ROLE OF SULFUR IN THE CELL DIVISION OF *CHLORELLA*, STUDIED BY THE TECHNIQUE OF SYNCHRONOUS CULTURE

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SUMMARY

1. *Chlorella* was grown synchronously under controlled supply of S- and N-sources so as to make the processes of nuclear and cellular division proceed stepwise, and by using ^{35}S as a tracer the fates of S-compounds in various fractions of cell material (extracts with 70% ethanol, with 10% trichloroacetic acid, and the residue) were followed.

2. In the cells grown in the normal medium, the S-content (in %) of the ethanol- and TCA-extracts decreased in the growing stage and increased markedly at the ripening stage.

3. The ethanol extract contained an unknown S-compound and a non-S-containing peptide-like substance, the latter substance appearing only at certain stages of cell ripening. When chromatographed on paper the TCA extract gave only 1 radioactive (but ninhydrin-negative) spot, which, on hydrolysis with HCl, turned ninhydrin-positive owing to the liberation of several amino acids including cyst(e)ine.

4. In the cells, whose nuclear and cellular division were controlled by limited supply of S- and N-sources, the quantity of the S-containing substance in the TCA-extract increased markedly before nuclear division, and the unknown non-S-containing substance in the ethanol extract appeared only before and concomitantly with, the occurrence of cellular division.

INTRODUCTION

Using the technique of synchronous culture of *Chlorella* we have shown[1] that the processes of nuclear and cellular division of algal cells can be controlled in a specific manner by regulating the supply of sulfur and nitrogen sources to the cells. The phenomena observed are shown schematically in Fig. 1; the events occurring in the S-N-controlled culture are compared with those of the normal life cycle of the alga. The successive developmental stages of cells and their relative sizes are shown by circles; the white arrows indicate photochemical steps (occurring under photosynthesizing conditions), while black arrows show the light-independent (aerobic) processes. The black dots represent nuclei, their number representing the average number of FEULGEN-stained particles observed in each cell, and their sizes and numbers showing the relative quantities of DNA determined by chemical analysis.

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The definitions and characteristics of successive developmental stages in normal life cycles (D_n, D_a, D∼L, L_1, L_2, L_3 and L_4) are given in previous reports[2,3]. The starting materials used in both normal and S-N-controlled cultures were D_a-cells ("active dark cells") which had been grown in normal (complete) nutrient medium. When such cells were grown synchronously in an S-deficient medium under photosynthesizing conditions, the cells showed some increase of DNA-content followed by division of the nucleus into two. At this stage the cells were stalemated, being unable to perform cellular division, but when they were transferred to a medium containing potassium sulfate only, further synthesis of DNA and other cellular substances as well as the division of nuclei occurred, although cellular division still did not. This could be evoked by supplying nitrate and sulfate simultaneously. It thus became possible to separate the processes of nuclear and cellular division by controlling the supply of sulfur and nitrogen sources to cells that had previously been made deficient in sulfur.

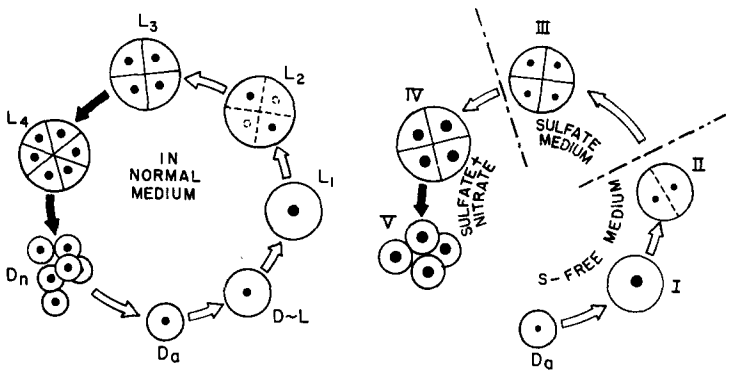

Fig. 1. Events occurring in the S-N-controlled culture (right) as compared with the normal life cycle (left). For full explanation see text.

For convenient reference, the successive stages observed in the S-N-controlled culture may be called Stages I, II, III, IV and V, as indicated in the figure*. The observations showed that in the life cycle of *Chlorella* sulfur plays, with nitrogen, an essential role in the processes of DNA-formation and nuclear division and of cellular division. The present work was undertaken to investigate, with radioactive sulfur as a tracer, the fate and behavior of sulfur in the cell material at the various stages of controlled or uncontrolled development illustrated in Fig. 1. It was found that nuclear division was preceded by an accumulation in the cells of some S-containing nitrogenous substance(s) which was soluble in 10% trichloroacetic acid solution, whereas the process of cellular division was accompanied by an accumulation of a non-S-containing nitrogenous substance(s) soluble in 70% ethanol.

METHODS

Procedures of synchronous culture

The experimental organism used was *Chlorella ellipsoidea*, and the methods of synchronous culture, which was started from the stage of "dark cells" (grown in

* As was remarked in our previous paper[1], it was notable that the cells at Stage V contained a larger amount of DNA/cell than the original Da-cells. In Fig. 1 these cells are tentatively represented as mononucleate cells having larger DNA-contents than the D_a-cells.

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normal nutrient medium) were the same as those described in our preceding paper[1]* In the culture with radioactive sulfate tracer, 0.3 to 0.7 mC of [^{35}S]sulfate was added to a liter of culture medium, the total concentration of sulfate being 10^{-3} mole/l.

Fractionation and analysis of cell material

To trace the distribution of S-containing substances in the cell material by radioactive measurements, the algal cells at different developmental stages were fractionated into 3 portions after being washed 3 times with distilled water. (a) An ethanol-soluble fraction was obtained by extracting fresh cells 3–4 times with boiling 70 % ethanol. The extract was evaporated to dryness under reduced pressure in a nitrogen atmosphere; the substance obtained was dissolved in a small amount of 70 % ethanol before further analysis. (b) A TCA-soluble fraction was obtained by treating the ethanol-extracted cells twice with 10 % trichloroacetic acid (TCA) at room temperature. The extracted substances were dried under reduced pressure of nitrogen as before and washed repeatedly with ether to remove TCA. After removal of ether, the substances were dissolved in a small amount of distilled water before further analysis. (c) A residual fraction after the above two extractions.

Each fraction was subjected to paper chromatographic analyses, either directly or after hydrolysis with hydrochloric acid. These procedures are described under the individual experiments. To measure the relative content of ^{35}S in each fraction, the material was oxidized by the method of PIRIE[5], non-labeled sulfuric acid (50 μM) was added to the mixture, and the total amount of sulfuric acid was precipitated as benzidine sulfate, collected on a filter paper and assayed for radioactivity.

RESULTS

Substances found in cells during the normal course of life cycle

S-compounds in 3 major fractions in cells growing in normal medium containing [^{35}S]sulfate: D_a-cells which had been grown in the normal medium containing radioactive sulfate were cultured synchronously in the same medium, and the distribution of ^{35}S in the 3 fractions at different developmental stages was followed. The results are illustrated in Fig. 2.

As has been observed in our earlier work[2], the total S-content in percent of cell dry wt. was highest in D-cells and L_4-cells and decreased progressively during the growing stages (from D$\sim$L to L_3). This trend was mainly due to the change in the ^{35}S-content of the ethanol and TCA extracts, for the percentage of ^{35}S in the residual fraction (about 60–70 % of the total) remained relatively constant during the life cycle. The characteristic changes of ^{35}S-contents in the first 2 extracts suggest that the S-compounds or related substances may play an important role in the ripening or cell division of algal cells. Most remarkable was the change in the ^{35}S of the TCA extract, although it occupied only a few percent of the total ^{35}S. It increased rapidly

* The normal (complete) medium contained/l: 5.0 g KNO_3, 2.5 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.003 g $FeSO_4 \cdot 7H_2O$, 1 ml of ARNON's "A5" solution[4]. The S-deficient medium contained/l: 5.0 g KNO_3, 2.56 g $Mg(NO_3)_2 \cdot 6H_2O$, 1.25 g KH_2PO_4, 0.002 g $FeCl_2 \cdot 4H_2O$, 1 ml of ARNON's "A5" soln. containing no sulfate. The pH of both media was adjusted to 5.2. In the S-N-controlled culture, the transfer of algal cells from one medium to another was effected only after washing the cells 3 times with distilled water.

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in the L_2–L_4 stages where the processes of DNA-formation and nuclear (as well as chloroplastic) division occurred most significantly.

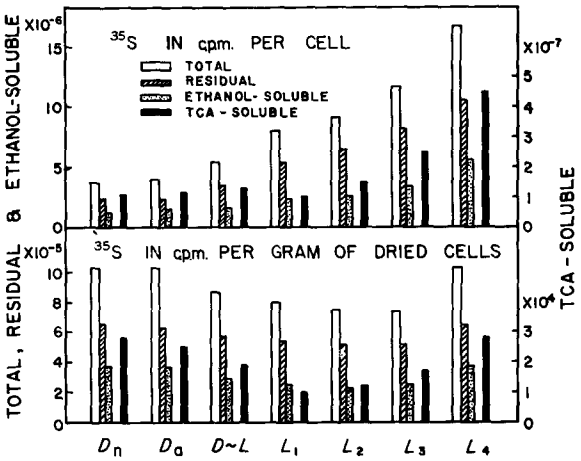

Fig. 2. Distribution of ^{35}S in 3 fractions of cell material at different stages in normal life cycle. Synchronous culture was effected in a complete medium containing ^{35}S-labeled sulfate as the S-source.

Substances in the ethanol-soluble fraction: The ethanol-soluble fractions obtained from the cells at various stages were subjected to paper chromatography, the developing solvent being butanol–acetic acid (10:1) saturated with water. The spots of ^{35}S-containing substances were determined by autoradiography, and those of nitrogenous substances by the ninhydrin test. The results obtained are shown in Fig. 3.

In the autoradiographic tests, cells at different developmental stages gave the same qualitative results showing the existence of 2 groups of sulfur compounds; one

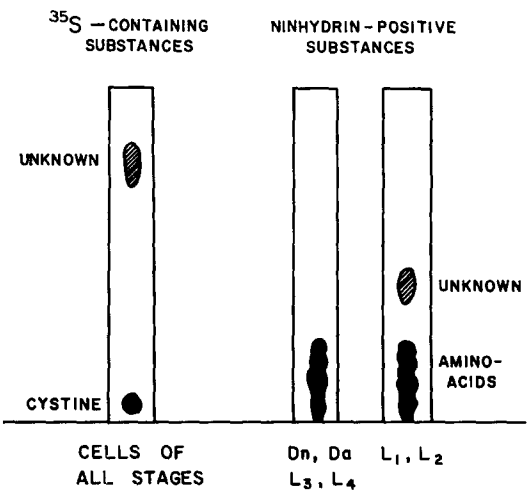

Fig. 3. Paper chromatograms of ethanol extract obtained from normally grown cells.

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with a high R_F-value could not be identified*, and the other, from its R_F-value, appeared to be cystine. The coexistence of glutathione with cystine could not be ascertained from the results.

The results of the ninhydrin test were rather different. With the cells at stages D_n, D_a, L_3 and L_4 the chromatogram gave a group of spots corresponding to known amino acids; these were revealed by a 2-dimensional paper chromatogram as alanine, glycine, serine, glutamic acid, aspartic acid and cystine. With the cells at the stages L_1 and L_2, on the other hand, a spot of a specific non-S-containing ninhydrin-positive substance appeared, whose nature could not be identified by 2-dimensional paper

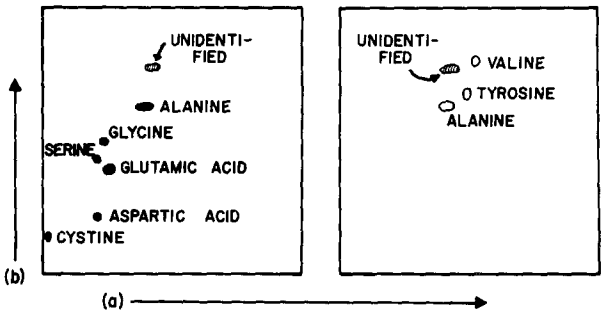

Fig. 4. Ninhydrin-positive spots on the paper chromatograms of the ethanol extract obtained from L_1 and L_2 cells. Developing solvents: (a) *n*-butanol–acetic acid–water (4:1:1). (b) 75% phenol–NH_3 (0.15%)–water. Unshaded spots of valine, tyrosine and alanine, which were not detectable in the cell extract were those of standard [^{14}C]amino acids (detected on autoradiogram).

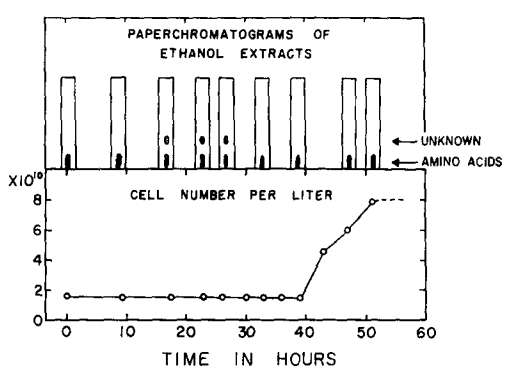

Fig. 5. Appearance of the unknown ninhydrin-positive substance on the paper chromatograms of the ethanol extract at the L_1 and L_2-stages in normal life cycle.

chromatography (Fig. 4)**. The characteristic events of appearance and disappearance of this peptide-like substance (when hydrolysed, this substance gave 2 separate spots

* After completion of this manuscript, we received a personal communication from Dr. A. A. BENSON of the Pennsylvania State College, stating that this substance might be acylated glycerol-1-β-galactoside-6-sulfate. The writers take this opportunity to thank Dr. BENSON warmly for his valuable suggestion.

** These amino acids were identified not only from the R_F values of individual spots, but also by comparing the spots with those of ^{14}C standard amino acids which were simultaneously chromatographed in sufficiently small amounts and detected by autoradiography. The [^{14}C]amino acids were kindly supplied to us by Mrs. T. WADA of the Institute of Applied Microbiology, University of Tokyo.

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on a paper chromatogram, suggesting a peptide nature) during the course of the synchronous culture are shown in Fig. 5.

Substances in the TCA-soluble fraction: The TCA-soluble fraction gave on the paper chromatogram (developed as before) only 1 radioactive spot which remained at the origin even after prolonged reaction. This spot gave no positive color reaction with ninhydrin reagent. However, after hydrolysis with 6 *N* HCl for 21 h in a boiling water-bath the substance became ninhydrin-positive, and gave spots of cystine, arginine, glutamic acid, aspartic acid, glycine, alanine and serine (but not methionine) on the chromatogram. The relative quantities of these components seemed to vary according to the stage of cell development. Detailed studies on these problems are reserved for some future occasion. Work now in progress in our laboratory shows that the extracted substance contains, besides the above components, a sizable amount of nucleotides; this work will be published later.

S-containing substances in the residual fraction: The residual fraction was paper chromatographed after hydrolysis with 6 *N* HCl in a boiling water-bath for 21 h. An autoradiogram of the paper proved the existence of 2 sulfur compounds which were shown, by their R_F values, to be cystine and methionine. In some cases, 1 or 2 more spots appeared on the autoradiogram; these were probably derived from methionine during the hydrolysis or subsequent treatments of the substance. No attempt has been made to analyse the components of this fraction or to follow the changes in their concentrations during the life cycle.

Sulfur-containing substances found in cells grown in S-N-controlled cultures

To get further insight into the fates of S-compounds in the process of cell development, synchronous cultures (with ^{35}S as a tracer) were performed under S-N-controlled conditions as illustrated in Fig. 1, and the distribution of ^{35}S in the 3 major fractions was followed. Three different methods were used to investigate the events during the periods from D_a to Stage II and from Stage II to Stage V.

Fates of the S-compounds in normal D_a-cells during the subsequent period of S-starvation: The D_a-cells, which had been grown in a complete medium containing [^{35}S]sulfate, were further grown synchronously in a medium containing no sulfate (but containing all other nutrient salts), and the fates of the S-compounds during the period from the D_a-stage to Stage II were followed. During this period the cells, as mentioned already, were incapable of cell division, but grew to some extent, performing some synthesis of DNA followed by nuclear division. The change of distribution of ^{35}S occurring during this period is illustrated in Fig. 6.

The total ^{35}S/cell remained unaltered throughout the experiment, but in terms of percentage of cell dry wt. it decreased progressively with the increase of the total cell material. In percentage of the total ^{35}S, the ^{35}S in the residual fraction eventually increased to a level of 90 % while that in the ethanol-soluble fraction decreased. The curves representing the time courses of these changes are almost mirror images, indicating that the S-compounds in the alcohol-soluble fraction were transformed gradually into those in the residual fraction. Similarly, the ^{35}S in the TCA-soluble fraction decreased as a whole with the lapse of time, but showed an abrupt temporary increase before reaching a steady low level. This increase is thought to be related to the process of nuclear and chloroplastic division occurring during the period under investigation.

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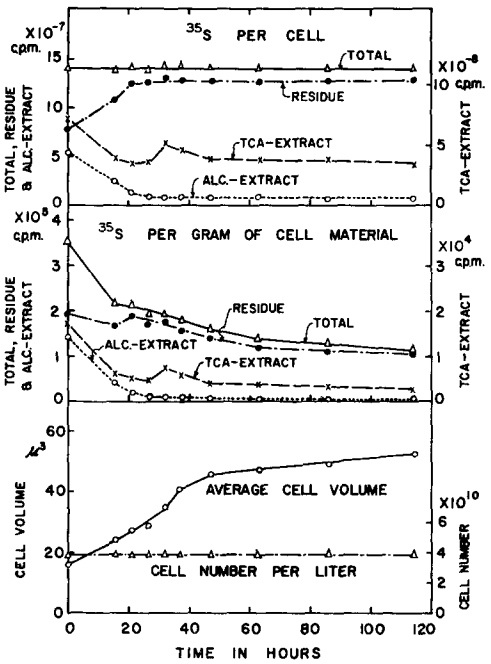

Fig. 6. Fates of the S-compounds in normal D_a-cells during the subsequent period of S-starvation.

Fates of the S-compounds during the period from Stage II to Stage V: The D_a-cells which had been grown in a complete medium containing [^{35}S]sulfate were subjected to S-deficiency (in the presence of all other nutrient elements) until they became stalemated at Stage II. Then, the cells were transferred to a solution containing [^{35}S]-sulfate only. As mentioned already (Fig. 1), the content of DNA increased and nuclear (and chloroplastic) division occurred but not cellular division. When potassium nitrate was then added to the sulfate solution, further increase of DNA occurred followed by the completion of cellular division.

During these processes (Stage II – III – V) the changes in ^{35}S-contents of the 3 major fractions of cell material were followed. The results are summarized in Figs. 7 and 8. When the S-starved cells at Stage II were placed in the sulfate soln., sulfur was abruptly absorbed primarily in the alcohol-soluble fraction*. The ^{35}S in this fraction decreased gradually, however, while that in the residual fraction increased, indicating (with the data in Fig. 6) that the incorporation of ^{35}S in the residual (proteinous) fraction proceeded mainly via substances appearing in the alcohol-soluble fraction. Similarly, the ^{35}S in the TCA-soluble fraction also increased abruptly at first, but soon decreased and eventually attained a steady low level as the ^{35}S in the residual fraction reached its steady high level.

At the 70th hour of the experiment, when the cells became stalemated at Stage III and the distribution of S-compounds in different fractions had attained steady states, potassium nitrate was added to the suspension, so that the cells could proceed from

* The subsequent decrease of the total S-content on prolonged incubation in the sulfate solution shows that the S which was first vigorously absorbed was later partially released into the medium.

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Stage III to Stage V. For about 20 h after the addition of nitrate, DNA-formation and S-assimilation—mostly in the residual fraction—were enhanced and this was followed by cellular division (but not by nuclear division). The ^{35}S-content of the alcohol-

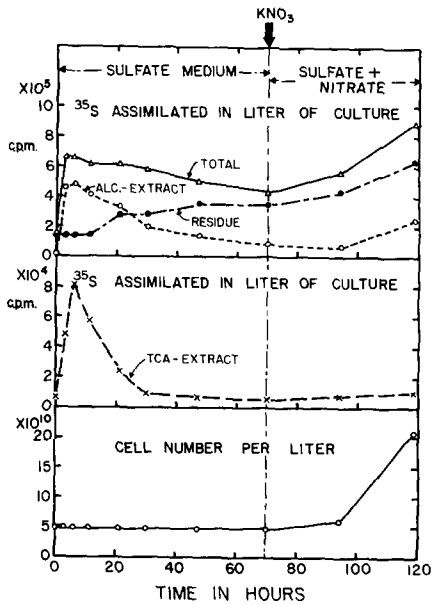
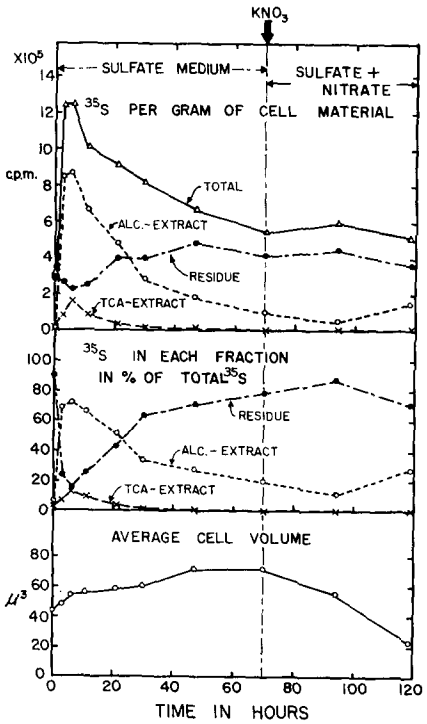


Fig. 7.



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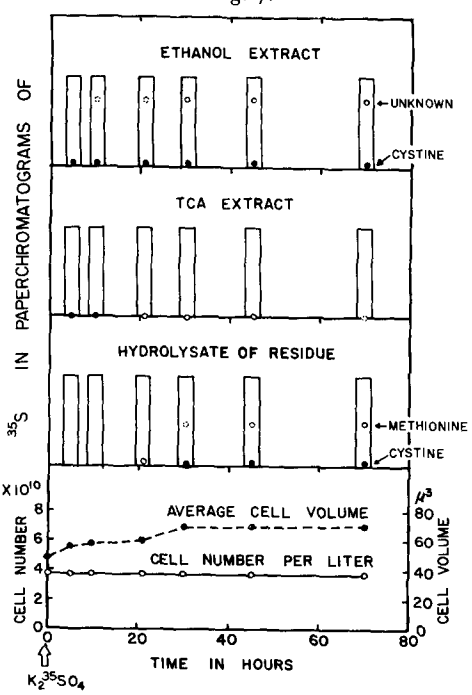

Fig 9.

Fig. 7. Fates of the S-compounds during the period from Stage II to Stage V.

Fig. 8. Fates of the S-compounds during the period from Stage II to Stage V.

Fig. 9. Fates of the ^{35}S newly assimilated during the period from Stage II to Stage III.

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soluble fraction increased when cell division started, whereas that of the TCA-soluble fraction remained at a low level throughout the whole period after the addition of nitrate. These results, with the data presented in Fig. 6, demonstrate clearly that the temporary increase of the S-compound in the TCA-extract is causally related to the process of nuclear (and/or chloroplastic) division, but not to the processes of DNA-formation and cellular division.

Fates of the ^{35}S newly assimilated during the period from Stage II to Stage III: To obtain further information about the sequence of S-incorporation, [^{35}S]sulfate was given to the S-deficient cells containing no radioactive sulfur, and at different stages of transformation from Stage II to Stage III, the S-compounds in each fraction were traced by paper chromatography followed by autoradiography. The results obtained are shown in Fig. 9.

The ^{35}S appeared most rapidly and mainly in the ethanol extract; only cystine was found at first, and the unknown S-containing substance with the high R_F-value (Fig. 3) appeared at later stages when the cells grew to some extent. The TCA-extract showed, throughout the whole experiment, only 1 spot at the origin; as in the preceding experiment, this was largest in the earlier stages and then decreased progressively with the increase in cell mass. The hydrolysate of the residual fraction, in which no radioactivity was detected in the earlier stages, gradually incorporated ^{35}S first in cystine and later in methionine.

The unknown peptide-like substance in the ethanol extract found in cells grown in S-N-controlled culture

The data presented in Figs. 3, 4 and 5 showed that at a certain stage of "light cells" an unknown non-S-containing peptide-like substance appeared in the ethanol extract of the cell material. With S-starved cells (at Stage II) containing no radioactive sulfur, the fate of this substance was investigated at stages II–V. The results are presented in Fig. 10. The unknown substance did not appear during the period from Stage II to Stage III, where formation of some DNA followed by nuclear (and chloroplastic) division took place*. When, at the 45th hour of the experiment, potassium nitrate was added to the sulfate medium, the DNA content increased further, un-

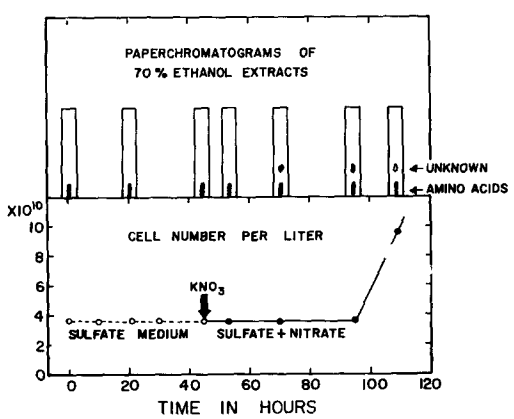

Fig. 10. Fate of the ethanol-soluble peptide-like substance during the course of cell transformation from Stage II to Stage V.

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accompanied by nuclear division, and after an induction period of about 50 h cellular division took place. It is worth noticing that about 25 h after the addition of nitrate a large quantity of the peptide-like substance appeared and was present, although gradually decreasing in quantity, until the completion of cellular division. The formation of this substance thus seems to be somehow related to the process of cellular division. A point of interest is that its formation required the simultaneous presence of sulfur and nitrogen sources, although the substance itself did not contain sulfur.

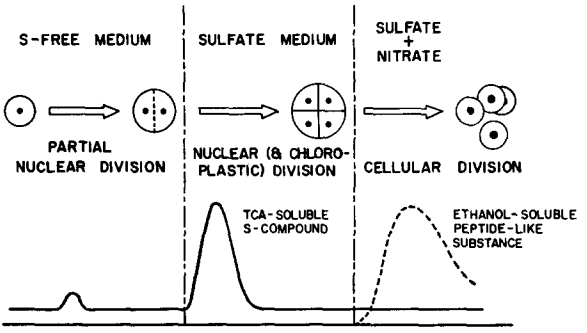

Fig. 11. Schematic representation of the fates of the TCA-soluble S-compound and the ethanol-soluble peptide-like substance in relation to nuclear (and/or chloroplastic) and cellular division, respectively, during the course of S-N-controlled synchronous culture of algal cells.

DISCUSSION

By controlling the supply of sulfur and nitrogen sources to the synchronous culture of *Chlorella* it was demonstrated that there were at least 2 substances which seemed to play important roles in the process of cell division. From their behavior in algal cells at different developmental stages in normal as well as in S-N-controlled cultures, it was inferred that the TCA-soluble substance was significant in the process of nuclear division, and that the ethanol-soluble substance played some important part in the process of cellular division. The main points of our observations on the behavior of these substances in the S-N-controlled synchronous culture are schematically illustrated in Fig. 11. Although the actual biochemical role played by these substances is still obscure, it seems worth while to elucidate their chemical nature. Details of our work along this line will be published elsewhere in the near future.

ACKNOWLEDGEMENT

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REFERENCES

[1] E. HASE Y. MORIMURA, S. MIHARA AND H. TAMIYA, *Arch. Mikrobiol.*, 32 (1958) 87.
[2] E. HASE, Y. MORIMURA AND H. TAMIYA, *Arch. Biochem. Biophys.*, 69 (1957) 149.
[3] T. IWAMURA, E. HASE, Y. MORIMURA AND H. TAMIYA, *Ann. Acad. Sci. Fennicae Ser. A*, II, 60 (1955) 89.
[4] D. I. ARNON, *Am. J. Botany*, 25 (1938) 322.
[5] N. W. PIRIE, *Biochem. J.*, 26 (1932) 2044.

* Even on prolonged incubation in the sulfate solution (more than 100 h) the cells at Stage II were incapable of forming the substance in question.