 μg. The smallest quantity that can be determined with an experimental error of 1.5 % is 1 μg of $N(NO_3')$ (5 cm cuvette).

Total nitrogen

The determination of the soluble organically bound nitrogen (N^{sol}_{bound}) and the in algal cells incorporated nitrogen (N^{cell}_{tot}) was carried out by means of digestion after Kjeldahl.

An aliquot of the material to be analysed is heated with 2 ml of H_2SO_4 96 % and 10 drops of a 10 % $CuSO_4$ solution in H_2O till the digest has been colourless for at least 15 minutes. When much carbon appears during the destruction, the product is left to cool after complete carbonisation, and 10–20 drops of a 30 % solution of H_2O_2 are added to it, after which it is heated again until it is colourless. In the digest the NH_3 is determined in the usual way; for the alkalinisation a 10 % NaOH solution is used.

The difference between the total amount of nitrogen in the solution (N^{sol}_{tot}) and the amount of $N(NH_3)$ is the quantity of the soluble bound $N(N^{sol}_{bound})$; the difference between the amount of nitrogen in a suspension (N^{susp}_{tot}) and in its filtrate (N^{sol}_{tot}) is the total quantity in the cells (N^{cell}_{tot}).

Inorganic phosphate

Originally the inorganic phosphate (PO_4''') was determined by measuring the extinction of the blue colour of phosphomolybdate, reduced with $SnCl_2$, in 1.0 N H_2SO_4. However, as we wanted to determine phosphate in the presence of labile phosphate-esters, and also in digests with a varying amount of H_2SO_4, this method was not satisfactory. Good results were obtained by the procedure after Berenblum and Chain, as modified by MARTIN and DOTY (1949). They extracted the phosphomolybdate formed with an isobutylalcoholbenzene mixture, after which an aliquot of the product was diluted with ethanol and subsequently reduced with $SnCl_2$.

Instead of extracting with a mixture of isobutylalcohol and benzene we used a mixture of n-butanol and benzene, as this appeared to separate quicker after shaking. We considered it simpler and more reliable to reduce the whole quantity of the extract. Moreover this makes the method more sensitive.

It is indispensable to determine the most favorable n-butanol-

benzene ratio. A mixture of about 80 % n-butanol and 20 % benzene (v/v) gave the best results (Exp. I). The extraction time was 15 seconds; longer periods did not yield a higher extinction, whatever ratio between n-butanol and benzene was chosen.

EXP. I. *The effect of the n-butanol-benzene ratio in the extraction solution on the* PO_4'''*-determination.*

6 ml of a 5 % ammoniummolybdate solution in 2.5 N H_2SO_4 was added to 25 ml of H_2O, containing 60 μg of P (PO_4'''). The solution was extracted during 15 seconds with 25 ml of a mixture of n-butanol and benzene. The yellow upper layer was washed carefully with H_2O, without shaking, and shaken during 1 minute with 6 ml of an $SnCl_2$ solution (0.5 g of $SnCl_2$ and 2 g of hydrazine-sulphate dissolved in 1 liter of 0.6 N H_2SO_4). The extinction of the blue upper layer was determined, after dilution with ethanol to a suitable known volume.

extraction-medium n-butanol	benzene	extinction × 10^3			
100 %	0 %	250			
50	50	275			
75	25	284			
77.5	22.5	307			
79	21	305	308		
80	20	306	301	308	308
81	19	305	308		
82.5	17.5	299			
85	15	298			
89	11	290			

The extinction did not vary, when during the procedure 3 to 10 ml instead of 6 ml of ammoniummolybdate reagent was added, neither when 5 to 10 ml of $SnCl_2$ solution was used. The maximal value of the extinction could only be obtained by shaking the solution during at least one minute with the $SnCl_2$ reagent. The yellow butanol-benzene layer could also be reduced in a homogeneous phase with a solution of $SnCl_2$ in ethanol or by diluting the mixture with ethanol and adding one drop of a 40 % solution of $SnCl_2$ in fuming HCl. Under these conditions the non-converted molybdate must be removed very carefully, which is possible but laborious.

The requirement was made above, that the method should also be suitable for both Kjeldahl-digests and eluates of ion-exchangers, containing varying amounts of H_2SO_4 and Na_2SO_4. Therefore the influence of both compounds was tested (exp. IIa and IIb).

These experiments demonstrate, that an amount of H_2SO_4 up to 28.5 maeq. in 30 ml of the reaction solution had no disturbing effect on the PO_4'''-determination. The same conclusion is valid for the effect of adding 2 g of Na_2SO_4, provided the reaction mixture is extracted repeatedly by the n-butanol/benzene solution.

Besides its relative independence of the H_2SO_4 or Na_2SO_4 concen-

EXP. IIa. *The influence of varying amounts of* H_2SO_4 *on the* PO_4'''*-determination.*
Experimental data, see Exp. I. 5 ml of a 5 % ammoniummolybdate solution in H_2O was used and varying amounts of H_2SO_4 were added.

μg P (PO_4''')	H_2SO_4 present in the reaction medium	extinction × 10^3/100 μg P			
50	12.5 maeq.	516	520		
	17.8	515			
	28.5	513			
100	12.5 maeq.	510	516	513	515
	17.8	516			
	20.5	520			
	28.5	514			
	35	450			

EXP. IIb. *The influence of varying amounts of* Na_2SO_4 *on the* PO_4'''*-determination.*
Experimental data: See Exp. I. — 5 ml of a 5 % ammoniummolybdate solution in 2.5 N H_2SO_4 was used as reagent. — Varying amounts of Na_2SO_4 were added.

μg P (PO_4''')	Na_2SO_4 added	Extinction × 10^3/100 μg P after 1 extraction with 25 ml 80 % butanol/20 % benzene				after 2 extractions with 25 ml 80 % butanol/20 % benzene			
50	0.0 g	516	520						
	1.2	513							
100	0.0	510	516	513	515				
	1.2	490					515		
	2.0	480					508	516	

tration of the reaction mixture, this method has the advantage, that small quantities of PO_4''', in relatively low concentrations, can be determined, if the solution is repeatedly shaken with the n-butanol-benzene mixture. In this way 2 μg of P(PO_4''') could be determined in 250 ml of H_2O with an error of 2 %.

As the H_2SO_4 needs to be present in the reaction medium only for a very short time (less than one minute), the inorganic phosphate can be determined beside labile phosphate-esters. (LINDBERG and ERNSTER 1956).

The procedure for the PO_4'''-determination runs as follows:

To 10 to 35 ml of PO_4'''-solution is added 5 ml of a 5 % solution of ammoniummolybdate in 2.5 N H_2SO_4, if the solution does not contain more than about 14 maeq. of H_2SO_4, and 5 ml of a 5 % solution of ammoniummolybdate in H_2O if the solution contains 14 to 28 maeq. of H_2SO_4. 12 to 15 ml of a mixture of n-butanol and benzene (8 : 2,

v/v) are added. After shaking for 15 seconds and complete separation of both layers the lower layer is removed. Some water is squirted inside the separatory funnel and let out again without shaking, by which the molybdate reagent, still adhering to the glass, is removed. The phosphomolybdate-solution is then shaken during one minute with a reducing agent, which is prepared by dissolving 2 g of hydrazine-sulphate through heating in 1 liter of 0.6 N H_2SO_4 and adding, after cooling to about 15° C, 0.5 g of $SnCl_2$ (this solution can be stored in a refrigerator for months).

The lower layer is removed completely and the upper layer diluted to a suitable known volume with ethanol 96 %. The extinction is determined with filter 72.

It is advisable to make the separatory funnel water-repellent with Desicote and to use a silicone-grease for the cocks.

The amount of phosphate, that can be determined in this way, is 1 to 200 μg of $P(PO_4''')$. The calibration curve is straight in this area. the experimental error is about 1.5 %.

Finally we draw attention to a disturbance, which can occur in some kinds of water or of cell-suspensions, when the aqueous lower layer also colours blue after reduction. This could be avoided by adding to the sample 20 maeq. of H_2SO_4 and so many drops of 0.1 N $KMnO_4$ that, after being left standing for 15 minutes, the last 2 or 3 drops did not lose colour. Subsequently the mixture of n-butanol and benzene is added and the two layers are shaken together for a moment, by which the excess of $KMnO_4$ is removed. After this the solution of ammoniummolybdate in H_2O is added and the determination carried out as described.

Total phosphate

The material, in which originally bound phosphate occurs, is destructed in the usual way with 2 ml of H_2SO_4 96 %. The digestions were carried out in Kjeldahlflasks of 25 to 50 ml with a neck of about 30 cm to counteract the evaporation of P_2O_5 with the H_2SO_4. The neck serves as a reflux-condenser.

After cooling the solution is boiled with about 10 ml of H_2O and kept hot for 10 minutes to bring about decomposition of the possibly formed pyrophosphates, after which the solution is cooled and diluted to 30 ml. 5 or 10 ml of this product, containing resp. 12 and 24 maeq. of H_2SO_4, can be used directly for a PO_4'''-determination with an ammoniummolybdate solution in H_2O.

When H_2O_2 has been used as a catalyst for the digestion, the digest is treated twice with water. In the first treatment it is distilled off to remove the H_2O_2 that had not been decomposed and in the second one the pyrophosphate is decomposed.

The difference between the total amount of phosphate in the solution to be tested (P^{sol}_{tot}) and the amount of PO_4''' is the quantity of the soluble bound P (P^{sol}_{bound}); the difference between the amount of phosphate in a suspension (P^{susp}_{tot}) and its filtrate (P^{sol}_{tot}) is the total quantity in the cells (P^{cell}_{tot}).

Silicate

SiO_3'' was determined according to the method of MILTON (1951). It is converted by Na-molybdate to silicomolybdate, which is reduced with $SnCl_2$ to a blue coloured compound.

Milton recommends to form the silicomolybdate complex at 100° C, as in his experiments this reaction was not complete at 20° C and higher temperatures lead to higher extinction values. We found, however, that at 20° C the reaction was complete and resulted in a constant extinction value, although a lower value was obtained than at 100° C. Contrary to Miltons findings heating has to our experience the disadvantage of colouring the blank far more intensively. For these reasons we carried the reaction out at room temperature, as the method was moreover sufficiently sensitive for our investigations.

The determination is carried out as follows:

An aliquot of the liquid to be tested (pH 5–8), containing 1 to 200 μg of $Si(SiO_3'')$ is diluted with water to 15 ml. 2 ml of a molybdate solution is added, containing 5 g of $Na_2MoO_4.2H_2O$ in 100 ml of 0.5 N H_2SO_4. This reagent can not be used sooner than 48 hours after it has been prepared, as this would lead to varying extinction values.

15 minutes later 2 ml of H_2SO_4 (s.g. 1.84) is added and the solution cooled to room temperature. Finally 1 ml of a 1:100 with water diluted $SnCl_2$ solution is added in drops. The stock solution contains 40 g of $SnCl_2$ dissolved in and diluted to 100 ml with fuming HCl, and can be stored in a refrigerator for months.

The extinction of the reduction product, constant after 10 minutes, is measured with filter 75. Its value is proportional to the quantity of $Si(SiO_3'')$ up to 200 μg. The smallest quantity, that can be determined with an experimental error of 1.5 % is 1 μg (5 cm cuvette).

As a standard we used $Na_2SiO_3.9\ H_2O$, which was 99.7 % pure according to acidimetric and gravimetric (crystal-water content) determinations.

The SiO_3''-determination may be disturbed by the phosphate, present in the solution. This was rectified by determining the extinction of phosphate-standards, treated according to the procedure for SiO_3''-determinations, and correcting the obtained extinctions of the tested samples in this respect. It appeared, that this correction depends on the $Na_2MoO_4.2\ H_2O$ used, possibly through the presence of varying amounts of $Mo_7O_{24}^{6-}$. The cause of these differences has not been traced further; it is very probable, that the differences in Milton's and our findings can also be attributed to the Na-molybdate, although perhaps the difference between his and our standards is of importance too.

Total silicium

For the determination of the total amount of Si present in cells, a known quantity of the diatom suspension is evaporated till dryness with 2.50 g of $NaHCO_3$ in a nickel crucible and heated subsequently

in an electric furnace to 1000° C. After cooling the contents are dissolved in H_2O, neutralized with HCl and diluted with H_2O to a known volume (100–1000 ml), depending on the expected Si-content. Then Si_{tot}^{susp} is determined with the method described above.

The value of Si_{tot}^{cell} is calculated as the difference of Si_{tot}^{susp} and SiO_3''.

FRACTIONATION OF THE COMPOUNDS IN SCENEDESMUS QUADRICAUDA

As it is not feasible, nor necessary, to carry out a complete analysis of the conversions of all compounds involved in the chemical cycle of algal cells, the investigation was restricted to a division into groups of compounds. Then it can be followed up, how the ecologically equivalent compounds are distributed over these groups.

The following fractionation technique proved to be very useful in practice for P- and N-compounds:

The algal cells were boiled after adding ethanol to the suspension to a concentration of 60 %. The alcohol-soluble compounds (fraction alc 60 %) were separated by centrifugation from the insoluble residue (residue A), which was suspended at 4° C in a 5 % trichloroacetic acid (TCA)-solution. The fraction soluble in it (Fraction TCA-c) was separated from the second residue (residue B). Some of the compounds in this residue could be dissolved under partial hydrolysis either with a 5 % TCA-solution at 90° C (fraction TCA-w) or with 0.25 N KOH at room temperature. All extractions could be carried out in such a way that reproducible and sharply defined fractions were obtained; repetitions of each extraction did not result in an increase of the quantity of the compounds extracted.

Boiling of the cells with alcohol, to obtain a first fraction, has the additional advantage of stopping instantaneously all processes, occurring in the cells, either before or after their breakdown.

Table 1 (Page 19) demonstrates, that under different culturing conditions the relative amounts of the P-compounds in the different fractions may vary considerably. The same holds true for other compounds.

In the following sections is described more in detail which P-compounds are extracted in each of the fractions.

a. *Alcohol-soluble fraction*

This fraction is obtained by boiling the cells in alcohol of 60 to 65 % for 5 minutes and setting the suspension aside at room temperature for 1 to 2 hours, to give the compounds time to leave the cells (exp. IIIb). It makes no difference whether the cells are left at 0° C or at 25° C.

The cells are centrifuged off and in the supernatant the amount of P-compounds, P_{alc}^{cell}, is determined. This amount does not depend on the alcohol percentage as long as this remains between 60 and 70; outside this range it diminishes quickly.

Boiling of the cell suspensions for a time as short as 5–15 minutes does not cause a noticeable hydrolysis of insoluble to soluble material, the P-content of the extract remaining constant. Over longer boiling-

periods the yield does increase and reaches a second level (Exp. IIIa). The extra amount of phosphate, dissolved in the alcohol after a 24 hours' boiling period (in exp. IIIa 43 % — 20 % = 23 %) is formed through hydrolysis out of phosphate from the fraction TCA-w. In accordance with this the fraction TCA-c also increases, viz. from 16 % after 5 minutes to 45 % after 24 hours boiling. Owing to this hydrolysis the obtained extracts are not suitable for further analysis.

Attempts were made to avoid the heating of the suspension by disintegrating the cells mechanically either in an ultrasonic homogenizer or in a blendor at 50000 r.p.m. or in a mortar with quartz or carborundum. These experiments yielded no results, as only part of the cells were damaged.

EXP. IIIa. *Influence of boiling time on the amount of* P-*compounds extracted with alcohol* 60 %.

To cell suspensions, which did not contain PO_4''' any more, ethanol was added to a concentration of 60 %. In an aliquot P_{tot}^{susp} was determined. Samples of these alcoholic suspensions were boiled in a waterbath under a reflux condenser during different periods, after which they were cooled off quickly to 20° C. 24 hours later the samples were centrifuged and the P-content of the supernatant, P_{tot}^{alc}, was determined.

Boiling period	P_{tot}^{alc} in % of P_{tot}^{susp}	
	susp. a	susp. b
5 min	21.4	20.0
10 min	21.0	20.0
15 min	21.5	21.0
20 min	—	21.0
25 min	23.6	—
180 min	40.2	—
6 hrs	48.5	39.0
20 hrs	—	43.0
24 hrs	—	43.0

EXP. IIIb. *Influence of the extraction time on the amount of* P_{alc}^{cell}.

In this experiment the alcoholic cell suspension was heated for 15 minutes. The extraction period at 20° C varied from 0.5 to 24 hours. Other data as in experiment IIIa.

Extraction time	P_{alc}^{cell} in % of P_{tot}^{cell}	
	susp. c	susp. d
0.5 hr	18 20	—
1 hr	20	—
2 hrs	20	23.7
4 hrs	—	24.2
6 hrs	—	23.7
24 hrs	—	23.8

Some of the P-compounds of fraction alc 60 % – the phospholipids – can be separated from it by shaking out with an equal volume of $CHCl_3$, followed by a few hours' standing. They move quantitatively into the $CHCl_3$ after one extraction.

Addition of H_2O during this extraction is not allowed, for it appeared that $CHCl_3$, which can take up most of the alcohol – and with it the phospholipids and pigments – from an equal volume of a 60 % solution, can not do so from a larger volume of a 30 % solution.

In the $CHCl_3$-fraction total phosphate can be determined, when all $CHCl_3$ is removed very carefully by distilling and subsequently producing a vacuum in the still very hot Kjeldahl-flasks. The residue is digested with H_2SO_4 and $CuSO_4$ in the usual way.

The PO_4''' of fraction alc 60 %, which includes the PO_4''' originally present in the cells, can not be determined as long as alcohol or pigments are present. This determination is therefore carried out in the water-layer, remaining after the $CHCl_3$-extraction, which must be repeated twice after the extraction of the phospholipids to remove the last traces of alcohol. The organic P-compounds present in the water-layer can be determined as described above.

b. *Fraction, soluble in* TCA *at* 4° C

Residue A is suspended in a 5 % TCA solution at 4° C. Experiment IV shows, that a certain amount of P-compounds dissolves within 1 hour. As the concentration in the solution does not increase further, we may assume that a definite fraction of P-compounds has been extracted. The residue (residue B) is separated from the TCA-c extract by centrifugation.

EXP. IV. *Influence of the extraction time on the extraction with* TCA *at* 4° C.

Residue A was suspended in 50 ml of a 5 % TCA solution at 4° C. After 0.5, 1 and 2 hours samples were centrifuged, and the P-content of the supernatant, $P^{cell}_{TCA\text{-}c}$, was determined. P^{cell}_{tot} had been determined before.

Extraction time	$P^{cell}_{TCA\text{-}c}$ in % of P^{cell}_{tot} susp. a	susp. b
0.5 hr	—	11.0
1 hr	20.0	20.5
2 hr	21.0	20.8

Fraction TCA-c contains no PO_4''', from which follows that the PO_4''', originally present in the cells, has been extracted quantitatively with alcohol 60 %. It contains compounds, which will form PO_4''' through hydrolysis, when the temperature of the TCA-solution rises to room temperature.

The TCA-c extract shows a definite U.V. absorption spectrum with a maximum between 245 and 250 mμ when all the TCA has been previously removed.

At 4° C a 0.3 N $HClO_4$ solution extracts from residue A more compounds than a 5 % TCA-solution, as appeared from the U.V. absorption spectrum, although the quantity of P-compounds is equal.

A further fractionation of these extracts was not carried out, but might be done chromatographically or by precipitation with $BaCl_2$ at different pH's (ALBAUM 1950).

Increasing the TCA concentration to 10 % yielded no more P-compounds in this fraction. The polyphosphates, mentioned by WIAME (1949, 1958) can not be extracted quantitatively with a 5% TCA solution, from which can be concluded, that no polyphosphates are present in fraction TCA-c.

c. *Fraction soluble in* TCA *at* 90° C

From residue B a third fraction can be obtained by heating it with a 5 % TCA solution during 20 minutes at 90° C and subsequently leaving it to stand for 1 hour at 20° C.

Experiment v shows, that under these conditions amost all P-compounds of residue B are dissolved, ($P^{cell}_{TCA\text{-}w}$), under partial hydrolysis to PO_4'''.

EXP. V. *Influence of heating and extraction time on the extraction with* TCA *at* 90° C.

Residue B was suspended in 50 ml of a 5 % TCA solution. In an aliquot P^{susp}_{tot} was determined. Samples were heated to 90° C for different periods. The suspensions were filtered after a 1 and 2 hours' standing at 20° C. In the filtrates P (PO_4''') and $P^{cell}_{TCA\text{-}w}$ were determined.

Suspension heated at 90° C for	$P^{cell}_{TCA\text{-}w}$ in % of P^{susp}_{tot}		P (PO_4''') in % of P^{susp}_{tot}	
	1 hr at 20° C	2 hrs at 20° C	1 hr at 20° C	2 hrs at 20° C
5 min	59	57	2	4
10 min	82	79	6	7
15 min	92	94	9	10
20 min	95	93	12	12

As is known, the TCA-w extract contains the nucleophosphates. Large quantities of P-compounds of other origin, called "polyphosphates" for the present, can also occur, as appeared from the fact, that different extracts had been obtained with the same extinctions, but a difference in P-contents of 500 %. As it could be expected that the nucleophosphates would react ecologically quite differently from the polyphosphates, it was important to determine the amounts of substances of both groups separately.

The quantity of nucleophosphate present was derived from the extinction values of the TCA-w extracts at 260 mμ. As, however, other compounds may occur in this extract with an absorption at the same wave-length (digestion products of proteins or plant pigments), it remains still necessary to find a more exact method for quantitative analysis of mixtures of nucleo-and polyphosphates.

CHAYEN (1955) has developed a method based on their different rates of hydrolysis to PO_4'''. This method, however, can not be used for our material, because in one experiment the nucleophosphate content determined after Chayen was about 4/5 of the value obtained by measuring the extinction at 260 mμ, while in another experiment it was about 2.7 times as high. In these two experiments the TCA was removed – together with the pigments – from the TCA-w extracts by shaking out twice with an equal volume of ether after addition of H_2SO_4 (0.1 aeq. per liter).

We discovered this method of removal after our series of experiments on autolysis had been closed.

We have not made use of the method of determination of polyphosphates by means of the metachromatic colour reaction with toluidineblue or of the precipitations with $BaCl_2$ at pH 2 and 4, because we were not sure that the phosphates, not belonging to the nucleophosphates, were indeed polyphosphates, and because we considered none of the methods, based on this supposition, sufficiently specific.

Table I demonstrates the varying ratios between the amounts of P-compounds present in the algal cells, depending on the species investigated and the culturing conditions.

TABLE I. *The distribution of the* P-*compounds over the fractions examined.*
The results are expressed as percentage of P^{cell}_{tot}. Data from experiments described in Chapter IV.

Exp.	Alga	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$	P^{cell}_{rest}
3	Scen.q.	54% — —	15%	30% —	—
5	id.	28 — —	9	63 —	4
6	id.	27 — —	11	56 —	3
7	id.	25 — —	32	38 —	4
9	id.	25 — —	12	54 (26) [3]	8
10	id.	33 — —	13	57 (24) [3]	0
16	id.	9 — —	5	82 —	2
17^{I}	id.	23 (12) [1] + (11) [2]	7	64 (32) [3]	3
17^{II}	id.	39 (19) [1] + (20) [2]	13	48 (18) [3]	0
17^{III}	id.	32 (16) [1] + (16) [2]	20	34 (10) [3]	7
18	Ankis.f.	16 — —	2	79 —	3
19	Clado.g.	10 — —	10	67 (23) [4]	13
20	id.	11 — —	17	68 (45) [4]	4
21	Steph.H.	19 — —	41	32 —	5
22	id.	23 — —	28	42 —	7
23	id.	19 — —	5	73 —	3

[1]) soluble in chloroform.
[2]) insoluble in chloroform.
[3]) P (PO_4''') after hydrolysis during 20 minutes.
[4]) P (PO_4''') after hydrolysis during 75 minutes.

CHAPTER IV

LIBERATION AND MINERALISATION

INTRODUCTION

The processes of liberation and mineralisation of elements play an important part in the chemical cycle in fresh water, when phytoplankton dies off. Ecologically, a distinction can be made between the decomposition processes through autolysis, effected only by the organism itself and through metabolic activity of other organisms, e.g. bacterial putrefaction. Consumption is, for the moment, left out of consideration.

The decomposition process starts with autolysis, which leads to an increase of permeability, enabling several compounds, already present in a dissolved state, to leave the cell. Moreover compounds, present in undissolved state, may dissolve. The temperature may be expected to exert a great influence, which can easily be determined, when the physico-chemical rate of the dissolution processes is the limiting factor. But it is also possible that the diffusion through the cellwall and protoplasm membranes limits the rates of dissolution and liberation. Here the size and nature of the molecule is of great importance. Nearly always these rates are increased by preceding hydrolysing processes. These conversions can either happen spontaneously or under influence of the cell enzymes present. Here, therefore, all kinds of factors, which can influence the enzymatic activity, may be of importance.

In order to study experimentally the part, that these autolysis processes have in the chemical cycle, it is necessary to kill the algae in a suspension completely, as quickly as possible and all at the same time, to prevent surviving cells from taking up liberated compounds from the dead cells. Furthermore the structure of the cells must be impaired as little as possible, because otherwise the diffusion rates of the various compounds may undergo inextricable changes. For this reason boiling of the cells in alcohol and other very vigourous means were rejected.

Three methods were used to kill the cells in a suspension: irradiation in an intensively shaken open glass dish (ø 25 cm) with U.V. light from three Philips T.U.V. lamps (30 Watt) mounted at a distance of about 10 cm for 0.5–4 hours; saturation with chloroform; heating during at least 2 hours at 60° C.

It appeared that the amounts of and the rates at which the P- and N-compounds leave the cells were equal when these three methods of killing were compared (Exp. 1, 3, 4 and 5). The only difference found was that the liberated P-compounds were hydrolysed to PO_4''' at a somewhat lower rate when U.V. light was used.

From this accordance can be concluded that the disturbing factors, mentioned above, were absent.

It might be considered whether some other poison than chloroform could be used. Mostly, however, lower yields were then obtained. When butanol, toluene or carbontetrachloride instead of chloroform were used, the same yields of extracted P-compounds were obtained eventually, but the starting rates of the decomposition processes were lower (Exp. 1–3). We did not investigate whether this was caused by a later penetration of the cells by these poisons, by a delayed dying off of the cells, or by an actually lower autolysis rate.

In principle U.V. irradiation is preferable, but, as the chloroform saturation prevents bacterial contamination, we usually made use of the latter method, as we carried out our experiments under sterile conditions. From one experiment it appeared that the autolysis continued normally, when the chloroform was removed after about one hour.

Liberation of P from Scenedesmus quadricauda

Table II gives an orientation about the rates of the different processes, which lead to the liberation of P-compounds. The cells were killed by chloroform treatment and the concentrations of the liberated P-compounds determined after different periods.

TABLE II. *Liberation of* P-*compounds from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period the liberated P (PO_4''') and P_{tot}^{water} were determined. For the experimental data see exp. 5 and 7, page 37 and 38.

Autolysis during	Experiment 5		Experiment 7	
	P (PO_4''')	P_{tot}^{water}	P (PO_4''')	P_{tot}^{water}
	in % of P_{tot}^{cell} at t = 0			
0 hrs	0 %	0 %	0 %	0 %
3.5 hrs	—	—	18	28
5 hrs	43	49	—	—
6 hrs	—	—	29	43
1. days	—	—	48	60
2 days	50	50	—	—
5 days	—	—	65	68
6 days	62	65	—	—
28 days	65	70	—	—

An important part of the P-compounds leaves the cells in a short time. In order to decide whether this is due to simple dissolution or to the action of hydrolysing factors, and whether the diffusion is limited or not, we investigated which P-compounds, grouped as described in Chapter III, contribute chiefly to this result.

a. *Liberation of the* P-*compounds of fraction alc* 60 %

Table III shows, that the P-compounds which can be extracted

from the cells with alcohol 60 %, consisting for about one half of phospholipids, have usually left the killed cells within 24 hours.

Never during or after autolysis can chloroform-soluble P-compounds be detected in the medium, so we concluded that the phospholipids are hydrolysed, probably to PO_4'''. Other cell compounds contributed too to the PO_4'''-liberation as the increase of PO_4''' exceeds the decrease of the phospholipids-phosphate. In the section on the influence of Mg^{++} and F' on the liberation (section e) is demonstrated that the hydrolysis comes about under influence of enzymatic activity.

TABLE III. *Liberation of* P-*compounds of fraction alc* 60 % *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period P^{cell}_{alc} was determined. For the experimental data see exp. 4, 5, 6 and 7, page 35 etc.

Autolysis during (*days*)	P^{cell}_{alc} in % of P^{cell}_{tot} at t = 0			
	Exp. 4	Exp. 5	Exp. 6	Exp. 7
0	23 %	28 %	27 %	25 %
1	—	8	1	3
4	0	—	—	—
5	—	8	1	2

b. *Liberation of the* P-*compounds of fraction* TCA-c

Table IV shows, that the P-compounds in the cell, soluble in a 5 % TCA solution at 4° C after the extraction with alcohol 60 %, are liberated in the same short time (1–2 days) as the P-compounds of fraction alc 60 %.

The same compounds leave the cells in 1–2 days, when residue A – cells boiled in alcohol, in which therefore all enzymatic activity has stopped – is suspended in H_2O at 4° C or at 20° C. This proves, that the liberation of fraction TCA-c can take place without any enzymatic conversions.

The quantities, dissolved from residue A in H_2O after 1 day at 4° C, gave the impression, that the same liberation rate occurred, as is found in normally autolysing cells, which suggests, that indeed enzymatic processes do not play a role in the liberation of these compounds.

At 4° C these compounds dissolved nearly without hydrolysis to PO_4'''; at 20° C hydrolysis was considerable, which is quite in accordance with our findings that the P-compounds of fraction TCA-c are hydrolysed very rapidly to PO_4''' in a 5 % TCA solution at 20° C.

c. *Liberation of* P-*compounds of fraction* TCA-w

Some of the compounds in the cells, which can be dissolved in a 5 % TCA solution at 90° C after extraction with alcohol 60 % and

TABLE IV. *Liberation of* P-*compounds of fraction* TCA-c *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period $P^{cell}_{TCA\text{-}c}$ was determined. For the experimental data see exp. 6, 7, and 8, page 38.

Autolysis during (*days*)	$P^{cell}_{TCA\text{-}c}$ in % of P^{cell}_{tot} at t = 0		
	exp. 6	exp. 7	exp. 8
0	11 %	32 %	10 %
1	—	2	7
2	3	—	—
5	—	2	7
12	—	2	—
14	—	—	4

with a 5 % TCA solution at 4° C, appear to leave these cells slowly after they are killed, while another part remains (Table v).

The liberation of the P-compounds of fraction TCA-w appears to be very variable. In experiment 7 the liberation took place very slowly, in some other experiments half of the fraction has left the cells in 4 days, while in experiment 6 only a small part is still present in the cells after 6 days.

TABLE V. *Liberation of* P-*compounds of fraction* TCA-w *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period $P^{cell}_{TCA\text{-}w}$ was determined. In some experiments the PO_4''' content of the TCA-w extracts was determined (numbers between brackets). For the experimental data see exp. 4, 6, 7, 8, 9, 10 and 17^{II}, page 35 etc.

Autolysis during (*days*)	$P^{cell}_{TCA\text{-}w}$ in % of P^{cell}_{tot} at t = 0						
	exp. 4	exp. 6	exp. 7	exp. 8	exp. 9	exp. 10	exp. 17^{II}
0	42%	56%	38%	60%	54% (26%)	57% (24%)	48% (18%)
1	—	—	36	52	—	—	—
4	21	—	—	—	20 (4)	21 (4)	23 (7)
5	—	—	25	32	—	—	—
6	—	10	—	—	—	—	—
12	—	—	26	—	—	—	—
14	—	—	—	25	—	—	—

Various causes can be suggested, of which we mention here especially, that fraction TCA-w might consist of several compounds, present in varying amounts and liberated at unequal rates. An indication in this direction is found in the fact, that the amount of PO_4''' of this fraction (formed through hydrolysis from $P^{cell}_{TCA\text{-}w}$), fluctu-

ates between 35 % and 50 % before, and between 19 % and 30 % after autolysis.

In fraction TCA-w polyphosphates (WINTERMANS 1955, TAIICHI NIHEI 1955, 1957) and derivatives of nucleic acids can be present.

We have carried out some preliminary experiments to investigate whether the decrease of the amount of P-compounds in the fraction TCA-w during autolysis of the cells can be attributed to the nucleic acids diffusing out of the cells. For this purpose the extinction of the TCA-w extract at 260 mμ was determined before and during autolysis, the blank being a 5 % TCA solution which had been subjected to the same treatment.

We suppose that the decomposition of the TCA during the extractions is not influenced by compounds from the cells.

Apart from the extinctions, also $P^{cell}_{TCA\text{-}w}$ and P^{cell}_{tot} at $t = 0$ were determined (Table VI).

TABLE VI. *The fate of nucleic acids in Scenedesmus during autolysis at room temperature.* The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period the extinction of the TCA extract at 260 mμ, $E^{cell}_{TCA\text{-}w}$, and $P^{cell}_{TCA\text{-}w}$ were determined.

For experimental data see exp. 8, 9, 10, 11 and 12.

mg of $P^{cell}_{TCA\text{-}w}$ per mg of P^{cell}_{tot} at $t = 0$.

$E^{cell}_{TCA\text{-}w}$ per mg of P^{cell}_{tot} at $t = 0$ per ml, and per mg of $P^{cell}_{TCA\text{-}w}$ per ml.

Autolysis during (*days*)	exp. 8			exp. 9			exp. 10		
	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$
0	0.60	71	120	0.54	52	96	0.57	52	91
1	0.52	71	137	—	—	—	—	—	—
4	—	—	—	0.20	51	255	0.21	52	250
5	0.32	71	222	—	—	—	—	—	—
14	0.25	71	284	—	—	—	—	—	—

Autolysis during (*days*)	exp. 11			exp. 12		
	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$
0	0.38	74	195	0.42	100	238
3	0.185	44	240	0.21	59	280
4	—	—	—	—	—	—
7	—	—	—	0.21	—	—

It appeared, that in some experiments (8, 9 and 10) the quantity of nucleic acids did not change during autolysis. So we have to assume that the nucleic acids did not leave the cells. Somewhat divergent results were obtained with the experiments 11 and 12, both carried out with cells of the same culture.

It is possible in both latter experiments that the extinction at 260 mμ before

autolysis is caused not only by nucleic acids, but also by other compounds, and that those compounds leave the cells during autolysis. An indication, that this might happen, is found in the fact that in an experiment, not further mentioned, the extinction at 235 mμ decreased less than the extinction at 260 mμ which decreased by 20–30 %. For the determination of the extinction at 235 mμ the TCA present must be removed, which was achieved by extraction with ether.

That the disturbing compounds at 260 mμ do not occur in experiment 8, 9 and 10, may be a fortunate coincidence, dependent on the previous history of the cells.

Therefore it can not be excluded that in these experiments part of the nucleic acids liberated through unusual circumstances.

The values obtained in table VI for the extinction per mg $P^{cell}_{TCA\text{-}w}$ per ml after autolysis are in accordance with those, reported in literature, for the extinctions of nucleic acids per mg of nucleophosphate per ml. Thus CHAYEN (1955) gives 260–275 for native nucleic acid, WIAME (1949) 350, from data of DI CARLO et al. (1948) is calculated 275, BEAVEN et al. (1955) give 225–320. These statements vary, but Chayen has pointed out that the highest extinctions are obtained by working with commercial nucleic acid instead of native nucleic acid. By the alkaline extraction during the preparation of the commercial product a part of the phosphate is supposed to be split off.

Although it must be borne in mind, when comparing our own values with those cited, that the cited ones refer to yeast- instead of algal nucleic acid, we feel justified in concluding on the strength of the accordance between both groups of values, that the P-compounds remaining after autolysis consist of nucleophosphate.

We conclude from these preliminary experiments that the nucleic acids do not leave the cells during autolysis.

The values obtained in table VI for the extinction per mg $P^{cell}_{TCA\text{-}w}$ per ml before autolysis showm, that at that moment still other P-compounds, "polyphosphates", are present in the cells, also belonging to fraction TCA-w, but which do leave the cells. They are hydrolysed to PO_4''' more easily than the nucleic acids (Chayen). The method of quantitative analysis, based on this difference, did not yield good results for our material (Chapter III, page 19). It could be established, however, (Table V, exp. 9, 10 and 17^{II}) that the part of the P-compounds, hydrolysed during extraction with a 5 % TCA solution at 90° C to PO_4''', is smaller after autolysis than before.

d. *Liberation of fraction rest*-P

The P-compounds which can be extracted from the cells neither by ethanol 60 % nor by a 5 % TCA solution, at 4° C or at 90° C, are not liberated during autolysis. They constitute about 5 % of P^{cell}_{tot}. According to TAIICHI NIHEI (1957) these compounds are phosphoproteins. Their phosphate, like that of the nucleic acids, is only liberated by means of bacterial digestion, which is discussed in section 3b.

e. *Influence of external factors on the liberation of* P-*compounds*

In section c was shown, that variation in the nucleophosphate-polyphosphate ratio can be a cause of variability of the liberation of the P-compounds of fraction TCA-w.

It might also be considered, whether a variation in activity of the enzymatic systems, connected with it, could lead to similar effects, not only for fraction TCA-w, but for all fractions. To check this, the influence of the temperature, the pH and of Mg-, Ca- and F-ions on the liberation of P-compounds was determined.

Influence of temperature

As the dying off of algae in nature can occur at different temperatures, it is important to investigate the dependence of the liberation of P-compounds during autolysis on this factor.

In exp. 4, 5, 8 and 15 the liberation at different temperatures was determined. These experiments show, that during autolysis the liberation of P-compounds at 0° C does not differ significantly from that at 10° C. At higher temperatures both the amount of PO_4''' and that of P_{tot}^{water} increase, reaching a maximum at 30° C. At these high temperatures practically all the P liberated was present as PO_4'''.

From these data it can not be concluded on which of the mechanisms mentioned on page 20 the temperature exerts its influence. For this purpose the different fractions must be examined separately —preferably by short during experiments.

The temperature is an important ecological factor for the liberation of P-compounds in nature. It is of some importance to know, whether the liberation of P-compounds during autolysis at low temperatures finally equals that at higher temperatures, and if such is not the case, to know what happens, when the cells are brought from 0° C to 20° C after a long period. This was investigated in experiment 14. The liberation of P-compounds at different temperatures was compared. It appeared that in 12 days at 10° C about 2/3 was liberated of the amount of P-compounds liberated at 20° C. When the autolysis took place only a few days at 0° C and proceeded afterwards at 20° C finally the same amount of P-compounds was liberated as in the sample in which the cells remained constantly at 20° C. It appeared from this experiment that the enzymes connected with the liberation of P-compounds had not been damaged after a ten days' storage at 0° C. Longer periods have not yet been tried.

Influence of the pH

Several experiments were carried out, in which the influence of the pH on the liberation of P-compounds was investigated. In these experiments the pH was kept constant by means of an 1/20 M succinate-buffer (pH 3–6) or a 1/20 M tris-maleic acid buffer (pH 5–10). No differences were observed whether one of these buffer solutions had been added or not, when they had the same pH as the suspension medium, from which appears that they had no specific

effect. Between pH 5 and 9 the liberation does not depend on the pH, outside this range it decreases rapidly.

Influence of Ca^{++} *and* Mg^{++}

As it is probable that the phosphatases play a role in autolysis, we investigated whether Mg^{++} has a stimulating effect on the liberation of P-compounds. This could be expected as Mg^{++} is a constituent of many phosphatases and it might be possible that it is split off from the phosphatases during autolysis. Moreover the influence of Ca^{++} was studied, to find out whether a non-specific ion-effect also occurs. Table VII gives a survey of the obtained results.

TABLE VII. *Influence of* Mg^{++} *and* Ca^{++} *on the liberation of P-compounds from Scenedesmus.* $P(PO_4''')$ and P_{tot}^{water} were determined during autolysis under sterile conditions at room temperature, and expressed as percentage of P_{tot}^{cell} at $t = 0$. To some samples 1 g/l $MgSO_4.7\,H_2O$ or 1 g/l $Ca(NO_3)_2.4\,H_2O$ had been added. For experimental data see exp. 9 and 10.

	Experiment 9				Experiment 10			
Added	$MgSO_4$				$Ca(NO_3)_2$			
Autolysis during	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0 h	0%	0%	0%	0%	0%	0%	0%	0%
5 h	33	42	51	58	33	39	30	48
4 d	59	60	71	74	59	60	63	71
15 d	—	72	—	78	—	67	—	72

This table shows that Mg^{++} accelerates the liberation of the P-compounds that are hydrolysed to PO_4''', while Ca^{++} accelerates the liberation of the P-compounds that appear in the medium as P_{bound}^{water}. This might be explained by the supposition that Ca^{++} increases the permeability of the cells. With Mg^{++} no similar effect is observed, possibly through the accelerated hydrolysis to PO_4''', which can leave the cells unhampered.

In the presence of both Mg^{++} and Ca^{++} the extinction of the TCA-w extract (per mg P_{tot}^{susp}) during autolysis decreased (Exp. 9 and 10). This can be explained by assuming, that in the presence of these ions the nucleic acids could not be recovered quantitatively, either through a lowering of the extinction of the nucleic acids (BEAVEN 1955), or through the formation of unsoluble precipitates in the cell.

An indication, that such a precipitate may occur, is the fact that Mg^{++} must be added when nucleophosphate has to be precipitated from a solution, and that Ca^{++} must be added for the isolation of nuclei, by means of which a higher yield of nucleic acids is obtained (DOUNCE 1955). Some investigators assume here an inhibition of autolysis. We are rather inclined to suppose, that during those isolations a – perhaps temporarily – less soluble product is formed.

These experiments are to be repeated, but not before the determination of the nucleophosphates yields completely satisfactory results.

Like the temperature, the Ca^{++} and Mg^{++}-contents of a lake are important ecological factors regarding an eventual autolysis occurring in it.

Influence of NaF

It appeared that F′ has an inhibiting effect on the liberation of P-compounds during autolysis of *Scenedesmus*, which inhibition has its maximum at a concentration of 10^{-2} M. NaF (exp. 11, 12 and 13), and is still observed at 60° C (exp. 5).

In higher concentrations this action of NaF decreases, probably because the solubility of the nucleic acids is increased. In accordance with this the P-content of fraction TCA-w decreases (exp. 12 and 13). High concentrations of NaCl had a similar effect only after a longer duration of the experiment (exp. 13).

Especially the liberation of the P-compounds from fraction alc 60 % and fraction TCA-w appears to be inhibited (exp. 11 and 12), which proves, that both these fractions are broken down by means of enzymes, as had already been stated on page 22 for fraction alc 60 %.

From exp. 4 appears that the extent of inhibition (in this experiment 15 % of P^{cell}_{tot}) does not depend on the method of killing the cells, from which follows once more that the processes studied here are not specific for any particular method of killing.

The influence of NaF on the course of the extinction of the TCA-w extract during autolysis (exp. 11 and 12) can not be explained, before the action of Mg^{++} and Ca^{++} has been elucidated, because it is quite possible that NaF counteracts the influence of these ions by blocking them.

LIBERATION AND MINERALISATION OF N FROM SCENEDESMUS QUADRICAUDA

a. *Under sterile conditions*

In the previous paragraph was shown, that 70–80 % of the P-compounds leave *Scenedesmus* cells during autolysis under sterile conditions in a few days. Of the N-compounds on the other hand only 20–30 % are liberated.

We applied the same method of fractionation for N- and for P-compounds, which is unusual for N-compounds, but justified in our case by the results. For, it appeared that during autolysis the N-compounds of fraction alc 60 % and fraction TCA-c (i.e. the N-compounds present in the cell in dissolved state) leave the cells. The N-compounds of fraction TCA-w only do so in part; the remaining part belongs without doubt to the nucleic acids (5–10 % of N^{cell}_{tot}). The bulk of the N-compounds, that can not be extracted with ethanol 60 % or with a 5 % TCA solution at 4° C and 90° C, remains also

in the cells (fraction rest-N, 70–80 % of N_{tot}^{cell}). These are the proteins which form about 50 % of the dry weight of the cell.

The nitrogen of nucleic acids and proteins can only be restored to the cycle of elements through the influence of other organisms.

See for experimental data exp. 4, 5, 6, 8, 16 and 17.

b. *Under influence of bacteria*

We have tried to digest the N-slag – remaining after sterile autolysis – by means of bacteria. To this purpose *Scenedesmus* cells were killed with chloroform, left to stand with water during one week and finally washed carefully, all under sterile conditions, to remove all dissolving N-compounds. These cells, which from now on will be referred to as leached cells, were suspended in lake water, containing no detectable amounts of nitrogen or phosphorus, the concentration of N^{cell} being 0.10 mg per ml.

After 5 days 45 % of the added N^{cell} appeared in the solution as NH_3, after 7 days 50 %; then this quantity remained constant. Of the *Scenedesmus* cells only a detritus was left, which could not be identified microscopically. Addition of 0.05 mg of KH_2PO_4/ml to the suspension of leached cells in lake water had a somewhat delaying influence on the production of NH_3. After addition of glucose no production of NH_3 was observed.

From this type of experiments it can not be concluded whether the amount of nitrogen, not converted to NH_3, is still in the *Scenedesmus* cells, or has been consumed by bacteria, because bacteria and *Scenedesmus* residue clot together so strongly that they can not be separated.

Therefore the inhibiting effect of glucose can not be elucidated, as it might be due to an increased bacterial growth, but also to a decreased digestion of *Scenedesmus*. An indication in this direction is the fact, that in the presence of glucose a larger amount of *Scenedesmus* cells remains identifiable.

Moreover the obtained results were rendered unreliable by the growth of ciliates. Therefore in a second experiment the leached cells were suspended in filtered lake water, which had been heated to 45° C during one hour. The growth of ciliates was strongly suppressed by this, while the clotting was rendered less intensive.

25 % of the added N^{cell} was now recovered as NH_3. Besides this about 50 % of the total nitrogen was present in the supernatant after the cells had been centrifuged off. This was due to the fact that in this experiment the *Scenedesmus* cells had been digested largely, and the remaining smaller particles of the *Scenedesmus* detritus were present in the supernatant together with the bacteria, as was confirmed by microscopic examination. Because of this fact the ratio between the nitrogen in *Scenedesmus* detritus and in bacteria could once more not be determined. The sediment contained about 20 % of the nitrogen and practically no bacteria.

As experiments of this type appear to present to many technical

difficulties, we investigated the action of bacteria in pure cultures on the decomposition of the algal cells.

As the slag to be digested consists chiefly of proteins, we chose bacteria which excrete proteolytic enzymes. Our choice fell on *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Proteus vulgaris* and *Bacillus subtilis;* the proteinases of those bacteria have been studied extensively a.o. by GORINI (1950–51) and HAINES (1931, 1932, and 1933).

When to this types of bacteria proteins are given as a substrate they may either be hydrolysed to amino acids, which are incorporated immediately by the growing bacteria, or be broken down by oxidation under formation of NH_3. This can be incorporated as long as the bacteria grow, but is liberated when bacterial growth is inhibited, e.g. by external circumstances or special poisons.

We have tried to achieve oxidative breakdown with liberation of NH_3 as follows:

The four previously mentioned bacteria were cultured on bactopeptone, centrifuged, washed with and finally suspended in a solution containing all the salts that are added to peptone water.

Peptone water: NaCl 5 g/l; K_2HPO_4 0.5 g/l; $MgSO_4.7H_2O$ 0.1 g/l; $CaCl_2$ or $Ca(NO_3)_2.4H_2O$ 0.1 g/l; NaOH to pH = 7.5.

To this suspension leached algae were added, by which the uptake of O_2 and the release of CO_2 of *Proteus vulgaris* and *Bacillus subtilis* increased a little, while those of *Pseudomonas fluorescens* and *Ps. aeruginosa* increased to 5–6 times the original value. Adding of dinitrophenol (DNP, 5×10^{-4} M.) caused a slightly greater increase. After some hours the exchange of gases returned to the original level. A production of NH_3 could, however, never be demonstrated.

In the second place we have tried to obtain bacterial growth on a medium with leached algae as the only source of nitrogen. This has not yet succeeded, not even when a concentrated suspension – cultured on bactopeptone – was added.

More promising results were obtained after partial hydrolysis of the algal proteins with alkali to prepare an "algal peptone".

Scenedesmus cells were extracted with ethanol 60 % and a 5 % TCA solution at 4° C, to remove all soluble substances, and subsequently suspended in twice their own volume of 0.25 N NaOH at 30° C during 24 hours. The cells were centrifuged off and washed with twice their volume of water, which was added to the NaOH extract. This diluted extract was filtered, first through a Schott G 4 and then through a Schott G 5 filter, after which the pH was brought to 7.5 by addition of HCl. The concentration of NaOH had been calculated so as to give the resulting NaCl the concentration, needed for the culture solution of the bacteria (0.5 %). After addition of 0.5 g of K_2HPO_4, 0.1 g of $MgSO_4.7\,H_2O$ and 0.1 mg of $CaCl_2$ per liter, the solution was sterilised, during which process a precipitate (probably proteins) was formed.

On this medium *Proteus vulgaris* and *Bacillus subtilis* did not grow, while *Pseudomonas aeruginosa* and *Ps. fluorescens* grew very well, causing the precipitate to disappear.

Part of the bacterial suspension cultured on algal peptone was added to a suspension of leached algae. Although the number of the bacteria clearly increased, we did not yet succeed in demonstrating a decrease of the N-content of the *Scenedesmus* cells.

Mineralisation of Si from Stephanodiscus Hantzschii

We will now discuss some preliminary experiments, bearing upon the mineralisation of silicium (exp. 21, 22 and 23).

The mineralisation of Si-compounds proceeds slowly. After killing of the diatoms with chloroform only 0.5–1.5 % of the bound Si is liberated in 1–2 days. This amount increases to 20–30 % only after several weeks, and the process is certainly not finished after 5 weeks. As the rate of mineralisation can, therefore, be dependent only on the hydrolysis rate of the bound Si, or on the dissolution rate of the formed product, we investigated the influence of the pH.

In exp. 22 adding of 0.1 g of $NaHCO_3$ per liter appeared to have no effect, possibly because the, already rather high, pH of the suspension (pH = 7) was not altered. In exp. 23 the mineralisation was clearly increased by the addition of 0.5 g/l of $NaHCO_3$ by which the pH of this suspension had increased from 6.4 to 7.8.

After 3 weeks samples of both suspensions of exp. 23 were filtered off and resuspended in water and in a $NaHCO_3$ solution (0.5 g/l). 2 weeks later the quantity of silicium mineralised in the old and new suspensions was determined. It appeared then, that in the second suspension in water as much silicium had been dissolved as in the first one, but that in the second suspension in a $NaHCO_3$ solution considerably more silicium had been dissolved, than in the first one. We feel justified to conclude, that the liberation of silicium in water is not limited by the solubility, whereas this might be the case in the suspensions with $NaHCO_3$. It will therefore be necessary to renew the suspending fluid regularly when these experiments will be further continued.

In exp. 23 the amount of silicium, still present in the cells after 3 weeks, was also determined, to find out whether Si-compounds, which do not react with the molybdate reagent, are dissolved. This is not the case, for the sum of the silicium, dissolved and present in the cells, is equal to the originally added quantity.

Mineralisation of Fe from scenedesmus quadricauda

A few experiments were carried out to investigate the liberation of iron during autolysis of *Scenedesmus* under sterile conditions. In preliminary experiments no iron could bedem onstrated in the suspension medium. As this could be due to precipitation of alkaline ironphosphate, caused by the relatively high pH of the solutions (pH = 5.5) and the large amount of phosphate present (mg P_{tot}^{susp}/ mg Fe_{tot}^{susp} = 20–30), we added EDTA to the autolysing suspensions (1 mg of Na_2EDTA per 10 ml) to convert the liberated iron to a stable complex. Moreover, as the PO_4''' disturbs the determination of iron and perhaps even the formation of the complex with EDTA, it was

removed for the greater part by transferring the cells to an EDTA solution after a 24 hours' autolysis in water. It appeared than that in one experiment 65 % of the iron present had been dissolved after 47 days, in another one 50 % after 10 days (Fe_{tot}^{susp} was resp. 2 and 3mg/l).

Liberation and mineralisation from other organisms

Finally we investigated whether the results, so far obtained, are restricted to *Scenedesmus* cells or are of a more general importance. Therefore the following organisms were killed with chloroform to observe the liberation and mineralisation of N- and P-compounds: *Ankistrodesmus falcatus (Corda) Ralfs* (exp. 18), *Stephanodiscus Hantzschii Grun.* (exp. 21, 22 and 23), and *Cladophora glomerata (L.) Kuetzing* (exp. 19 and 20).

We need not describe these results in detail. An exception has to be made for exp. 19 and 20. In exp. 19 the large amount of P_{bound}^{water} liberated during autolysis is striking. This sample was collected from a ditch, as the organism is not cultured in the laboratory. In exp. 20 a sample was used that had been collected on the same spot one week later. In the meantime a large amount of Vecht-water, rich in phosphate, had entered the ditch, with the result, that the composition of this sample was different, as appeared from the amounts of PO_4''' in the TCA-w extracts, which had been kept at 90° C during 1 hour. The large amount of P-compounds that are easily hydrolysed in the second sample indicates a high polyphosphate content.

From laboratory experiments it appears also, that addition of phosphate to a P-deficient culture leads to an important increase of the non-nucleophosphate of fraction TCA-w.

In these circumstances the percentages of $P(PO_4''')$ and P_{bound}^{water} liberated during autolysis do not differ from those obtained with *Scenedesmus*.

The addition of $NaHCO_3$ (0.5 g/l) in exp. 23 had an accelerating influence on the liberation of the P-compounds, but did not cause an increase of the total amount. As it has been observed that a change in the pH does not alter the liberation rates of the P-compounds it must be assumed that this accelerating effect is caused by the increase in concentration of the anions present.

Experimental

EXP. 1. *Influence of the method of killing on the liberation of* P-*compounds from Scenedesmus quadricauda.*

Scenedesmus was cultured at 20° C, as has been described in Chapter II. A culture of 4–8 weeks, in which growth had stopped, was centrifuged off, washed several times with H_2O and finally suspended in H_2O. — Two samples (200 ml), A and B, were irradiated in a sterile room in an open glass dish (ø 25 cm) during 4 hours, with the U.V. light of three Philips T.U.V. lamps (30 Watt), mounted 10 cm

above the suspension. Sample C was diluted with sterile H_2O, after which 5 ml of chloroform per liter suspension was added, and was shaken during 15 minutes. Sample C was diluted with sterile H_2O and killed with formaldehyde (1 %). All samples were brought up to the same final volume as accurately as possible. In the four suspensions P^{cell}_{tot} at t = 0 (equal to P^{susp}_{tot}) was determined. — All actions were carried out under sterile conditions at room temperature. — At different moments $P(PO_4''')$ and P^{water}_{tot} were determined in the filtrates of the suspensions. Results as percentage of P^{susp}_{tot}.

Cells killed by	irradiation		irradiation		chloroform		formaldehyde	
P^{susp}_{tot} (mg/l)	4.5		4.9		4.7		4.9	
Autolysis during (*days*)	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}
0	0%	0%	0%	0%	0%	0%	0%	0%
1	42	64	41	62	53	66	20	28
2	52	69	56	64	63	71	45	52

EXP. 2. *Influence of the density of the suspension and the method of killing on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared and divided as in exp. 1. The cells were killed: in sample A by saturation with chloroform, in sample B in the same way after dilution 1:5, in sample C by saturation with toluene. — The remaining sample was used for the fractionation of the P-compounds, as has been described in chapter III. — In all suspensions P^{susp}_{tot} (= P^{cell}_{tot} at t = 0) was determined. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P^{susp}_{tot}.

Sample	Cells killed by	Autolysis during (*days*)	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c} + P^{cell}_{TCA\text{-}w}$	Recovered	P^{susp}_{tot} mg/l
D	—	—	0%	0%	29%	70% [1]	99%	—
A	chloroform	1	52	55	3	43	101	4.9
		6	68	74	—	—	—	
B	chloroform	1	50	54	4	41	99	1.1
C	toluene	1	32	35	6	53	94	4.8
		6	69	72	—	—	—	

[1]) at t = 0: $P^{cell}_{TCA\text{-}c}$ = 8 %.

EXP. 3. *Influence of the method of killing and of* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared, as in exp. 1, and divided into 6 samples. In each sample P_{tot}^{susp} was determined, after which the cells were killed: in sample A by U.V. irradiation from t = 0 till t = 4, as in exp. 1; in sample B by addition of 5 ml of chloroform per liter at t = 2; in sample C by addition of 1 ml of carbon tetrachloride per liter at t = 2; in sample D as in sample A, 10^{-2} M. NaF being present; in sample E as in sample B, 10^{-2} M. NaF being present; the remaining sample F was used for the fractionation of the P-compounds. — Autolysis at room temperature. — Results as percentage of P_{tot}^{susp}. — All actions under sterile conditions.

Sample	Cells killed by	Autolysis during (*days*)	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	Recovered	P_{tot}^{susp} mg/l
F	—	—	0%	0%	54%	15%	30%	99%	
A	irradiation	1	38	45	—	—	—	—	8.7
		3	—	51	6	3	39	99	
B	chloroform	1	53	55	—	—	—	—	7.5
		3	—	59	10	3	34	106	
C	carbon tetrachloride	1	32	38	—	—	—	—	7.5
		3	—	54	4	3	36	97	
D	irradiation + NaF	1	9	19	—	—	—	—	8.0
		3	—	24	6	4	71	105	
E	chloroform + NaF	1	9	19	—	—	—	—	7.8
		3	—	22	8	5	66	101	

EXP. 4. *Influence of the temperature and of* NaF *on the liberation of* P- *and* N-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into five samples: A_1 was killed by a 4 hours' U.V. irradiation as in exp. 1; A_2 as A_1, 10^{-2} M NaF being present per liter; B_1 with chloroform; B_2 as B_1, 10^{-2} M NaF being present per liter. — The four samples were divided into three equal parts, which were rapidly brought resp. to 0° C, 20° C and 30° C. Sample C was used for the fractionation of the P- and N-compounds. — All actions under sterile conditions.
$P_{tot}^{susp} = 6.9$ mg/l; $N_{tot}^{susp} = 44$ mg/l; mg N/ mg P = N/P = 6.4.

A) P-*compounds.*

Autolysis at during (*days*)	Liberated $P(PO_4''')$ in % of P_{tot}^{susp}																	
	0° C						20° C						30° C					
	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
1	21	10	*11*	32	13	*19*	32	16	*16*	48	27	*21*	36	18	*18*	63	43	*20*
4	31	16	*15*	41	22	*19*	43	24	*19*	59	44	*15*	—	33	—	75	62	*13*

Autolysis at during (*days*)	Liberated P_{tot}^{water} in % of P_{tot}^{susp}																	
	0° C						20° C						30° C					
	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
1	45	32	*13*	48	29	*19*	61	48	*13*	65	45	*20*	71	56	*15*	75	67	*8*
4	57	43	*14*	59	37	*12*	74	61	*13*	74	63	*11*	—	70	—	85	77	*8*

Autolysis at during (*days*)	$P_{TCA\text{-}c}^{cell}$ [1]) + $P_{TCA\text{-}w}^{cell}$ in % of P_{tot}^{susp}								P_{rest}^{cell} in % of P_{tot}^{susp}							
	0° C				20° C				0° C				20° C			
	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2
0	66%	66%	66%	66%	66%	66%	66%	66%	8%	8%	8%	8%	8%	8%	8%	8%
4	40	44	33	53	21	29	21	29	6	12	12	—	11	11	10	12

EXP 4 *(Continued)*

Autolysis at during (*days*)	Recovered in % of P^{susp}_{tot}							
	0° C				20° C			
	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2
0	97%	97%	97%	97%	97%	97%	97%	97%
4	103	99	104	—	106	101	105	104

[1]) At t = 0 $P^{cell}_{TCA\text{-}c}$ amounted to 24 %; after autolysis the amount was so small, that it has been determined together with $P^{cell}_{TCA\text{-}w}$. At t = 0 P^{cell}_{alc} amounted to 23 %; after autolysis to less than 1 %.

B) N-*compounds*

The quantity of N-compounds, liberated in the suspensions to which NaF had been added (A_2 and B_2) was equal to that, liberated in the suspensions without NaF (A_1 and B_1). Only the results of autolysis in suspensions A_1 and B_1 are given.

Autolysis at during (*days*)	N^{water}_{tot} in % of N^{susp}_{tot}				$N^{cell}_{TCA\text{-}w}$ in % of N^{susp}_{tot}				N^{cell}_{rest} in % of N^{susp}_{tot}				Recovered in % of N^{susp}_{tot}			
	0° C		20° C		0° C		20° C		0° C		20° C		0° C		20° C	
	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1
0	0%	0%	0%	0%	10%	10%	10%	10%	79%	79%	79%	79%	101%	101%	101%	101%
4	16	15	21	20	7	7	7	6	80	86	82	81	96	101	103	101

$N^{cell}_{TCA\text{-}c}$ was before autolysis 2 %, after autolysis less than 1 %.
N^{cell}_{alc} was before autolysis 10 %, after autolysis less than 1 %.

EXP. 5. *Liberation of* N-*compounds and influence of temperature and* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into 6 samples. The cells were killed: in sample A, B and C with chloroform, at resp. 20° C, 30° C and 60° C; in sample D by heating to 60° C; in sample E with chloroform at 60° C after adding 10^{-2} M. NaF. — During further autolysis these temperatures were maintained. — The remaining sample was used for the determination of P_{tot}^{susp} and N_{tot}^{susp} and for the fractionation of N- and P-compounds. — All actions under sterile conditions. — The results for the P-compounds are given as percentage of P_{tot}^{susp} (a); for the N-compounds (only sample A) as percentage of N_{tot}^{susp} (b). P_{tot}^{susp} = 4.2 mg/l; N_{tot}^{susp} = 27 mg/l; N/P = 6.5.

A) *Librration of* P-*compounds.*

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell} + P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}	Recovered
Beginning of experiment:							
	0%	0%	28%	72%	(9%) + (63%)	4%	104%
Autolysis with chloroform at 20° C during:							
5 h	43	49	—	—		—	—
2 d	50	50	8	27	(5) + (22)	—	—
6 d	62	65	8	19	— —	11	103
28 d	65	70	—	—		—	—
Autolysis with chloroform at 30° C during:							
5 h	50	58	—	—		—	—
2 d	62	68	6	18	(4) + (14)	—	—
6 d	73	75	6	10	— —	10	101
Autolysis with chloroform at 60° C during:							
5 h	51	57	—	—		—	—
2 d	69	74	6	13	(1) + (12)	—	—
6 d	73	76	4	10	— —	6	96
Autolysis at 60° C during:							
5 h	48	—	—	—		—	—
2 d	63	68	6	14	(1) + (13)	—	—
6 d	66	71	4	10	— —	12	97
Autolysis with chloroform and 10^{-2} M. NaF at 60° C during:							
5 h	18	—	—	—		—	—
2 d	37	60	10	20	(2) + (18)	—	—
6 d	45	72	5	16	— —	13	106

B) *Liberation of* N-*compounds.*

	N_{tot}^{water}	N_{all}^{cell}	$N_{TCA\text{-}c}^{cell} + N_{TCA\text{-}w}^{cell}$		N_{rest}^{cell}	Recovered
Beginning of experiment:						
	0%	6%	7%	(0%) + (7%)	86%	99%
Autolysis with chloroform at 20° C during:						
6 d	10	4	10	— —	80	104
28 d	25	—	—		70	—

EXP. 6. *Liberation of* N- and P-*compounds from Scenedesmus quadricauda.*

A 12 days' old culture of *Scenedesmus* was treated as sample A, exp. 5. — P_{tot}^{susp} = 5.4 mg/l; N_{tot}^{susp} = 36 mg/l; N/P = 6.7.

All actions under sterile conditions.

A) *Liberation of* P-*compounds.*

Autolysis during	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA-c}^{cell}+P_{TCA-w}^{cell}$		P_{rest}^{cell}	Recovered
0 h	0%	0%	27%	67%	(11%)+(56%)	3%	97%
5 h	51	65	—	—		—	—
2 d	63	74	1	20	(3) (17)	—	—
6 d	74	80	1	10	— —	8	99
28 d	76	81	—	—		—	—

B) *Liberation of* N-*compounds*

Autolysis during (*days*)	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA-c}^{cell}+N_{TCA-w}^{cell}$		N_{rest}^{cell}	Recovered
0	0%	12%	4%	(0%) + (4%)	82%	98%
6	14	7	6	— —	72	99
28	20	—	—		72	—

EXP. 7. *Liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1, and killed with chloroform at room temperature. — Further data as exp. 6. — All actions under sterile conditions. — P_{tot}^{susp} = 6.2 mg/l.

Autolysis during	$P(PO_4''')$	P_{bound}^{water}	P_{tot}^{water}	P_{alc}^{cell}	P_{TCA-c}^{cell}	P_{TCA-w}^{cell}	P_{rest}^{cell}	Recovered
0 h	0%	0%	0%	25%	32%	38%	4%	99%
3.5 h	18	10	28	—	—	—	—	—
6 h	29	14	43	—	—	—	—	—
1 d	48	12	60	3	2	36	—	—
5 d	65	3	68	2	2	25	—	—
12 d	68	4	72	1	2	26	3	104

EXP. 8. *Liberation of* N-*compounds and influence of the temperature on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into four samples. The cells were killed: in sample A, B and C with chloroform at resp. 4° C, 20° C and 30° C. During further autolysis these temperatures were maintained. — The remaining sample was used for the determination of N_{tot}^{susp} and P_{tot}^{susp}, and for the fractionation of the N- and P-compounds. — Of the TCA-w extracts the extinction was determined at 260 mμ, and E_{TCA-w}^{cell} (the extinction per mg P_{tot}^{susp} per ml per 1 cm cuvette) calculated. — Results for the

P-compounds as percentage of P^{susp}_{tot} (a), for the N-compounds as percentage of N^{susp}_{tot} (b). — All actions under sterile conditions.
P^{susp}_{tot} = 8.8 mg/l; N^{susp}_{tot} = 72 mg/l; N/P = 8.2.

A) *Liberation of* P-*compounds.*

	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$	P^{cell}_{rest}	Recovered	$E^{cell}_{TCA\text{-}w}$
Beginning of experiment:								
	0%	0%	22%	10%	60%	5%	97%	71
Autolysis with chloroform at 4° C during:								
1 d	21	24	9	6	50	—	—	71
5 d	—	26	9	6	50	—	—	72
14 d	—	30	8	7	51	4	100	71
Autolysis with chloroform at 20° C during:								
1 d	32	34	7	7	52	—	—	70
5 d	—	43	7	7	32	—	—	71
14 d	—	55	8	4	25	3	95	72
Autolysis with chloroform at 30° C during:								
1 d	33	38	9	8	40	—	—	65
5 d	—	62	6	2	20	—	—	71
14 d	—	63	5	0	26	5	99	74

B) N-*compounds.*

	$N(NH_4^+)$	N^{water}_{tot}	N^{cell}_{alc}	$N^{cell}_{TCA\text{-}c} + N^{cell}_{TCA\text{-}w}$	
Autolysis with chloroform at 20° C during:					
0 d	0%	0%	10%	17%	(10%) + (7%)
1 d	5	11	4	13	— —
5 d	6	11	1	14	— —
14 d	4	10	2	14	— (9)

EXP. 9. *Influence of* Mg^{++} *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into three samples. The cells were killed: in sample A with chloroform, in sample B with chloroform, after 1 g of $MgSO_4 . 7\ H_2O$ per liter had been added. — The remaining sample was used for the determination of P^{susp}_{tot} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P^{susp}_{tot}. For the determination of $E^{cell}_{TCA\text{-}w}$ vide exp. 8. — P^{susp}_{tot} = 4.0 mg/l; N/P = 6.5.

	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$\frac{P^{cell}_{TCA\text{-}w}}{P(PO_4''')}$	$\frac{P^{cell}_{TCA\text{-}w}}{P_{bound}}$	P^{cell}_{rest}	Recovered	$E^{cell}_{TCA\text{-}w}$
Beginning of experiment:									
	0%	0%	25%	12%	26%	28%	8%	99%	52
Autolysis with chloroform during:									
5 h	33	42	—	—	—	—	—	—	—
4 d	59	60	6	6	4.4	15.4	8	100	51
15 d	—	72	—	—	—	—	—	—	—
Autolysis with chloroform in the presence of $MgSO_4$ *during:*									
5 h	51	58	—	—	—	—	—	—	—
4 d	71	74	5	3	2	10	7	101	37
15 d	—	78	—	—	—	—	—	—	—

EXP. 10. *Influence of* Ca^{++} *on the liberation of* P*-compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was treated as in exp. 9, but sample B was killed with chloroform after addition of 1 g of $Ca(NO_3)_2.4\,H_2O$ per liter. — For further data vide exp. 9. — All actions under sterile conditions. — P^{susp}_{tot} = 3.8 mg/l; N/P = 8.

	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$\frac{P^{cell}_{TCA\text{-}w}}{P(PO_4''')}$	$\frac{P^{cell}_{TCA\text{-}w}}{P_{bound}}$	P^{cell}_{rest}	Recovered	$E^{cell}_{TCA\text{-}w}$
Beginning of experiment:									
	0%	0%	33%	13%	24%	33%	0%	103%	52
Autolysis with chloroform during:									
5 h	33	39	—	—	—	—	—	—	—
4 d	59	60	5	4	4	17	7	97	52
15 d	—	67	—	—	—	—	—	—	—
Autolysis with chloroform in the presence of $Ca(NO_3)_2$ *during:*									
5 h	30	48	—	—	—	—	—	—	—
4 d	63	71	2	7	5	14	5	104	38
15 d	—	72	—	—	—	—	—	—	—

EXP. 11. *Influence of* NaF *on the liberation of* P*-compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension, prepared as in exp. 1, was divided into six samples. The cells were killed with chloroform: in sample A, B, C, D and E after addition of resp. 0, 1, 4, 40 and 400 mg/l of NaF. The remaining sample was used for the determination of P^{susp}_{tot} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — For the determination of $E^{cell}_{TCA\text{-}w}$ vide exp. 8. — Results as percentage of P^{susp}_{tot}.

P^{susp}_{tot} = 5.4 mg/l; N/P = 9.4.

A) *Influence of* NaF *on liberation of* P (PO_4''') *and of* P_{tot}^{water}.

NaF mg/l	0		1		4		40		400	
Autolysis during	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
5 h	33	51	30	46	27	46	30	40	14	25
3 d	47	58	—	—	—	—	40	52	23	32

B) *Relation between the concentrations of* NaF *added and of the* P*-compounds of the different fractions.*

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered	$E_{TCA\text{-}w}^{cell}$
Beginning of experiment:	0%	0%	29%	27%	38%	8%	102%	74
Autolysis during 3 days with NaF*:*								
0 mg/l	47	58	3	9	19	7	96	44
40 mg/l	40	52	5	9	23	5	94	48
400 mg/l	23	32	15	17	27	6	97	67

EXP. 12. *Influence of* NaF *on the liberation of* P*-compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1, and divided into five samples. The cells in samples A, B, C and D were killed with chloroform, after addition of resp. 0, 400, 1200 and 4000 mg of NaF per liter. — The remaining sample was used for the determination of P_{tot}^{susp} and for the fractionation of the P-compounds. Autolysis at room temperature. All actions under sterile conditions. For further experimental data vide exp. 11. — P_{tot}^{susp} = 11 mg/l; N/P = 9.0.

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered	$E_{TCA\text{-}w}^{cell}$
Beginning of experiment:	0%	0%	32%	19%	42%	4%	97%	100
After a 3 days' autolysis with chloroform:								
0 mg NaF/l	58	59	5	7	21	10	102	59
400 mg NaF/l	26	32	15	7	33	11	98	92
1200 mg NaF/l	21	31	15	9	32	10	97	83
4000 mg NaF/l	21	46	16	11	15	10	98	40
After a 7 days' autolysis with chloroform:								
0 mg NaF/l	67	67	3	4	21	7	102	—
1200 mg NaF/l	30	49	10	5	35	5	104	—

EXP. 13. *Influence of* NaF *and* NaCl *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into 8 samples. The cells were killed: in sample A–F with chloroform, after addition of resp. 0, 0.13, 0.48, 1, 4 and 10 g of NaF per liter; in sample G with chloroform after addition of 15 g of NaCl per liter. The remaining sample was used for the determination of P_{tot}^{susp}. — Autolysis at room temperature. — All actions under sterile conditions. — P_{tot}^{susp} = 6.5 mg/l; N/P = 6.3.

Autolysis during (*days*)	NaF (mg/l) added												NaCl added	
	0		0.13		0.48		1		4		10		15 mg/l	
	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
2	47	59	12	24	10	21	11	24	18	43	17	66	50	60
4	58	69	22	37	21	39	22	44	29	61	21	70	61	78

EXP. 14. *Effect of a preceding cold treatment on the liberation at 20° C of the* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into two samples. The cells were killed with chloroform: in sample A at 0° C; autolysis proceeded at the same temperature–parts of the suspension, A_2, A_3 and A_4, were placed at 20° C after resp. 1, 3 and 10 days-; in sample B at 20° C; autolysis proceeded at the same temperature. — $P(PO_4''')$ and P_{tot}^{water} were determined in the filtrates and given as percentage of P_{tot}^{susp}. — All actions under sterile conditions. — P_{tot}^{susp} = 5.0 mg/l; N/P = 13.6.

Sample	A		A_2		A_3		A_4		B	
Autolysis during (*days*)	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
	autolysis at 0° C		*autolysis at 0° C*		*autolysis at 0° C*		*autolysis at 0° C*		*autolysis at 20° C*	
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
1	29	39	29	39	29	39	29	39	50	55
1							*autolysis at 20° C*			
2	36	43	36	43	36	43	54	61	58	62
3	—	—	—	—	—	—	59	64	59	63
3					*autolysis at 20° C*					
5	—	—	—	—	60	63	—	—	66	66
10			*autolysis at 20° C*							
12	45	49	65	65	61	68	—	—	—	—

EXP. 15. *Influence of the temperature on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared and killed by irradiation at 20° C, as described in exp. 1. Afterwards the suspension was divided into four samples, which were placed resp. at 2° C, 10° C, 20° C and 30° C. — $P(PO_4''')$ and P_{tot}^{water} were determined in the filtrates and given as percentage of P_{tot}^{susp}. — Results as percentage of P_{tot}^{susp}. — All actions under sterile conditions.

P_{tot}^{susp} = 3.0 mg/l; N/P = 9.8.

Autolysis at during (*days*)	2° C		10° C		20° C		30° C	
	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%
1	12	16	13	17	22	33	41	56
2	20	26	18	27	32	51	46	66
3	23	30	23	30	40	58	51	68
4	23	32	23	35	41	61	50	65
6	28	38	32	45	41	58	54	67
14	37	54	35	49	49	56	59	75

EXP. 16. *Liberation of* N- *and* P-*compounds from Scenedesmus quadricauda.*

To a *Scenedesmus* culture phosphate was added. After 24 hours a suspension was prepared as in exp. 1. — The cells were killed with chloroform at room temperature, after N_{tot}^{susp} and P_{tot}^{susp} had been determined, and the fractionation of the N- and P-compounds had been carried out in an aliquot. — The autolysing suspension was kept at room temperature for 63 days. Results for the P-compounds as percentage of P_{tot}^{susp} (a), for the N-compounds as percentage of N_{tot}^{susp}. (b). — All actions under sterile conditions.

P_{tot}^{susp} = 53.5 mg/l; N_{tot}^{susp} = 177 mg/l; N/P = 3.3.

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered
0	0%	9%	5%	82%	2%	98%
4	76	0	3	18	1	98
18	86	0	0	12	1	99
36	85	0	0	16	1	102
63	89	1	1	7	1	99

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N^{water}_{tot}	N^{cell}_{alc}	$N^{cell}_{TCA\text{-}c}$	$N^{cell}_{TCA\text{-}w}$	N^{cell}_{rest}	Recovered
0	0%	5%	2%	8%	82%	97%
4	8	1	2	6	81	98
18	11	2	1	7	73	94
36	14	2	0	6	70	92
63	17	6	0	7	71	101

EXP. 17. *Liberation of* P-*compounds from Scenedesmus quadricauda.*

In a 10 liter flat bottomed flask 8 liters of *Scenedesmus* suspension were cultured. After a 4, 11 and 31 days' growth two samples were siphoned out; one was used for the fractionation of the P-compounds (A), the other one was washed and killed with chloroform; after a 4 days' autolysis at room temperature P^{susp}_{tot} and the distribution of the phosphate over the different fractions were determined (B). The results appeared to be independent of the age of the culture, therefore only those after an 11 days' growth are given (as percentage of P^{susp}_{tot}). — All actions under sterile conditions.

A) *Fractionation of the* P-*compounds in Scenedesmus quadricauda.*

Growth during (*days*)	P^{cell}_{alc} soluble in $CHCl_3$	P^{cell}_{alc} insoluble in $CHCl_3$	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$ $P(PO'''_4)$	$P^{cell}_{TCA\text{-}w}$ P_{bound}	P^{cell}_{rest}	Recovered	P^{susp}_{tot} mg/l	N/P
4	12%	11%	7%	32%	32%	3%	97%	1.2	8.4
11	19	20	13	18	30	0	100	1.4	11
31	16	16	20	10	24	7	93	1.8	14

B) *Liberation from Scenedesmus quadricauda after an 11 days' growth.*

Autolysis during (*days*)	$P(PO'''_4)$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$ $P(PO'''_4)$	$P^{cell}_{TCA\text{-}w}$ P_{bound}	P^{cell}_{rest}	Recovered
0	0%	0%	39%	13%	18%	30%	0%	100%
4	58	63	1	4	7	16	4	95

EXP. 18. *Liberation of* P- *and* N-*compounds from Ankistrodesmus falcatus.*

Ankistrodesmus was cultured in a 10 l flat bottomed flask. After a 5 weeks' growth a suspension was prepared as in exp. 1. — The cells were killed with chloroform, after N^{susp}_{tot} and P^{susp}_{tot} had been determined and the fractionation of P- and N-compounds had been carried out (Schott glass filter G 5 M) in an aliquot. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P^{susp}_{tot} and N^{susp}_{tot}.

P^{susp}_{tot} = 23 mg/l; N^{susp}_{tot} = 84 mg/l;N/P = 3.6.

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$	P^{cell}_{rest}	Recovered
0	0%	16%	2%	79%	3%	100%
4	47	5	1	42	1	96
44	80	0	0	20	2	102

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N^{water}_{tot}	N^{cell}_{alc}	$N^{cell}_{TCA\text{-}c}$	$N^{cell}_{TCA\text{-}w}$	N^{cell}_{rest}	Recovered
0	0%	6%	3%	10%	80%	99%
4	9	3	2	6	77	97
44	15	2	0	6	82	105

EXP. 19. *Liberation of* P-*compounds from Cladophora glomerata.*

From a ditch *Cladophora* was collected. This alga was washed with sterile water; all other organisms were removed as well as possible. Only some diatoms and a little amount of Euglena remained, but they certainly did not surpass 1 % of the material. Bacteria were practically not present any more. — Immediately afterwards a sample of the material was drawn and used for the fractionation of the P-compounds (Schott glass filter G 5 M), the rest was suspended in 1 l of sterile water and killed with chloroform. — Autolysis at room temperature. — After 7 days the suspension was fractionated again. — Both obtained TCA-w extracts were kept at 90° C for one hour after the cells had been removed, to determine that part of the P-compounds, which is easily hydrolysed. — All actions under aseptic conditions. — Results as percentage of P^{susp}_{tot}, which was calculated by addition of the different fractions. — P^{susp}_{tot} = 7.3 mg/l; N/P = 8.4.

Autolysis during	$P(PO'''_4)$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$		P^{cell}_{rest}
					$P(PO'''_4)$	P_{bound}	
0 h	0%	0%	10%	10%	23%	44%	13%
2 h	—	22	—	—	—	—	—
4 h	—	25	—	—	—	—	—
21 h	12	49	—	—	—	—	—
3 d	26	71	—	—	—	—	—
7 d	48	85	0	1	4	7	3

EXP. 20. *Liberation of* N- *and* P-*compounds from Cladophora glomerata.*

At the same place, but 1 week later than in exp. 19 *Cladophora* was collected again; the ditch had been filled in the meantime with water, rich in PO'''_4. — Further data as in exp. 19. — Moreover the liberation of the N-compounds after a 9 days' autolysis was determined. — P^{susp}_{tot} = 8.4 mg/l; N^{susp}_{tot} = 71 mg/l; N/P = 8.4

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}
					$P(PO_4''')$	P_{bound}	
0	0%	0%	11%	17%	45%	23%	4%
7	80	88	—	—	—	—	—
9	—	88	0	0	3	10	—

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA\text{-}c}^{cell}$	$N_{TCA\text{-}w}^{cell}$	N_{rest}^{cell}
0	0%	17%	1%	10%	72%
9	25	2	0	8	65

EXP. 21. *Liberation of* Si- *and* P-*compounds from Stephanodiscus Hantzschii.*

Stephanodiscus was cultured in Fernbach flasks without aeration, as described in chapter II. A suspension of about 5 weeks old cells was prepared as in exp. 1 and divided into two samples. — The cells in sample A were killed with chloroform, after which the liberation of Si- and P-compounds at room temperature was followed up. — Sample B was used for the determination of P_{tot}^{susp}, N_{tot}^{susp} and Si_{tot}^{susp} and for the fractionation of the P-compounds (Schott glass filter G 5 M). — Results as percentage of P_{tot}^{susp} and Si_{tot}^{susp}. — All actions under sterile conditions. P_{tot}^{susp} = 11 mg/l; Si_{tot}^{susp} = 97 mg/l; N_{tot}^{susp} = 33 mg/l; Si/P = 8.9; N/P = 3.1.

Autolysis during (*days*	$Si(SiO_3'')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered
0	0.0%	0%	19%	41%	32%	5%	97%
1	0.5	59	—	—	—	—	—
2	1.6	60	—	—	—	—	—
11	15	66	—	—	—	—	—
21	27	78	1	3	10	4	96

EXP. 22. *Influence of* $NaHCO_3$ *on the liberation of* Si- *and* P-*compounds from Stephanodiscus Hantzschii.*

A suspension of *Stephanodiscus* was prepared as in exp. 21 and divided into three samples. The cells were killed with chloroform in samples A and B after addition of $NaHCO_3$ to sample B. The remaining sample was used for the determination of P_{tot}^{susp}, N_{tot}^{susp} and Si_{tot}^{susp} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — P_{tot}^{susp} = 8 mg/l; Si_{tot}^{susp} = 56.5 mg/l; N_{tot}^{susp} = 27 mg/l; Si/P = 7; N/P = 3.3. — P_{alc}^{cell} = 23%; $P_{TCA\text{-}c}^{cell}$ = 28%; $P_{TCA\text{-}w}^{cell}$ = 42%; P_{rest}^{cell} = 7% of P_{tot}^{susp}.

$NaHCO_3$ added	0 mg/l			100 mg/l		
Autolysis during (*days*)	$Si(SiO_3'')$	P_{tot}^{water}	pH	$Si(SiO_3'')$	P_{tot}^{water}	pH
0	0%	0%	7	0%	0%	7
7	2	65	7	2	70	7
14	6	70	7	6	71	7
28	15	—	—	15	—	—
42	19	72	7	18	74	7

EXP. 23. *Influence of* $NaHCO_3$ *on the liberation of* Si-, P- *and* N-*compounds from Stephanodiscus Hantzschii.*

Data as in exp. 22. — After a 3 weeks' autolysis 50 ml of both suspensions (susp. A_1 and A_2) were filtered off and the cells were suspended anew in 50 ml of H_2O and of $NaHCO_3$ solution (susp. B_1 and B_2). — The liberation of Si- and P-compounds was followed up in suspensions A_1, A_2, B_1 and B_2, of N-compounds only in suspensions A_1 and A_2. — Results as percentage of Si_{tot}^{susp}, P_{tot}^{susp} and N_{tot}^{susp}. — All actions under sterile conditions.

$P_{tot}^{susp} = 20$ mg/l; $N_{tot}^{susp} = 63$ mg/l; $Si_{tot}^{susp} = 132$ mg/l; Si/P = 6.6; N/P = 3.2.
$P_{alc}^{cell} = 19$ %; $P_{TCA\text{-}c}^{cell} = 5$ %; $P_{TCA\text{-}w}^{cell} = 73$ %; $P_{rest}^{cell} = 3$ % of P_{tot}^{susp}.
$N_{alc}^{cell} = 18$ %; $N_{TCA\text{-}c}^{cell} = 1$ %; $N_{TCA\text{-}w}^{cell} = 12$ %; $N_{rest}^{cell} = 65$ % of N_{tot}^{susp}.

$NaHCO_3$ added	0 mg/l						
	suspension A_1				suspension B_1		
Autolysis during (*days*)	$Si(SiO_3'')$	P_{tot}^{water}	N_{tot}^{water}	pH	$Si(SiO_3'')$	P_{tot}^{water}	pH
0	0%	0%	0%	6.4			
7	4	59	—	6.4			
14	8	77	—	—			
21	12	84	20	6.4			
					0%	0.0%	6.4
35	17	76	—	6.4	4	1.5	6.4
70	21	84	28	5.5	7	1.0	5.4
$NaHCO_3$ added	500 mg/l						
	suspension A_2				suspension B_2		
Autolysis during (*days*)	$Si(SiO_3'')$	P_{tot}^{water}	N_{tot}^{water}	pH	$Si(SiO_3'')$	P_{tot}^{water}	pH
0	0%	0%	0%	7.8			
7	11	86	—	7.8			
14	16	86	—	—			
21	20	81	30	7.8			
					0%	0.0%	7.8
35	23	82	—	7.8	17	1.5	7.8
70	25	86	39	7.9	23	1.0	8.2

CHAPTER V

DISCUSSION

In principle the problem of determining the rate of liberation of elements from dying phytoplankton in natural surroundings can be studied with two methods, either by comparing the chemical composition of the dead plankton cells with that of the living ones, or by measuring the quantity of liberated compounds when the algae have died off. The method, first mentioned, was applied by KLEEREKOPER (see page 54). It has the advantage that the results are hardly affected by a possible uptake of the liberated compounds by the living organisms, but it requires either a rather constant composition of the living or the dead plankton, or a thorough investigation into the changes in composition and into the interval between the dying off and collecting of the algae.

The second method yields the best results when all algal cells die off rapidly, which happens rarely in nature; it has the disadvantage that the uptake of the liberated compounds by other organisms is a seriously disturbing factor.

In both methods the mineralisation is determined, as it is effected by the total biocoenosis, which yields a result dependent on many and various factors.

We wished to study the liberation processes during autolysis separately. In order to eliminate disturbing conversions – e.g. a reuptake either by other organisms or by still living algal cells – we carried out our experiments with pure uni-algal cultures, and killed the cells all at once, which had to be done in such a way that the results were comparable to natural situations. However, very little is known as yet about the circumstances under which algae die off in nature. The numerous causes, that are of no interest within the scope of our investigation, because they do not lead to autolysis, are left out of consideration.

Frequently the dying off of algae is attributed to a depletion of nutrients, but it is questionable whether this is the most important factor. For, in a culture depleted of nutrients many algae can live on for months, provided the light intensity and the temperature are not too high.

In an extensive investigation into the periodicity of *Asterionella* and *Melosira*, LUND found (1949, 1950, 1954 and 1955), that the growth had been stopped by a Si-deficit (1950), and that *Asterionella* died off eventually. He assumed, that the organism divided once more after Si-depletion, but that the cell-walls could no longer be silicified. This supposition does, however, certainly not hold for all diatoms, because all our cultures can live on for months after Si-depletion, provided they are placed in dim light. Lund's observations were not sufficiently supported by laboratory experiments. Especially the joined influence of high light intensity and temperature ought to be investigated.

It is not unlikely that often the dying off of algae is indeed caused

by exposure to the combination of strong light and high temperature, as is mentioned by RODHE (1948).

Consequently we choose irradiation with U.V. light as a way to kill the cells rapidly. It is important that we could demonstrate that almost the same autolytical conversions occurred whether the cells were killed by chloroform treatment, by irradiation with U.V. light or by heating to 60° C. It strengthened our opinion that the results are not restricted to the particular circumstances of our experiments, but might be helpful to elucidate the phenomena observed in nature.

Experiments on the course of autolysis of plankton are reported in literature. STEINER (1938*a*, 1938*b*) killed zoöplankton with chloroform and noted the liberation of phosphate and phosphatases from the cells. WAKSMANN (1937) found, that diatoms, suspended in seawater and placed in the dark, die and give off phosphate to the water. HARVEY (1955) found, that about 40 % of the total amount of P-compounds present in *Skeletonema costatum* was liberated in three hours as P^{water}_{bound} and 35 % as PO_4''', when the cells had been pulverized in the presence of chloroform.

Our experiments resemble most those of HOFFMANN (1951, 1953 and 1956), who killed phytoplankton with chloroform. We find for *Scenedesmus* a liberation rate of the same order of magnitude as reported by him. He fractionated his material before autolysis with hot water, and discerned three fractions: inorganic phosphate, bound phosphate soluble in hot water and insoluble bound phosphate. He calculated that in case of autolysis 70 % of the insoluble bound phosphate leaved the cells, under complete hydrolysis to PO_4'''. He supposed that the soluble bound phosphate present in the cell was liberated without hydrolysis to PO_4''', because he found no decrease of the bound phosphate in the water. We can not share his conclusions, that the insoluble phosphate is hydrolysed to PO_4''' during liberation, while the soluble bound phosphate is not, because they are based on the ratio between the liberated amounts of PO_4''' and bound phosphate. We found in our experiments, that this ratio depends on the methods of killing. We obtained a larger amount of PO_4''' after killing with chloroform than after killing with U.V. light.

70–80 % of the element phophorus leaves the killed *Scenedesmus* cells, in case of autolysis under sterile conditions, in a few days.

The PO_4''', which constitutes 0–5 % of P^{cell}_{tot} and forms a part of the first fraction obtained in the fractionation (fraction alc 60 %), leaves the cells rapidly. Another part of this fraction, the phospholipids (10–20 % of P^{cell}_{tot}), is broken down enzymatically and liberated as PO_4'''.

10–30 % of the P-compounds, originally present in the cells, can be extracted by a 5 % TCA solution at 4° C (fraction TCA-c) from the residue, obtained after removal of the compounds soluble in ethanol 60 %. During autolysis at 0° C this fraction is liberated without hydrolysis to PO_4''', at 20° C they are partly hydrolysed to PO_4'''. It is not unlikely, that the rate at which these compounds

diffuse out of the cells is limited by the membranes of the cell. The stimulating effect of Ca-ions on the liberation of these compounds points in this direction. Moreover, the observation that at 0° C these compounds leave the cells in H_2O in 48 hours, but in a 5 % TCA solution in 1 hour, indicates a limited diffusion. It is remarkable, that the liberation in water at 0° C can happen after the cells have been boiled with ethanol 60 %, which therefore evidently does not abolish the limitation of the diffusion. The enzymes, however, are destroyed, from which follows, that the liberation of these compounds can be brought about without enzymes.

The P-compounds, which can still be extracted – after the removal of fraction alc 60 % and fraction TCA-c – with a 5 % TCA solution at 90° C (fraction TCA-w) and constitute 30–70 % of P^{cell}_{tot}, belong to at least two groups: the nucleophosphates, which are not liberated, and the other P-compounds, "polyphosphates" which are liberated, probably under influence of enzymes.

The polyphosphates have, besides the function of energy-storage (vide e.g. WIAME, 1958), a second, ecological, function: that of phosphate-storage. In our experiments it appeared, that P-deficient cells, when phosphate was replenished, could store, in the form of polyphosphate, in one hour three times as much phosphate as was already present in them.

In case of autolysis the P-compounds of the fraction alc 60 % and fraction TCA-c and the polyphosphates are rapidly liberated, thus forming an ecologically important group of substances. The nucleic acids and the protein-phosphates (fraction rest-P, about 5 % of P^{cell}_{tot}) are not liberated. Their phosphate becomes only available for the autotrophic organisms after a bacterial breakdown of the killed cells.

Quite a different picture is found in the liberation and mineralisation of the element nitrogen.

The N-compounds, present in the cell in dissolved state, are rapidly liberated in case of autolysis, but they represent only a small percentage of the nitrogen present in the algae studied. The bulk, the protein-nitrogen, remains as a slag.

This nitrogen can enter the cycle by means of bacterial digestion. We extended our investigations to this phenomenon to acquire a quantitative impression of it. Therefore, "leached" *Scenedesmus* cells – i.e. cells from which the soluble P- and N-compounds had been removed through autolysis and repeated washing – were suspended in filtered lake water at 30° C. After 5 days the *Scenedesmus* cells had desintegrated and 50 % of the added nitrogen had appeared in the medium as ammonia, which amount further remained constant; nitrite and nitrate had not been formed. Microscopically it could be demonstrated that various types of bacteria and ciliates had developed.

The quantity of ammonia liberated can be used as a measure for the digestion by bacteria, when a constant ratio exists between this quantity and that of the N-compounds digested. The latter amount could not yet be determined, because of the strong clotting of *Scenedesmus* detritus and bacteria.

By keeping the lake water at 45° C during one hour the growth of ciliates was strongly suppressed and the number of species of developing bacteria reduced. After this treatment maximally 25 % of the *Scenedesmus* nitrogen was liberated as ammonia; moreover another 50 % of the nitrogen remained in the supernatant, when the *Scenedesmus* cells were centrifuged off. As the supernatant contained small fragments of *Scenedesmus* cells as well as bacteria, the digestion could once more not be measured.

Although this phenomenon may be important for a bacterial putrefaction in a lake – for, as the sedimentation rate of the nitrogen slag is considerably decreased, the slag can stay longer in the upper water layers – it meant a great technical difficulty in laboratory experiments, where we wished to follow the course of the protein digestion quantitatively.

Better results were expected from experiments, in which *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Proteus vulgaris*, which are known to excrete proteinases, were cultured on a medium with leached *Scenedesmus* cells as the only source of nitrogen.

Against expectation this has not succeeded. Both types of *Pseudomonas*, however, could be cultured on a medium with no other source of nitrogen than the N-compounds, present in an alkaline extract of leached cells ("algal peptone"). A protein-precipitate, formed after the pH of the extract was brought to 7.5, disappeared during the growth of the bacteria.

Starting from the supposition, that the inability to culture bacteria on the leached cells themselves is due to the fact that the bacteria are not adapted to the substrate, we tried to subculture bacteria, grown on algal peptone, on leached cells. The results of these experiments are as yet dubious.

A second observation, which justified further investigations in this direction, is the fact, that the same types of *Pseudomonas* also showed a five- to sixfold increase of O_2 uptake and CO_2 release, when leached cells were given to a suspension of bacteria as a substrate, whereas *Bacillus subtilis* and *Proteus vulgaris* did not do so. This increase was, however, not accompanied by a production of ammonia.

This fact can not be caused by growth of the bacteria, as addition of DNP, which increased the exchange of gases by a few per cent, did not alter it. Moreover, when aspartic acid was given as a substrate under exactly similar circumstances, it appeared that 56 % of its nitrogen was converted to ammonia, when no DNP was added, and 71 % when DNP was added.

This ammonia production was strongly inhibited by addition of glucose, which suggested, that perhaps a liberation of carbohydrates from *Scenedesmus* cells prevented the production of ammonia. Although it is improbable, that many carbohydrates are liberated from the leached cells, we have tried to find out, by means of the R. Q., whether carbohydrates, proteins or a combination of both were respired. However, interpretation of the R.Q. is not possible, as appeared from the following observations:

When aspartic acid, succinic acid, malic acid or acetic acid were given to *Pseudomonas fluorescens* and *Ps. aeruginosa* as a substrate, the products of oxidation were not only CO_2 and H_2O, but probably oxalic acid as well. The ratio between combustion and oxalic acid formation appeared not to be the same for the different substrates mentioned. Thus succinic acid yielded 2 Mol O_2, whereas malic acid yielded 1 Mol O_2 per Mol acid. These observations can be explained by the action of the glyoxylic acid cycle in the bacteria (KORNBERG 1958, SAZ 1956).

Summarizing we can mention four possible causes, why no ammonia is produced:

1) Growth of the bacteria, which moreover is inhibited insufficiently by DNP. In view of the results with respiration of aspartic acid this seems improbable.
2) Simultaneous respiration of carbohydrates. This seems also improbable, because the substrate has been washed thoroughly.
3) Insufficient adaptation.
4) Wrong choice of bacteria.

We hope to effect a necessary adaptation by culturing of the bacteria on algal peptone. As regards a wrong choice of bacteria, we have started isolations of bacteria from the material of the experiments, in which leached cells are broken down in lake water.

Our experiments partly resemble those of WAKSMANN (1937), partly those of VON BRAND *et al.* (1937–1942).

Waksmann killed diatoms by suspending them in seawater in the dark, and concluded from the appearance of bacterial growth, that nitrogen was liberated from the diatoms.

Von Brand felt justified to conclude from his experiments, that the nitrogen from phytoplankton is converted in seawater quantitatively to ammonia, subsequently to nitrite and finally to nitrate. His experiments provoke criticism. Sometimes considerably more $N^{water}_{soluble}$ was recovered, than had been added as N^{cell}_{tot}. Von Brand assumed, that this increase came from N^{water}_{bound}, but that is impossible, as it appeared from his later experiments, that N^{water}_{bound} also increased after the addition of the plankton. Moreover, it can not be decided from his experiments whether the $N_{particulate}$ is still in the plankton or already in the bacteria. Finally it is remarkable, that such an intensive production of nitrite and nitrate can occur in seawater, while in fresh water without doubt it would have been inhibited considerably, when so much organic material had been added. It would therefore be important to know, to which extent the organic material in his experiments had been broken down.

The mineralisation of Si is a non-enzymatic, slow process. In our experiments only 20–30 % of the Si-compounds present in *Stephanodiscus Hantzschii*, was liberated after some weeks. Here the solubility of the formed product plays an important role, as appears from the effect of the addition of $NaHCO_3$. The percentage of Si-compounds liberated can vary considerably even in this single species.

Our experiments partly resemble those of JØRGENSEN (1955) with *Nitzschia linearis* and *Thalassiosira nana*. He found the liberation of the Si-compounds to be dependent on the pH. He used mixtures of $KHCO_3$ and K_2CO_3 as buffers. Whether his results were influenced by other factors than the pH's of his buffers was not investigated.

A distinct difference between both diatoms appeared: for *Nitzschia* at pH = 10 a maximal amount of 20 % of dissolved SiO_3'' was found after 85 days, whereas at the same pH for *Thalassiosira* this amounted to 97 %, after 37 days. As, moreover, in experiments with *Nitzschia*, all with the same pH, the rates of mineralisation varied considerably, he concluded, that various Si-compounds occur, with different hydrolysis rates.

The observation of LEWIN (1958) that *Navicula pelliculosa* contains "stored" silicium, which might be chemically different from the "functional" silicium, points in the same direction. Little is known as yet about these compounds. It seems, however, probable, that the supposition of ENGEL (1953), that silicium is linked as silicic acid to galactose in rye straw, applies to diatoms as well. We intend to investigate whether, also from diatoms, two fractions can be isolated with a different silicium-galactose ratio, like Engel reported.

COOPER (1951) supposed the mineralisation of Si-compounds could be judged by means of microscopic examination of the cells. He concluded from HART's communication (1934), that some diatoms could be recognised in the gastric contents of the animals that feed on these organisms, whereas others could not, that the Si-compounds must have been liberated from the latter cells. We fear his conclusion might be wrong, because *Thalassiosira* remains identifiable after digestion (Hart), while it has been shown in the experiments of Jørgensen, that the mineralisation of Si-compounds from this organism can be considerable. Moreover, the observation of ENGEL (1953) must be taken into account, that in rye straw the structure of the Si-deposits is still clearly visible microscopically, when 82 % of the silicium has been removed by extraction with methanolic NaOH.

When *Scenedesmus* cells were suspended in water during autolysis, iron could not be found outside the cells. When, however, EDTA was added to the suspensions 50–65 % of the iron appeared in the medium.

The effect of EDTA can be explained by assuming either that it extracts the – usually not liberated – iron from the cells "actively", or that it forms a stable complex with the liberated iron, which is otherwise precipitated immediately after liberation as alkaline iron-phosphate. The formation of such a precipitate (EINSELE 1938) is certainly not improbable during autolysis, considering the high pH of the suspensions and the large amounts of phosphate liberated.

This precipitation might also be prevented by causing autolysis under anaerobic circumstances. In that case the iron might be liberated as ferrocompounds, which are better soluble. MORTIMER (1941 and 1942) found that in lake water iron could dissolve under

anaerobic conditions as ferrocompounds; when afterwards conditions had become aerobic, these compounds were converted to ferri-compounds, which were precipitated.

Whatever the function of the EDTA may be, it is clear, that, in case of autolysis in a lake, the "chelate-forming" capacity of the water, which depends probably on the humus content, is an ecologically important factor.

From an ecological point of view the results of our experiments can be summarized as follows:

When an alga is killed under sterile conditions, 70–80 % of the P-compounds leave the cell in a few days, 70–80 % of the N-compounds remain in the cell, silicium leaves the cell only for a small part and very slowly, and iron is only dissolved when a stable complex can be formed, e.g. with EDTA.

The process of the liberation of N- and P-compounds was similar for all algae investigated. Therefore we feel justified to consider our results important for the interpretation of the phenomena observed in the development of algae in lakes, where no nutrients are supplied from the outside. Apart from the amount of autolysing cells in these lakes, the quite different liberation rates of the various elements will determine the growth rate of the algae.

The question now arises, whether the rates of liberation and mineralisation, observed during autolysis, have to be considered when we investigate the breakdown processes in a lake, were bacterial putrefaction always occurs.

In this connection we wish to discuss the experiments, already mentioned, of KLEEREKOPER (1952*a*, 1952*b* and 1953) more in detail. He concluded from analysis of living plankton and dead plankton collected in a lake at a depth of 11 m, that the mineralisation of nitrogen takes place more rapidly than that of phosphorus, as the phosphorus content of the dead material was very high. In our experiments on autolysis under sterile conditions the rates were reversed. As we found in preliminary experiments, that at least an important part of the N-slag, remaining after autolysis, can be digested by bacteria very rapidly, the experiments of Kleerekoper, though carried out under different circumstances, give at first the impression, that autolysis does not play an important role in the lake, studied by him.

In a later paper (COOPER e.a. 1953) it is suggested that the high P-content might be caused by a precipitation of alkaline ferriphosphate on the dead plankton. The occurrence of such a precipitate in a lake had also been reported already by EINSELE (1936, 1938). The argument against autolysis in Kleerekopers experiments is refuted by this supposition, so that no conclusions can be drawn from this type of experiments as far as the ratio between the rates of autolysis and bacterial putrefaction are concerned.

This ratio, as found in laboratory experiments, may be used only

with the greatest reserve when judging similar phenomena in a lake, because bacterial breakdown in the laboratory will mostly be achieved with far higher concentrations of bacteria than in a lake.

We have tried in another way to acquire some understanding of the processes, occurring during the breakdown of cells in natura surroundings.

Algal growth was studied in concrete tanks with a capacity of 1–2 m^3, filled with lake water which had been filtered through plankton gauze. The concentrations of nitrogen, silicium and phosphorus were increased roughly to those of Rodhe's culture solution No. 8, to cause a sufficient bloom for quantitative chemical determinations.

Because of the bad summers of 1954 and 1956 no bloom of algae was obtained, but in 1955 a distinct growth was observed. Table VIII gives a survey of the variations in the concentrations of nitrogen, phosphorus and silicium and in the pH.

TABLE VIII. *Results of chemical analyses of an experimental pond during algal growth under semi-natural conditions.*

On the 2nd of June 1955 a concrete tank of 1.5 m^3 was filled with bog-water, filtered through plankton gauze (200 mesh). The inside of the tank was painted with Neodon-glas "S" (Neodon-Lackfabrik Helmut Sallinger Krumbach/Schwaben), to prevent dissolution of concrete into the water. The tank was dug into the ground and protected against the entrance of material by a glass roof, while gaps covered with nylon gauze prevented heating. — On the 4th of June quantities of NH_4NO_3, KH_2PO_4 and Na_2SiO_3 were added, till the concentrations mentioned below were reached. — In June a bloom of algae occurred; in July the algae died off. — The temperature and light intensity were nearly equal to those in a lake; they were registered, but are not given in the table. Evaporated water was replaced by distilled water. — Every morning the water was stirred. — The concentrations of the elements are expressed in mg/l.

	June 4		June 23		July 11		July 26		August 26	
$P(PO_4''')$	1.97		1.23		1.08		1.36		1.13	
P^{water}_{bound}	0.03		—		0.33		0.42		0.56	
P^{cell}_{tot}	0.00		—		0.67		0.39		0.29	
P^{susp}_{tot}		2.00		—		2.08		2.17		1.98
$N(NO_2')$	0.02		3.76		2.08		0.24		0.00	
$N(NO_3')$	5.18		1.19		0.47		1.11		0.54	
$N(NH_4^+)$	7.00		1.28		0.28		0.00		0.95	
Sum		12.2		6.23		2.83		1.35		1.49
N^{water}_{bound}	3.4		3.74		5.47		4.74		4.00	
N^{cell}_{tot}	0.00		—		4.35		3.76		2.66	
N^{susp}_{tot}		15.6		—		12.7		9.9		8.15
$Si(SiO_3'')$	1.78		0.54		0.34		0.67		1.21	
pH	7.8		8.2		8.4		8.1		8.2	

As we are discussing the liberation processes during autolysis, affected by bacterial activity, we will only consider the breakdown period (July 11th–August 26th).

During this period the dissolved SiO_3'' increases. In the preceding period 1.44 mg of silicium had been incorporated, 60 % of which is liberated during the breakdown period. Therefore about 70 % of the silicium originally present, is again available for the growth of diatoms. Both Jørgensen's and our laboratory experiments yielded far lower values, which is remarkable, because the pH, although rather high for bog-water, is much lower than that, at which Jørgensen observed only a Si-liberation of a few per cent, even from *Thalassiosira*.

In our tank experiments the conversions of P^{cell}_{tot} and N^{cell}_{tot} come about more slowly than might be expected.

The N-balance shows a considerable deficit, which without doubt must be ascribed to the release of ammonia at these high pH's. Hence the nitrogen liberated during breakdown can not be recovered.

Nitrite was formed from nitrate, as appeared from experiments where KNO_3 was added to the lake water instead of NH_4NO_3. This phenomenon could be reproduced excellently. It is not plausible, that the reduction of nitrate stops at the level of nitrite; therefore a possible release of other gaseous reduction products than ammonia must be taken into account.

The experiments here described have a too preliminary character to determine exactly the ratio between autolysis and bacterial putrefaction, because the samples were drawn at too long intervals.

The impression is warranted, however, that the liberation of the P-compounds takes place more rapidly than that of the N-compounds, which suggests that autolysis can indeed occur in a lake under certain circumstances.

Before long we hope to repeat these experiments with some modifications, in order to establish a biocoenosis, which is an intermediate form between laboratory experiments and a natural lake.

SUMMARY

This paper describes an investigation into the function of the breakdown of algae in the cycle of elements in a lake.

The liberation and mineralisation of phosphorus, nitrogen, silicium and iron during sterile autolysis of *Scenedesmus quadricauda* and some other algae were determined. Autolysis was induced by chloroform treatment, U. V. irradiation or heating to 60° C.

70–80 % of the P-compounds are liberated in a few days, only the phosphate of the nucleic acids and proteins not being liberated. The forms in which phosphorus is liberated, and the mechanisms involved, were followed up. Some compounds are liberated by enzymatic activity.

Only 20–30 % of the N-compounds are liberated. The rest of the N-compounds belongs to the proteins and the nucleic acids, and remains as a slag.

The liberation of P- and N-compounds does not depend on size or nature of the killed algae investigated.

. The liberation of Si-compounds is a non-enzymatic slow process. In 5 weeks 20–30 % of the Si-compounds were liberated from *Stephanodiscus Hantzschii*. This amount could be increased by adding $NaHCO_3$ to the suspensions of autolysing diatoms.

The mineralisation of iron, which is also a slow process, depends on the chelate-forming capacity of the milieu.

Preliminary experiments were carried out to digest the slag of sterile autolysis by means of bacteria. When the slag was suspended in lake water one half of the nitrogen was converted to ammonia in 5 days, while the other half was divided up into bacterial and *Scenedesmus* nitrogen, the mutual ratio of which could not yet be determined.

It appeared not yet to be possible to digest the slag by cultures of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Bacillus subtilis*. The fact, however, that *Ps. fluorescens* and *Ps. aeruginosa* showed an increased respiration, when the slag was given as a substrate, and could also be cultured on a lye extract of the slag, is a stimulus for further investigations in this direction.

The combination of high light intensity and high temperature as a cause of the dying off of algae in nature is suggested to be a more frequently occurring phenomenon than is generally believed.

A preliminary experiment is mentioned, which gave probably a positive answer to the question whether autolysis can play a part in a lake, where bacterial putrefaction always occurs.

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