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INTRACELLULAR DISTRIBUTION OF SULFUR DURING THE SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

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SUMMARY

Intermittent illumination was used to synchronize cells of *Chlorella pyrenoidosa*. The degree of synchrony of the cultures was evaluated during three consecutive generations of growth in continuous light following the synchronization procedure. The loss in synchrony, as measured by the coefficient of variation, was only 8.53% for the three generations.

The uptake and intracellular distribution of [^{35}S]sulfate was followed during synchronous growth. The total cellular-^{35}S (percentage of cellular dry wt.) remained essentially constant throughout cellular development. However, the percentage ^{35}S (percentage of total cellular-^{35}S) in the total cellular protein, cold trichloroacetic acid extract, and sulfolipid fractions of the cells showed dennite periodism.

Between the third and fifth hours of cellular development, during a 14-h synchronous growth cycle, the cold trichloroacetic acid-soluble fraction showed a marked increase in percent ^{35}S while the protein fraction showed a decrease of equal magnitude. The decrease in protein-^{35}S was traced to a decrease in the percentage of protein cystine–cysteine-^{35}S. The percentage of protein methionine-^{35}S increased during this period but not enough to compensate for the decrease in protein cystine–cysteine-^{35}S. Thus, the percentage of protein-^{35}S decreased. Between the fifth and seventh hours of cellular maturation, the percentage of ^{35}S in the protein and trichloroacetic acid-soluble fractions returned to their original levels. The protein–sulfur–amino acid composition likewise returned to its original value. The change in the ^{35}S content of the cold trichloroacetic acid-soluble fraction was attributed to a single spot on paper chromatograms which absorbed ultraviolet light and was ninhydrin negative. Acid hydrolysis of the compound(s) in this spot released six or seven ninhydrin-positive spots and two ultraviolet-absorbing spots. After acid hydrolysis, most of the radioactivity was associated with a ninhydrin-positive spot which had the characteristics of cysteic acid.

Immediately after the ^{35}S content of the protein and trichloroacetic acid-soluble fractions returned to their original values, nuclear division was initiated. A close relationship between these shifts in sulfur metabolism and the initiation of nuclear division seems possible.

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INTRODUCTION

HASE *et al.*[1] have studied the effect of the deficiency of the macronutrients (N, P, Mg, K and S) on the synchronous growth of *Chlorella ellipsoidea*. The effect of sulfur deficiency was found to be unique in causing complete cessation of cellular division. During sulfur deficiency, the synchronized cells were able to grow to some extent and showed some increase in DNA. However, the nucleus of each cell divided into only two daughter nuclei instead of the usual four. At this stage of development, the cells were unable to undergo further nuclear or cellular division unless sulfate–sulfur was supplied to the medium. Thus, it appeared that sulfur might play a key role in either nuclear or cellular division.

The uptake of [^{35}S]sulfate and the intracellular distribution of ^{35}S during normal and sulfur-deficient growth of synchronized *C. ellipsoidea* were also studied by HASE *et al.*[2]. To follow the intracellular distribution of ^{35}S, cells of each developmental stage were fractionated into: (a) a boiling 70 % ethanol-soluble fraction, (b) trichloroacetic acid-soluble fraction, and (c) a cellular residue remaining after stepwise removal of the above two fractions from the cells.

It was reported that the ^{35}S content (percentage of dry wt.) of the cells was highest in daughter cells, decreased during the growing stages, and returned to its initial level immediately prior to cellular division, *i.e.*, release of daughter cells. This trend was attributed to the ^{35}S content of the ethanol and trichloroacetic acid-soluble fractions since the percentage of ^{35}S in the residual fraction remained relatively constant throughout cellular development. The rapid increase in ^{35}S content of the trichloroacetic acid-soluble fraction during the stages of nuclear and chloroplastic division suggested a possible relationship of sulfur compounds in this pool to these processes. Nucleotide- or polynucleotide-polypeptides were isolated[2–4] as the major radioactive components of trichloroacetic acid-soluble fraction. Although these compounds have been partially characterized, their role in nuclear, chloroplastic, or cellular division is not clear.

As in the initial studies by HASE *et al.*[2,5], the present investigation was designed to gain insight into the possible role of sulfur in nuclear and cellular division by following the intracellular distribution of ^{35}S during normal synchronous growth of *Chlorella pyrenoidosa*.

MATERIALS AND METHODS

Organism and culture conditions

The alga used in these studies was the high temperature strain 7-11-05 of *C. pyrenoidosa* described by SOROKIN AND MYERS[6]. The cells were synchronized by a modification of the procedure of SCHMIDT AND KING[7] in which the length of the light and dark periods was increased from 9 to 10 h and the concentration of CO_2 in the aeration mixture was increased from 2.5 to 3.75 %. During synchronization, the cells were cultured on the medium described by SOROKIN AND MYERS[8] which was modified by: (a) doubling the levels of KNO_3 and KH_2PO_4 to permit the exponential growth of higher cell densities, (b) reducing the level of $MgSO_4 \cdot 7H_2O$ by 75 % to eliminate clumping of the cells during growth. A sulfur-deficient medium was designed by replacing all sulfate salts of the complete medium with chloride salts. This medium contained the following nutrients (g/l): KNO_3, 2.5; KH_2PO_4, 2.5; $CaCl_2 \cdot 7H_2O$, 0.1112;

$MnCl_2 \cdot 4H_2O$, 0.0144; $(NH_4)_6Mo_7O_2 \cdot 4H_2O$, 0.0089; H_3BO_3, 0.1142; $CoCl_2 \cdot 6H_2O$, 0.0108; $FeCl_3 \cdot 6H_2O$, 0.0489; KOH, 0.31. The final pH of the medium was 6.7. For radioactive tracer experiments, this medium was modified by the addition of [^{35}S]sulfate as [^{35}S]sulfuric acid plus sufficient $MgSO_4$ to meet the minimum sulfur requirement of the cells for one synchronous growth cycle.

Biochemical determinations

After synchronization, the cells were washed three times with sulfate-deficient medium and resuspended, to a density of 20–30·10^6 cells per millilitre, in the same medium. Approx. 0.4 mC of carrier-free [^{35}S]sulfate plus 10 μmoles of carrier $MgSO_4$ were added to 950 ml of this cell suspension. The suspension was then illuminated to start the synchronous growth cycle. Thereafter, samples were taken at 1- or 2-h intervals. At each sampling period, the cell number/ml of culture was determined using a Levy–Hausser hemacytometer; then, the cells were centrifuged from the culture medium and washed three times with sulfate-deficient medium. The amount of [^{35}S]sulfate per millilitre of culture medium was determined after removal of the cells. After washing, a portion of the cells was resuspended in distilled water to the original concentration in the culture medium and the total cellular-^{35}S per millilitre was determined. The remaining washed cells were fractionated by the following procedure: (a) acid-soluble-^{35}S was removed from the cells by three 10-min extractions with 10% trichloroacetic acid at 0°, (b) sulfolipid-^{35}S was removed from the residue of the preceding step by an overnight extraction with 70% ethanol at 3–5°, followed by three 20-min extractions with ethanol–ether (3:1, v/v) at 45°, (c) the residual-^{35}S remaining after the two previous extractions was assumed to be "protein-^{35}S".

Radioactivity (^{35}S) in the culture medium, whole cells, and cellular extracts and residue was determined after plating directly on stainless-steel planchets. Three replications of each sample were plated, with and without the addition of internal standard, and counted in a Nuclear-Chicago GM Counter. The addition of internal standard made it possible to correct for self-absorption in each sample.

The cellular residues (protein) were each made to volume with distilled water and aliquots were taken so that each contained the same amount of radioactivity. These aliquots were taken to dryness and hydrolysed with 6 N HCl under nitrogen for 24 h in sealed tubes at 110°. After hydrolysis, the HCl was removed by repeated drying *in vacuo*. The dried hydrolysates were made to a volume of 1 ml with water. To measure the radioactivity of each sulfur amino acid in the hydrolysates, two methods were used. The first method involved the separation of sulfur amino acids by paper chromatography on Whatman No. 3 MM filter paper strips using the following solvent system: ethanol–*n*-butanol–water–diethylamine (10:15:5:2, v/v). The radioactivity associated with each sulfur amino acid was measured using a Nuclear-Chicago "strip counter". The second method involved separation of the amino acids, by ion-exchange chromatography, with a Beckman-Spinco Amino Acid Analyzer. The eluate from the analyzer was collected in 2-ml fractions. Each set of fractions containing a sulfur amino acid or sulfur amino acid derivative was pooled, made to a given volume, and the radioactivity determined by plating and counting.

Aliquots of the cold trichloroacetic acid extract from each sample were extracted continuously with ethyl ether for 24 h to remove the trichloroacetic acid. The ether extract from each sample was reduced in volume, plated and counted. The aqueous

phase from each sample was taken to dryness and the resulting residue was dissolved in approx. 1 ml of distilled water. Aliquots of these aqueous solutions were chromatographed on Whatman No. 3MM paper strips with each of the following solvent systems: (a) ethanol–*n*-butanol–water–acetic acid (10:10:5:2, v/v), (b) *n*-butanol–water–acetic acid (65:34:1, v/v). After development, the chromatograms were viewed under ultraviolet light and then scanned for radioactive spots with the strip counter. To detect amino acids, the chromatograms were sprayed with a solution of 0.4 % ninhydrin in ethanol. Radioactive spots were cut from unsprayed chromatograms and eluted from the paper with water. The water eluates were then taken to dryness, and the resulting residues were hydrolyzed with 6 N HCl. The hydrochloric acid was removed and aliquots of the hydrolysates were chromatographed in each of the above solvent systems. These chromatograms were scanned for ultraviolet-absorbing, radioactive, and ninhydrin-positive spots. An unhydrolyzed cold trichloroacetic acid extract from a 5-h sample was added to a Norit-A (activated charcoal) column (0.5 g) which had been pretreated with 20 ml of 1 N HCl followed by 20 ml of 10 % EDTA (pH 6.9). The sample, containing a known amount of radioactivity, was washed into the column. The column was then washed with 30 ml of 10 % aqueous pyridine. The water and the aqueous pyridine washings were collected separately and the radioactivity in each determined.

RESULTS AND DISCUSSION

Synchronous growth

Fig. 1 illustrates an experiment in which synchrony of the high-temperature strain of *C. pyrenoidosa* was followed for three consecutive generations in continuous light following the synchronization treatment. The culture was diluted to its original cell concentration after 12 and 27 h of growth to prevent low light intensity or low nutrient concentration from limiting the growth rate of the cells. For each synchronous growth cycle, the average generation time and average increase in cell number were determined. The coefficient of variation was determined by the method of SPENCER *et al.*[9] to evaluate the synchrony in each successive generation (Table I).

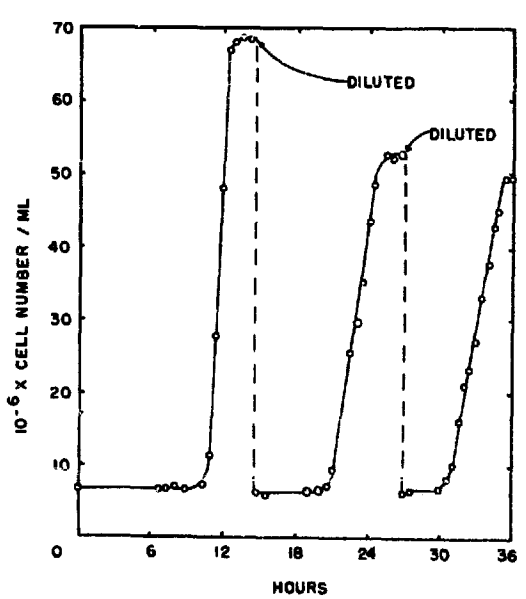

Fig. 1. Continuous synchronous growth of *C. pyrenoidosa* (strain 7-11-05).

The percentage loss in synchrony for the three generations, as indicated by the coefficient of variation, was only 8.53 %. Thus, cells of *C. pyrenoidosa*, synchronized by intermittent illumination, continue to divide with a high degree of synchrony for several generations in the absence of periodic exposure to the synchronization treatment.

TABLE I

ANALYSIS OF SYNCHRONY DURING THREE CONSECUTIVE GENERATIONS OF SYNCHRONOUS GROWTH OF *Chlorella pyrenoidosa* (STRAIN 7-11-05)

Generation	*Average generation time (h)*	*Cell number increase*	*Coefficient of variation (%)*
1	11.71	10.71	5.02
2	11.25	8.33	10.60
3	10.67	7.02	13.55

The reduction in the increase in cell number for each successive generation is difficult to interpret. Either the dark periods prior to the first synchronous growth cycle stimulate multiple nuclear divisions in the first cycle or continuous illumination inhibits multiple nuclear divisions in the second and third cycles. The results of preliminary experiments have favored the former interpretation.

[^{35}S]Sulfate uptake

In this laboratory, SCHMIDT[10] and SCHMIDT AND KING[7] have observed essentially linear logarithmic increases in total cellular dry weight, nitrogen, phosphorus, and sulfur during synchronous growth of *C. pyrenoidosa* when a cell concentration of 30–36·10^6 cells per millilitre was cultured at 38.5° and aerated with CO_2–air (2.5:97.5).

However, it was observed in the present study, at CO_2 concentrations above

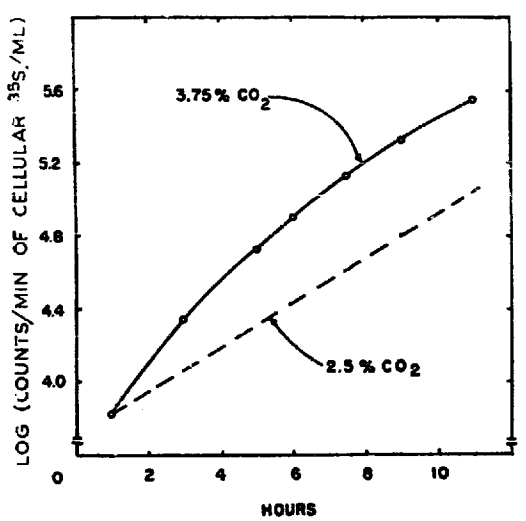

Fig. 2. Cellular dry wt. as affected by CO_2 concentration in the aeration mixture during synchronous growth of *C. pyrenoidosa* (strain 7-11-05). The dashed-line represents the slope experimentally determined by SCHMIDT[10].

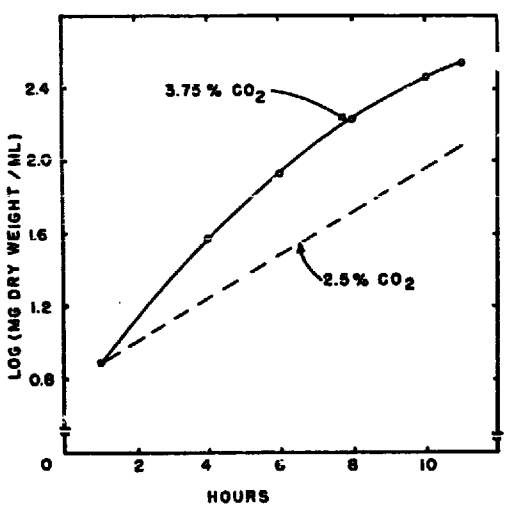

Fig. 3. [^{35}S]Sulfate uptake as affected by CO_2 concentration in the aeration mixture during synchronous growth of *C. pyrenoidosa* (strain 7-11-05). The curve at 3.75 % CO_2 was obtained by averaging data from two identical experiments. The dashed-line represents the slope experimentally determined by SCHMIDT[10].

2.5 % in the aeration mixture, that the accumulation of cellular dry wt. (Fig. 2) as well as the uptake of sulfate (Fig. 3) increased at a rate greater than the linear logarithmic rate previously reported.

It is apparent from Figs. 2 and 3 that at either 2.5 or 3.75 % CO_2 the curves for total cellular ^{35}S and cellular dry-weight accumulation can be superimposed. Since the rates of [^{35}S]sulfate uptake and dry weight accumulation are parallel at a given CO_2 concentration, the level of total cellular-^{35}S, if expressed as percent of cellular dry wt., will remain essentially constant throughout cellular growth.

HASE *et al.*[2], however, observed periodism in the level of total cellular-^{35}S (percentage of cellular dry wt.) during synchronous growth of *C. ellipsoidea*. The total cellular-^{35}S decreased from the beginning of growth until a stage prior to cellular division when it returned to its initial level. This apparent difference, in either sulfate uptake or dry weight accumulation, between the two organisms may be an inherent metabolic difference or merely a difference in culture conditions or synchronization procedure.

In addition to the greater rate of accumulation of cellular dry matter and of sulfate uptake, the higher CO_2 concentrations increased the average number of daughter cells yielded by each mother cell during cellular division. At a concentration of 2.5 % CO_2, each mother cell divided into eight daughter cells; whereas, at CO_2 concentrations between 3.75 and 5 %, the average mother cell divided into 10 or more daughter cells.

It seemed possible that conditions which stimulated sulfate uptake and cellular division would also tend to magnify any metabolic shifts or pools of sulfur-containing compounds; therefore, in all of the following experiments, the cells were aerated with CO_2–air (3.75:96.25). The incorporation of [^{35}S]sulfate into synchronized cells at a CO_2 concentration of 3.75 % is shown in Fig. 3.

Intracellular distribution of ^{35}S

The intracellular distribution of ^{35}S was examined during the first cycle of synchronous growth following the last dark period of the synchronization procedure.

Figs. 4A, B and C represent several ways in which the intracellular distribution of ^{35}S can be expressed for a synchronous culture. Although each figure aids in the interpretation of the metabolism of sulfur during synchronous growth, Fig. 4A is by far the most valuable for showing the relationship of the rate of increase in ^{35}S in each cellular fraction to the rate of increase in total cellular-^{35}S or [^{35}S]sulfate uptake.

Because the cellular dry weight and total cellular-^{35}S increase at the same rate (Figs. 2 and 3) at a given CO_2 concentration, during cellular growth, the same general trends shown in Fig. 4A would be obtained if the ^{35}S in these same cellular fractions had been expressed as percent of cellular dry wt.

Although differences in the rate of incorporation into total cellular-^{35}S, protein-^{35}S, acid-soluble-^{35}S, and sulfolipid-^{35}S can be seen in Figs. 4 B and C, it is very difficult to compare the magnitude and relationship of these rate changes between cellular fractions. Therefore, the data expressed as percent of total cellular-^{35}S in Fig. 4 A will be used solely in further discussions of the intracellular distribution of ^{35}S during synchronous growth.

The protein-^{35}S (percentage of total cellular-^{35}S) decreased dramatically and was accompanied by a simultaneous increase in the percent ^{35}S in the trichloroacetic acid-

soluble fraction of the cells between the third and fifth hours of cellular development. Between the fifth and seventh hours of growth, however, the ^{35}S in the protein fraction and the ^{35}S in the trichloroacetic acid-soluble fraction returned to nearly their initial

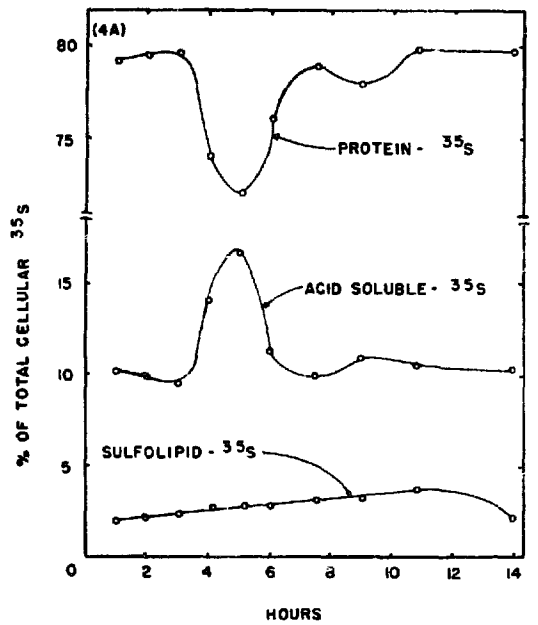

Fig. 4 A.

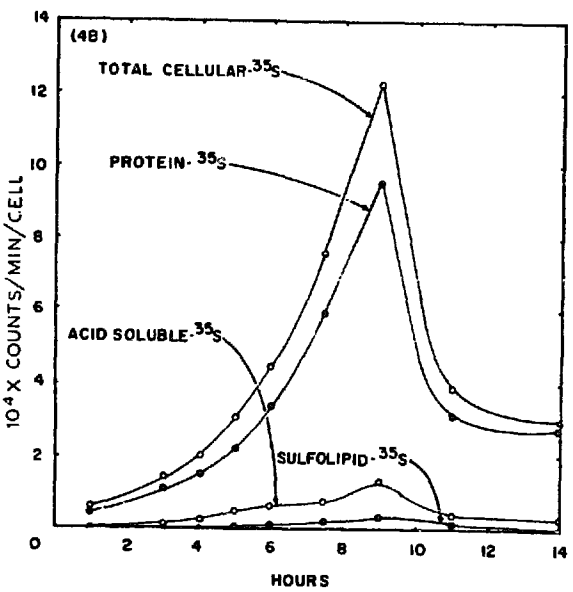

Fig 4 B.

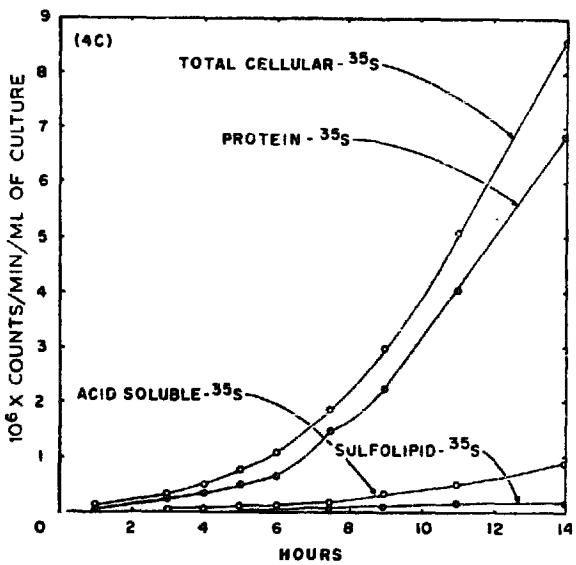

Fig. 4 C.

Fig. 4 A, B and C. Intracellular distribution of ^{35}S during synchronous growth of *C. pyrenoidosa* (strain 7-11-05).

levels. The percentage change in the protein-^{35}S exactly equaled, in each case, the percentage change in ^{35}S in the trichloroacetic acid-soluble fraction. After the seventh hour, the ^{35}S level of each fraction remained constant until the end of the synchronous growth cycle.

The sulfolipid fraction of the cells represented only a small portion of the total cellular-^{35}S (2–4 %). The percent ^{35}S in this fraction increased up to the eleventh hour of cellular development when it decreased to its initial level between the eleventh and fourteenth hours of growth.

Nuclear division began in the cells between the seventh and eighth hours of synchronous growth. Daughter-cell release was initiated 2–3 h after the initiation of nuclear division or between the ninth and tenth hours of cellular development. It is interesting to note that the shifts in the ^{35}S content of the acid-soluble and protein fractions of the cells occurred during a 4-h interval immediately prior to the initiation of nuclear division. The possible relationship of these shifts in sulfur metabolism to nuclear division remains to be elucidated.

HASE *et al.*[2] found essentially no periodism in the ^{35}S level of the cellular residue (protein fraction) during synchronous growth of *C. ellipsoidea*. Periodism was observed, however, in the ^{35}S content of the trichloroacetic acid and ethanol extracts of the cells. The ^{35}S level in the trichloroacetic acid extract appeared to increase dramatically during nuclear division while the ^{35}S level of the ethanol extract showed a significant but lesser increase during the same period.

Although a similar increase was observed in the ^{35}S level of the trichloroacetic acid extract of *C. pyrenoidosa*, it occurred before, instead of during, nuclear division. Thus, again there appears to be a significant difference between these two organisms or in the methods for studying them.

Distribution of ^{35}S in the total cellular protein

In order to explain the decrease in protein-^{35}S prior to nuclear division, the total cellular protein fraction was hydrolyzed and the amount of ^{35}S associated with each of the sulfur amino acids was determined (Fig. 5).

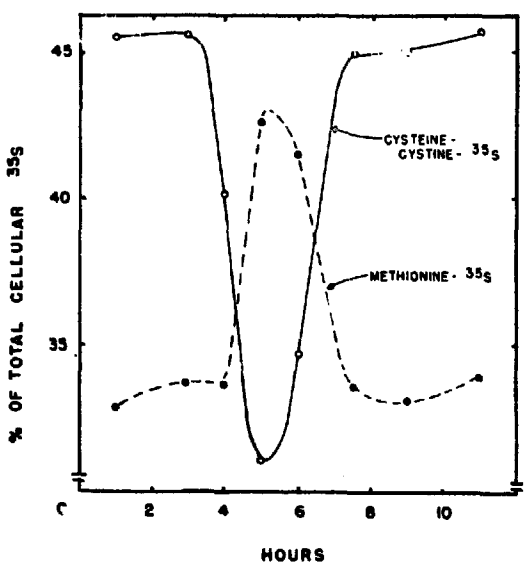

Fig. 5. Distribution of ^{35}S in the total cellular protein during synchronous growth of *C. pyrenoidosa* (strain 7-11-05).

Between the third and fifth hours of growth the level (percentage of total cellular-^{35}S) of cysteine–cystine-^{35}S decreased from 46 to 31 % and returned to 46 % between the fifth and seventh hours of growth. The decrease in cysteine–cystine-^{35}S was accompanied by a simultaneous increase in methionine-^{35}S. However, the methionine-^{35}S increased only 7.5 % (34–42.5 %); whereas, the cysteine–cystine-^{35}S decreased 15 %. Thus, the algebraic sum of the percentage changes in methionine-^{35}S and cysteine–

cystine-^{35}S accounts for a 7.5 % decrease in protein-^{35}S at the fifth hour of cellular development.

The dramatic shifts in protein-^{35}S may be explained in either of two ways: (a) the rate of incorporation of ^{35}S into protein cysteine–cystine may decrease between the third and fifth hours of cellular development due to an increased rate of incorporation into trichloroacetic acid-soluble components, or (b) the depression in protein-^{35}S may be due to an actual loss of ^{35}S (as cysteine–cystine) from the protein fraction into the trichloroacetic acid-soluble fraction while the rate of incorporation into protein is not affected.

Distribution of ^{35}S in the 0° trichloroacetic acid extract

Simultaneous with the studies on the protein fraction of the cells, experiments were initiated to identify the ^{35}S-containing compound(s) of the trichloroacetic acid-soluble fraction of the cells.

The trichloroacetic acid-soluble fraction of the cells was extracted continuously with ether to remove the trichloroacetic acid and any ether-soluble ^{35}S compounds. After extraction the ether phase contained no detectable ^{35}S. When the aqueous phase was chromatographed on paper in solvent systems which adequately separate sulfur amino acids and many of their derivatives, all of the radioactivity was found associated with a single, non-mobile, ultraviolet-light-absorbing, ninhydrin-negative spot. No detectable radioactivity was found associated with any free sulfur amino acids or their commonly occurring derivatives. Acid hydrolysis of the compound(s) in the ultraviolet-light-absorbing spot released six or seven ninhydrin-positive spots one of which was radioactive and had the color characteristics and R_F of cysteic acid. After acid hydrolysis the ultraviolet-light-absorbing spots moved independently and separately from the radioactive and ninhydrin-positive spots.

Most of the radioactivity (82 %) in the unhydrolyzed trichloroacetic acid extracts of the cells was Norit-A adsorbable (Table II). 55 % percent of the radioactivity which was adsorbed by Norit-A could be eluted with aqueous pyridine.

TABLE II

NORIT-A ADSORPTION OF ACID-SOLUBLE-^{35}S FROM EXTRACTS OF *Chlorella pyrenoidosa* (STRAIN 7-11-05)

	Total added to column	*Adsorbed by column after H_2O wash*	*Removed from column with aqueous pyridine*
Counts/min	64 500	52 800	29 040
Percent	100	82	55 (% of adsorbed)

Because (a) most of the radioactivity is Norit-A adsorbable, (b) the radioactivity is associated with an ultraviolet-light-absorbing spot on paper chromatograms, (c) acid hydrolysis of the radioactive spot releases six or seven ninhydrin-positive compounds, it seems probable that the ^{35}S is associated with a nucleotide or polynucleotide–peptide complex similar to those isolated by HASE *et al.*[2-4] from *C. ellipsoidea* and by WILKEN AND HANSEN[11] from bovine liver.

The radioactivity (20 %) which was not Norit-A adsorbable may consist of free peptides which may be precursors or hydrolysis products of nucleotide–peptide complexes.

At present studies are in progress to determine the metabolic significance of the shifts in ^{35}S content of the protein and cold trichloroacetic acid-soluble fractions of *C. pyrenoidosa*.

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