The cellular events which lead to growth and division, though highly integrated, can be resolved to the extent that growth may continue independently. Analysis of such uncoupled growth has served to define processes essential for the division of the cell. One biochemical syndrome whose interruption seems to cause uncoupled growth involves the metabolism of sulfur compounds. Nickerson and co-workers (19), for example, have discovered that a metabolic imbalance which results in the maintenance of an inadequate supply of reduced sulfur compounds, such as cysteine, is associated with the transformation of dividing cells of Candida albicans into non-dividing, filamentous forms. That thiol groups are somehow connected with cell division is generally accepted (16, 27), although the precise mechanism is still subject to debate. Methionine, another of the sulfur-containing amino acids, also has been implicated in the division mechanism. Spoerl et al found that this amino acid, as well as several other metabolites, occasioned the development of short, rod-like cells of the fungus Ustilago sphaerogena, instead of the long, mycelial-like form (25). Conversely, the growth of Chlorella vulgaris can be uncoupled from division by selenomethionine [CH3-Se-CH2CH2(NH2)COOH], a structural analogue known to competitively antagonize methionine (23). When these cells are cultured in the presence of the analogue and with sulfate as their only source of sulfur, division stops and many of the cells increase in size and dry weight. This uncoupled growth, whose duration depends on the concentration of selenomethionine, is followed by the resumption of exponential divisions as well as a return to normal size and weight. The addition of L-methionine at any time will terminate the uncoupled growth and reinstate exponential divisions. These data indicate that when L-methionine which normally originates from sulfate is antagonized by its selenium analogue, division but not growth is prevented, and that the amino acid may consequently have a biochemical function essential for the division mechanism of the Chlorella vulgaris cell.

Little more is known about the nature of the uncoupled growth beyond what has been mentioned above. In this paper changes in nitrogen and sulfur metabolism and O2 uptake have been examined in order to characterize the giant cells biochemically in greater detail.

**Materials and Methods**

**Growth Conditions:** The strain of Chlorella vulgaris, the composition and preparation of media, and most other aspects of growth conditions were identical with those described previously (22, 23). The following changes in procedure were made. A sulfate concentration of 3.1 \( \times 10^{-3} \) M was chosen in all experiments, since at this level maximum growth was attained with only a 20% depletion of the total sulfur, and a high specific activity could be effected in experiments with radioactive sulfate. Selenomethionine was supplied at a level of 3.1 \( \times 10^{-7} \) M which induced a period of uncoupled growth that lasted about 150 hours under the conditions of these experiments. The initial population density was increased to 0.4 million cells per ml to provide more cells for analysis. Culture tubes were sup-

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2 This investigation was supported by a grant, G2833, from the National Science Foundation, and by an Atomic Energy Commission Grant, AT (30-1)-1460, to Dr. David R. Goddard.
ported in a circular glass water bath surrounded by a bank of thirty 14-watt, white fluorescent lamps. The light intensity at the position of the culture tubes was approximately 750 ft-c as measured with a Weston illumination meter, model 756. A mixture of 3% CO₂ in air was supplied at a rate of about 500 ml/min through a gas mixing device as described by Biale (2).

Single dry weight and volume measurements were made because the water bath held a limited number of culture tubes, several of which frequently had to be pooled, particularly during the period of uncoupled growth, to obtain adequate quantities of cells for other analyses.

**Nitrogen Determinations:** A micro-Kjeldahl and Nesslerization assay, based on the methods described by Koch and McMeekin (14) was used. Four aliquots of cells that had been washed 3 times with distilled water were analyzed for total nitrogen. TCA-insoluble nitrogen was determined on 4 aliquots of cells suspended in an equal volume of cold 10% TCA for 0.5 hour at 2 to 5°C. The cells were then centrifuged and washed twice with cold 5% TCA prior to overnight digestion. The slopes of 8 standard curves, derived from the readings of digested and Nesslerized aliquots of (NH₄)₂SO₄ that contained 10 to 60 μg nitrogen, and calculated by the method of least squares, gave an average slope with its standard error of 3.3 ± .03. This value was used to calculate the nitrogen contents of the unknown samples from their Klett readings. The nitrogen per cell values are plotted with their 95% confidence intervals (standard error × t value).

**Oxygen Uptake:** Standard Warburg technique was used to measure the endogenous respiration at 25°C of the uncoupled and normal Chlorella cells.

**Sulfur Determinations:** Radioactive sulfur measurements and fractionations were performed as described by Roberts et al (20), and by Atkinson and McFadden (1). The filter apparatus used for the determination of cellular sulfur was generously supplied by Dr. Dean B. Cowie of the Carnegie Institution of Washington.

After filtration the cells were washed 3 times with sulfur-free medium for total nitrogen determinations or with 5% TCA for the protein determinations. For the TCA-insoluble sulfur, aliquots of cells were first suspended in an equal volume of 10% TCA at 2 to 5°C for 0.5 hour.

The following fractionation procedure, based on that described by Roberts et al for Escherichia coli (20), p. 13), was adopted. In each of the steps 10 ml of solvent were used. Where evaporation had occurred, the volume was adjusted after decantation. Known numbers of cells, washed 3 times with sulfur-free medium, were suspended in 10 ml of 5% TCA at 2 to 5°C for 0.5 hour, centrifuged, and the supernatant decanted. The cells were next suspended in 10 ml 75% alcohol for 30 minutes at 40 to 50°C. A 15-minute alcohol-ether extraction at the same temperature was followed by extraction with 5% TCA in a boiling water bath for 0.5 hour. A 1% HCl-alcohol wash for 5 minutes at room temperature followed, and the residue was then thoroughly suspended in 75% alcohol. Aliquots were withdrawn for counts of the remaining activity in the cells. Centrifugation yielded the final precipitate which is termed the residual protein sulfur and which was eventually analyzed for amino acid content. The supernatant fractions collected in each of the above steps were prepared for radioactivity measurement as described by Roberts et al (20), p. 9). Throughout the procedure samples for counting were prepared in duplicate.

Counts were made with a Nuclear model 163 scaler equipped with a counter whose mica end-window was approximately 1.4 mg per cm². Calculations were corrected for geometry, self-absorption and background. Since the ratio of radioactivity to the molality of the sulfate in the medium was known, the specific activity was used to express all calculations on a molar basis.

**Chromatography:** Residual protein fractions were hydrolyzed in 6 N HCl for 4 hours in sealed glass tubes at 105°C (20), p. 25). The hydrolysates were evaporated to dryness under vacuum at 30 to 40°C, resuspended in water and chromatographed. For the two-dimensional chromatograms the solvents consisted of n-butanol: glacial acetic : water, 100 ml : 22 ml : 50 ml (26), p. 36), and methanol : n-butanol : benzene : water, 100 : 50 : 50 : 50 (15, 21). After development the Whatman no. 1 sheets were placed on Kodak No-screen x-ray film for varying time periods. The films then were processed and compared with the ninhydrin-sprayed chromatograms.

**Results and Discussion**

**Growth Relationships:** Growth patterns were qualitatively similar to those observed in earlier experiments (23) but differed quantitatively in several respects. A maximum dry weight of 29 × 10⁻⁸ mg/cell and a maximum volume of 74 × 10⁻⁸ mm³/cell were recorded toward the end of the 150-hour period of uncoupled growth. These data are in contrast to those of the earlier experiments in which cells were exposed to half the concentration of selenomethionine. Previously, uncoupled growth lasted only about 100 hours, and the maximum weights and volumes attained were 17 × 10⁻⁸ mg/cell and 60 × 10⁻⁸ mm³/cell. Since these measurements are made on centrifuged, packed cells which consist of a mixture of giant as well as small cells, one cannot tell whether the higher maxima resulted from the development of a larger proportion of giant cells at the higher level of selenomethionine or from a continued enlargement of the same number of giant cells. In the present work a differential count showed that at the end of the period of uncoupled growth approximately 50% of the cells were larger than 12 μ in diameter, whereas in normal cultures, the maximum diameter of cells during early exponential growth rarely exceeds 10 μ.
Nitrogen changes in uncoupled cells of *Chlorella vulgaris*.

**Fig. 1.** (upper left). Total nitrogen content expressed on a per cell basis.

**Fig. 2** (upper right). Protein nitrogen content expressed on a per cell basis.

**Fig. 3.** (lower left). Data of figure 1 expressed on a dry weight basis.

**Fig. 4** (lower right). Data of figure 1 expressed on a packed cell volume basis.
Nitrogen Metabolism: The changes in nitrogen content of uncoupled cells and of untreated cells are shown in figure 1. The nitrogen contents of the control cells represent average values for a heterogeneous population of cells in all stages of growth and division. Studies of randomly dividing cells give little information about a single cell during its cycle of growth and division. One must resort either to techniques that result in synchronization, so that the majority of the cells grow or divide at the same time (6), or to the study of organs and tissues whose cells show such a synchrony under normal conditions of development (9, 27). In synchronized or uncoupled cultures of microorganisms, moreover, the cells soon reach different physiological ages than the controls, so that a comparison of the 2 types of cultures at any given time after inoculation has only limited meaning. In the Chlorella cultures, for example, at 100 hours the control had reached the end of its exponential phase of growth, and due to mutual shading of the cells, changes in pH, changes in salt concentration, etc., the cells were physiologically different from the giant cells which were still in their uncoupled phase of growth and still enlarging. For these reasons, no detailed comparison has been made between uncoupled and control cells. The important changes in the uncoupled cells are the ones that begin at the time of initial exposure to selenomethionine.

Under the influence of selenomethionine the uncoupled cells continued to absorb nitrates and to incorporate a large portion into protein (figs 1, 2). That the continued uptake and incorporation did not keep pace with the change in size and dry weight is illustrated in figures 3 and 4 in which the same data are plotted on a concentration basis per unit of dry weight and volume. During the 1st 20 hours of exposure to the seleno-analogue there was a sharp rise in cellular nitrogen which may have been due to the rapid uptake of the amino acid in addition to the uptake of nitrate. A steady decline in nitrogen concentration per unit weight and volume occurred for the remainder of the uncoupled growth period. Since the nitrogen content per cell had risen, there must have developed an imbalance between the rates of protein synthesis and the rates of other syntheses which lead to dry weight increase.

Chlorophyll synthesis appears to have been impaired too, judging from the pale green of the packed uncoupled cells. Whether the large increment in dry weight was due to the functioning of pre-existing chlorophyll or to a limited production of the pigment is not known.

At the end of approximately 150 hours, when the giant cells were resuming division, and cell weight and size were falling precipitously to the values of control cells, the nitrogen level per cell also fell rapidly (fig 1). This fall undoubtedly represents a partitioning of the existing nitrogen among the daughter cells and not a slackening in protein formation, since the dry weight and volume relationships (figs 3, 4) show that the nitrogen per unit weight and volume rose to the level of control cells that had reached the end of their exponential growth. Because the newly dividing cells were not synchronized, the data here again represent values for a heterogeneous population of cells. The rise in nitrogen concentration probably was not due to a decline in other syntheses that lead to dry weight increase, for concomitant with new division the cells became dark green again. Instead, the observed rise seems to have resulted from a resumption of the normal rates of protein formation.

Synthesis of cell material in Chlorella vulgaris during cell enlargement in the absence of division has also been observed by Finkle and Appleman (10, 11). Magnesium exhaustion from the medium was responsible for the halt in multiplication, in this instance, and increases in dry weight and nitrogen content of the enlarging cells were taken as indices of continuing metabolism.

The cells of the control population in the present studies exhibited an initial rise in nitrogen content (figs 1, 2) which attained its maximum very early during exponential growth. After a rapid drop, a nitrogen level was reached that remained constant. A similar pattern was detected by Fowden in C. vulgaris cells grown with nitrate and ammonium salts (12). When the data are expressed on a concentration basis (figs 3, 4) they show that the nitrogen per unit volume or per unit weight of packed cells continued to rise until exponential growth had ceased, and that the nitrogen level was maintained well into the stationary phase of growth. These quantitative changes associated with randomly dividing cultures show the necessity for studying cells along their entire growth curve rather than at some arbitrarily chosen time.

Oxygen Uptake: As another index of metabolic activity, the O₂ uptake of uncoupled cells was measured. Table I summarizes the data obtained with 44-hour-old cells, and although these data do not give a complete picture of the changes throughout the course of uncoupled growth, it is evident that the respiration of the giant cell rose markedly. By 44 hours cell size had increased 6-fold, cell dry weight 4-fold, protein nitrogen 4-fold, and O₂ consumption per cell had tripled. Once again, these data show that the various processes had not kept pace with each other. Unanswered by the data is whether the higher O₂ uptake resulted from continued synthesis of respiratory enzymes, or from a more rapid operation of the existing enzymes.

<table>
<thead>
<tr>
<th>AGE OF CELLS</th>
<th>µL/HR × CELL</th>
<th>µL/HR × MG</th>
<th>µL/HR × MM³</th>
<th>µL/HR × µG</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (inoculum)</td>
<td>0.176</td>
<td>5.47</td>
<td>2.18</td>
<td>0.160</td>
<td></td>
</tr>
<tr>
<td>44 normal</td>
<td>0.345</td>
<td>8.19</td>
<td>1.46</td>
<td>0.105</td>
<td></td>
</tr>
<tr>
<td>uncoupled</td>
<td>0.518</td>
<td>4.14</td>
<td>1.08</td>
<td>0.114</td>
<td></td>
</tr>
</tbody>
</table>

Table I

Oxygen Uptake by Normal and by Uncoupled Cells of Chlorella vulgaris
enzymes. The increase in protein nitrogen as the uncoupled cell enlarged would presuppose the maintenance of enzyme synthesis, unless these proteins were only structural. If the newly synthesized proteins are indeed enzymes then one is faced with the fact that they lack methionine (see next section). Altered proteins, however, are known, for Gross and Tarver (13) have isolated ethionine from the proteins of Tetrahymena cells that had been grown with this analogue; Munier and Cohen (18) found that β-F-phenylalanine partially replaced phenylalanine and tyrosine in the proteins of Escherichia coli; Yoshida (31) has reported the isolation, from a methionine-requiring mutant of Bacillus subtilis, of an α-amylase that not only contained ethionine but which also had the same activity as the unaltered enzyme; and there is strong evidence that selenomethionine can be built into the proteins of Escherichia coli in place of methionine (7, 8, 28).

Sulfur Metabolism: The changes in sulfur content and concentration were similar to those of nitrogen. The uncoupled cells absorbed considerable sulfate (fig 5), although not in proportion to the increase in dry weight (fig 6) or size. This sulfate was metabolized into a variety of fractions, as shown in table 2 which lists the distribution at 92 hours of the sulfur in giant cells as well as in randomly dividing cells. The giant cells incorporated sulfur into the same fractions as did the normal cells, with a large amount soluble in hot TCA which is commonly used to extract nucleic acids. In comparable studies with E. coli ((20), p. 320), in which the technique for radioactive fractionation was essentially the same as the one used here, very little radioactive sulfur was removed by hot TCA. The nature of this sulfur component in Chlorella vulgaris has not yet been characterized. A portion of the total sulfur found its way into the residual proteins, but radioactive methionine could not be detected in the acid hydrolysate (figs 7, 8). However, the sulfur amino acid was again found in the protein hydrolysate of cells that had resumed division (fig 9). The renewed ability to incorporate methionine into protein may have some bearing on the ability of these cells, when subcultured into fresh medium that contains selenomethionine, to divide after only a short period of uncoupled growth.

<table>
<thead>
<tr>
<th>Table II</th>
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<tbody>
<tr>
<td>Sulfur Fractions in Normal and in Uncoupled cells of Chlorella vulgaris</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>Uncoupled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM % of total</td>
<td>µM % of total</td>
</tr>
<tr>
<td>Total sulfur</td>
<td>16.59 100</td>
</tr>
<tr>
<td>Cold TCA soluble</td>
<td>308 18.5</td>
</tr>
<tr>
<td>75% ethanol soluble</td>
<td>138 8.2</td>
</tr>
<tr>
<td>Alcohol-ether soluble</td>
<td>13 0.8</td>
</tr>
<tr>
<td>Hot TCA soluble</td>
<td>298 17.9</td>
</tr>
<tr>
<td>Acid alcohol soluble</td>
<td>28 1.7</td>
</tr>
<tr>
<td>Insoluble (residual protein)</td>
<td>856 51.5</td>
</tr>
<tr>
<td>Recovery</td>
<td>98.6%</td>
</tr>
</tbody>
</table>

* Age of cultures, 92 hrs.
** Specific activity of culture medium: 6147 cts/min × µM sulfate for normal; 6818 cts/min × µM sulfate for uncoupled; cold sulfate conc. 3.1 × 10⁻³ µM/ml.

Sulfur changes in uncoupled cells of Chlorella vulgaris.
Fig. 5 (left). Total sulfur content per cell.
Fig. 6 (right). Data of figure 5 expressed on a dry weight basis.
The absence of radioactive methionine in the proteins of the uncoupled cell is consistent with other work in which selenomethionine was shown to compete with methionine. A competitive relationship between the 2 molecular species was first established in growth and absorption studies with *C. vulgaris* (23). In these experiments the exponential rate of growth and the selenium content of the cells were a function of the external methionine/selenomethionine ratio, an indication that the analogue pair had competed for passage across the cell membrane as well as for an enzyme site within the cell. More recently, Cowie and Cohen (7, 8) confirmed such a competition by the demonstration that selenomethionine decreased the incorporation of radioactive methionine into the proteins of a methionine-requiring mutant of *Escherichia coli*. Furthermore, the selenium analogue supported exponential growth of the mutant in the

Radioautographs of protein hydrolysates of *Chlorella vulgaris* cells grown with radioactive sulfate.  
**FIG. 7** (upper left). Normal Chlorella cells.  
**FIG. 8** (upper right). Uncoupled Chlorella cells. Note absence of methionine.  
**FIG. 9** (lower left). Chlorella cells that have resumed exponential divisions after uncoupled growth.
The complete absence of the sulfur compound. The conclusion from these data, that selenomethionine can be built into proteins in place of methionine, is borne out by the experiments of Tuve and Williams (28) with wild type E. coli, in which radioactive selenomethionine was identified in the proteins after the cells had been exposed to radioactive selenite.

Additional evidence for a competition can be derived from the relationships between other selenium compounds and the analogous sulfur-containing metabolites. Selenocystine prevents the influx of $^{35}$S-cystine into leukemic leukocytes and into Murphy lymphosarcoma cells of the rat (29, 30), and a competitive antagonism between the sulfate and selenate ions has been demonstrated in many biological systems (22, 24). There appears to be a general competitive relationship between many sulfur metabolites and their selenium analogues.

One can be reasonably certain that in the mutant studied by Cowie and Cohen, and in the uncoupled Chlorella cell, selenomethionine has been built into proteins and probably into functioning enzymes. But the critical experiment to show that an isolated enzyme, altered in this way, can still function has not yet been performed.

Inclusion into proteins is, however, not the only biochemical reaction in which selenomethionine and methionine can compete. Mudd and Cantoni have demonstrated that the enzyme system which transforms methionine into S-adenosylmethionine will also operate as well, if not more rapidly, with selenomethionine as substrate (17).

This last discovery raises the question of the biochemical site at which selenomethionine acts to prevent cell division. Methionine apparently has at least 2 general functions in organisms, one as a constituent of proteins, although some proteins have been isolated which seem to lack methionine ((3) pp. 252-337), the other as a methylating agent. In higher plant cells, more specifically, methionine has been implicated as a source of methyl groups for lignin (5) and for certain alkaloids (4). In Chlorella, however, a methylating role for methionine has not yet been established.

In an overall evaluation of the uncoupling effect induced by selenomethionine a possible transformation of this analogue into other selenium compounds must be considered. Since C. vulgaris cells can satisfy all their sulfur requirements with either D- or L-methionine, the enzymes that are involved in the utilization of these substances most probably also metabolize the DL-selenomethionine into other forms which conceivably might be more directly connected with the uncoupling. In addition, it must be recognized that secondary reactions that result from the metabolic derangement caused by selenomethionine or its products may be responsible for the uncoupling. As a first approximation though, the uncoupled growth appears to be correlated with the synthesis of altered proteins which may be incompatible with division.

**Summary**

The course of metabolic changes was analyzed in cells of Chlorella vulgaris that were exposed to selenomethionine whose uncoupling action allowed cell growth to continue without division. The giant cells continued to synthesize protein and to incorporate radioactive sulfur, derived from sulfate, into the protein as well as into a number of other fractions. Radioactive methionine could not be detected in the protein fraction. The increase in protein did not keep pace with the increase in cell weight and volume. The pale green of the packed giant cells indicated impairment to chlorophyll synthesis, in spite of which the dry weight of the uncoupled cells rose markedly. Oxygen uptake by the uncoupled cell tripled after 44 hours of growth in the presence of the seleno-analogue, but the rise was not in proportion to the increase in weight, volume, or protein.

With the return of exponential divisions after the period of uncoupled growth, the cells assumed the characteristics of normal Chlorella cells. Size, weight, protein nitrogen and protein sulfur reverted to the usual levels, and methionine could once more be detected in the proteins.

The author wishes to thank Miss Eva Konrad for technical assistance, and Mr. Michael Luskin for his help with the nitrogen assays.

**Literature Cited**


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TRANSLLOCATION OF CALCIUM IN THE BEAN PLANT 1, 2, 3

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There are a number of reports in the literature of translocation which indicate that calcium is immobile in the phloem, or relatively so (1, 2, 4, 5, 13). The majority of these are not quantitative studies but merely indicate that calcium translocation compares poorly with that of phosphorus, sulfur, potassium and some other elements. Contrasted with these reports on calcium immobility in the phloem are two which claim that the anesthetizing of leaves with diethyl ether (6) and the application of triiodobenzonic acid (TIBA) (8) cause a reversal of polarity in the phloem and thereby allow calcium to move from leaves. However, neither of these papers established that the calcium transport from the leaf was via the phloem.

The purpose of the present investigation is therefore 2-fold: to examine calcium translocation in more quantitative terms, and to investigate the effects of certain substances or treatments on calcium translocation. In addition we have carefully determined the tissue through which calcium movement occurs.

The quantitative study was made by appraising the mobility of cotyledonalary calcium, by observing the partition of the calcium absorbed from the nutrient medium between various plant parts, by observing the

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2 This investigation was supported in part by the U.S.A.E.C., Division of Biology and Medicine; Contract No. AT(45-1)-213.
3 The radioisotopes were acquired from the U.S.A.E.C., Oak Ridge, Tennessee.