INTRACELLULAR DISTRIBUTION OF NITROGEN DURING SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

by

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INTRODUCTION

During recent years, synchronized cultures of cells have been widely used to study the biochemical and physiological events accompanying the development of a cell during its growth and division cycle (1-6).

Sulfur-containing compounds have been implicated as playing a possible role in nuclear and/or cell division. For example, Rapkine (7) showed that acid-soluble sulfhydryl compounds increased, within synchronized sea urchin eggs, during the period of nuclear division and reached a maximum level at the onset of cell cleavage.

Mazia (8) and coworkers confirmed Rapkine's observation and further showed that the level of sulfhydryl-containing proteins exhibited an inverse relationship to that of the acid-soluble sulfhydryl compounds. As a result, Mazia suggested that the acid-soluble sulfhydryl-disulfide compound(s) may serve as (a) oxidants of certain sulfhydryl proteins, resulting in their polymerization to form the high molecular weight proteins of the mitotic spindle, and (b) reductants during disassembly of the

mitotic spindle after completion of its normal function within the cell. This proposal was further supported by the observation that the isolated mitotic spindle consisted primarily of protein which was solubilized by thioglycollate, a disulfide reducing agent.

Radioactive sulfate-^{35}S was used by Johnson and Schmidt (9) to trace the metabolism of sulfate during the synchronous cell cycle of Chlorella. Acid soluble-^{35}S (% of total cellular-^{35}S) was observed to increase markedly during early cellular development (0.26-0.43 fraction of cell cycle). During this same period, protein-^{35}S showed a decrease of equal magnitude. Following this period, the level of ^{35}S in the acid soluble and protein fractions exhibited opposite trends (0.43-0.60 fraction of cell cycle), reaching their initial values at approximately the onset of nuclear division. The changes in protein-^{35}S (0.26-0.60 fraction of cell cycle) were accounted for by the algebraic sum of inverse fluctuations of protein-cysteine (and cystine)-^{35}S and protein-methionine-^{35}S. These data suggested either that the protein sulfur amino acids were shifting in concentration or that their turnover rates were changing in the protein fraction during cellular development.

The changes in the level of acid-soluble-^{35}S appeared to be due almost entirely to fluctuations in the level of ^{35}S in peptides

or peptide derivatives. The inverse relationship observed between the levels of cellular protein-^{35}S and the peptide-^{35}S suggested that peptides (or peptide derivatives) may be functioning in the synthesis of and/or resulting from breakdown of cellular proteins. Likewise, because the reciprocal relationship between the protein-^{35}S and the acid-soluble-^{35}S occured prior to nuclear division, it is appealing to propose that acid soluble peptides serve as intermediates in the synthesis of proteins that function during nuclear division.

Hase et al. (10) and Tamiya (11) have also shown that sulfur is essential for nuclear division in Chlorella. They were able to control nuclear and cellular division by regulating the availability of sulfate and nitrate to the cells. In the absence of sulfate, synchronized cells were unable to complete nuclear division. When sulfate was added to these cells in the absence of nitrate, they completed nuclear but not cellular division. However, when supplied with both sulfate and nitrate they completed both of these cellular events. When cells were cultured in the presence of both sulfate and nitrate, an increase in certain sulfur-containing acid-soluble substances was observed prior to the period of nuclear division. These acid-extractable substances were reported to be nucleotide-peptides.

Subsequently nucleotide-peptides were reported from a wide variety of other organisms (12-38). The metabolic role of these compounds has not been established with the exception of those which function in the synthesis of the bacterial cell wall (33, 36, 39).

The objectives of the present research were:

1. To determine whether the changes in the levels of ^{35}S in the protein sulfur amino acids (observed by Johnson and Schmidt) reflect changes in the concentrations of these amino acids or simply changes in the turnover rates of these amino acids in the cellular protein during cellular development.
2. To determine the metabolic relationship, if any, between the peptide-S and protein-S fractions at different stages of cellular development.
3. To isolate and structurally characterize predominent nucleotide-peptides from Chlorella in preparation for use in studies to determine the metabolic role of these compounds.
4. To measure the distribution of the free common amino acids and those found in proteins and peptides during the cell cycle.
5. To establish the general patterns of change in nitrogen distribution during the cell cycle in preparation for studies

to elucidate specific metabolic control mechanisms that are operative during cellular growth and division.

MATERIALS AND METHODS

Synchronization and mass culture prodecures.

The high temperature strain 7-11-05 of the unicellular alga (40, 41), *Chlorella pyrenoidosa*, was cultured in small volumes and synchronized using techniques previously described (42, 43).

Two methods were used to mass culture this organism. The mass culturing technique of Baker and Schmidt (43) was employed to measure the cellular protein-amino acid composition during the synchronous growth and division cycle. Baker and Schmidt determined the maximum initial cell density that would allow exponential growth for 14 hours (1.2 cell cycles) in continuous light without dilution in a 35 l culture chamber. During this time interval, the light intensity and nutrient levels decreased continuously but always remained at sufficient levels to saturate the minimum growth requirements of the cells. However, shortly after the 14th hour, a cell density was reached which limited the availability of light and therefore the growth rate of the cells. Using this method of culturing, it was possible to obtain a maximum

of 1 g fresh weight of cells at hourly intervals by harvesting exponentially smaller volumes during the cell cycle. Although this amount of cellular material was sufficient for the accurate determination of the cellular protein-amino-acids, it was inadequate for measurement of the levels of free-amino acids and acid-soluble peptides during cellular development.

Therefore, a new mass culturing technique was developed which yielded approximately 3x as much cellular material as the previous method in the same time period and in less than one-tenth the culture volume. Using the new mass culturing technique, the cells were maintained at nearly a constant light intensity and nutrient concentration by continuous dilution with culture medium.

Synchronized cells of this organism increase their dry weight by 24.12% per hour. If culture medium is pumped into the culture at a constant rate such that the culture volume also is increased by 24.12% per hour; then, even though the ratio of dry weight to culture volume does not remain constant during the hourly interval (i.e., the cells increase their dry weight exponentially while the pump rate is constant), this ratio will be the same at the beginning and at the end of the interval. At the end of the one hour interval, that volume of culture in excess of the original

volume was harvested, allowing use of the same dilution rate for the subsequent hour interval. This dilution and harvesting procedure was repeated at hourly intervals throughout the synchronous cell cycle.

The apparatus and methods for the new mass culturing system are described below. The cells were cultured in a flat transparent acrylic plastic (0.8 cm.) chamber having the following internal dimensions: 71 cm length, 0.8 cm width, and 75 cm height. A CO_2-air mixture (4:96) was bubbled through the cell suspension using 11 sections of glass tubing distributed evenly across the length of the chamber. The temperature of the culture was controlled by placing the chamber into a glass constant-temperature water bath having the following internal dimensions: 120 cm length, 60 cm height, and 10 cm width.

The temperature of the water bath was maintained at 38.5° by 2 low-lag stainless steel heaters (750 watt), one in front of and one behind the culture chamber, each being independently controlled by a thermoregulator. Cold tap water was circulated through a coil of Teflon-coated copper tubing in the water bath at a rate such that the heaters operated approximately 50% of the time. Rapid circulation of the water around the culture chamber was provided by centrifugal pumps (Aminco Co-

Model 4-6035), one situated at each end of the water bath, positioned so that a jet of water passed over the heaters and thermoregulators in front of and behind the chamber.

A bank of 14-40 watt, cool white fluorescent lamps (backed by aluminum foil) was mounted approximately 0.2 cm from each side of the water bath. The light intensity at the middle of the water bath was approximately 5000 foot candles.

A synchronous growth experiment was initiated by placing 2417 ml of cell suspension (370×10^6 synchronous daughter cells per ml) into the culture chamber and allowing it to become equilibrated with the temperature of the water bath and the aeration mixture of CO_2-air. After equilibration, the lights were turned on and a Sigma-Motor (model no. T8-4002) pump was started. The pump was calibrated to deliver 583 ml per hour (583 ml equals 24.12% of the original culture volume). At hourly intervals, 583 ml of culture were harvested. Each 583 ml harvest volume contained 3 g fresh weight of cells.

Extraction Procedure.

Pellets of cells harvested hourly, by centrifugation, during synchronous cell cycles, were washed 3x with aqueous buffer (0.125% KH_2PO_4, 0.01% $CaCl_2$, pH7) and then made to a given volume with buffer. Aliquots of this suspension were analyzed for total

cellular nitrogen (44). The remaining cell suspension was quantitatively transferred to a stainless steel centrifuge tube, centrifuged, and the pellet stored at -15° until extraction. The frozen pellets were thawed in an ice bath and then extracted. Acid-soluble nitrogen was removed by an initial extraction with 10% trichloroacetic acid (TCA) at 0° for 10 min followed by three similar extractions with 5% TCA. Chlorophyll-N and lipid-N (along with the TCA) were extracted from the resulting residues with two one-hour extractions with 95% ethanol at 0° followed by six extractions with ethanol-ether (3:1) for 20 min at 45°. The above extracts were analyzed for their total nitrogen content (44). The resulting lipid-free cell residue was dried and stored in a vacumn des iccator over P_2O_5 until protein amino acid distribution determinations were performed.

Measurement of growth parameters.

Cell number-, cellular dry weight-, nitrogen-, and phosphorous- per ml of culture were determined by the procedures of Schmidt (44).

Amino acid analysis of cellular fractions.

The TCA extract was prepared for amino acid and peptide analyses by removing the TCA by continuous extraction (at 2-3°)

with ethyl ether in a Kutscher-Steudel liquid-liquid extractor (45).

The lipid-free cell residues and aliquots of the TCA extracts (after removal of the TCA) were hydrolyzed in 6N HCl for 24 hours at 120° in sealed tubes containing nitrogen gas. After hydrolysis, the HCl was removed by repeated drying *in vacuo*.

Aliquots of the hydrolyzed lipid-free residue and the TCA extract (before and after hydrolysis) were analyzed for their amino acid distribution, using a Beckman-Spinco amino acid analyzer, by the procedure of Spackman *et al.* (46). The difference between the amino acid content of the TCA extracts before and after hydrolysis indicated the levels of the peptide amino acids located therein.

Purification of sulfur-containing peptides.

The ion-exchange columns used to purify sulfur-containing peptides were either 5 X 38 cm(AG-1, X4, 200-400 mesh, formate form; Bio Rad laboratories), or 1 X 23cm (AG-50, X8, 200-400 mesh, hydrogen form). All ion-exchange resins were sized by the procedure of Hamilton (47). The resin particles ranged in size between 20 and 40 microns. Separation of the peptides was achieved using linear gradient elution with either formic acid (AG-1 columns) or hydrochloric acid (AG-50 columns) by the

procedure of Cherkin *et al.* (48).

The peptide samples subjected to ion-exchange column chromatography were extracted from cells cultured on sulfate-^{35}S (see "Determination of Norit-A adsorbable peptides" for TCA extraction and removal procedure). The pellets of cells extracted ranged in weight from 100 to 500 g fresh weight and were obtained at 0.4 and 0.7 fractions of the cell cycle.

Determination of average molecular weight distribution of TCA soluble peptides.

Three columns (1 X 23 cm) of Sephadex (G-25, medium; G-15; G-10) were used in a stepwise fashion to separate TCA-soluble peptides on the basis of their molecular weights. The peptides were eluted from these columns with 0.005 N formic acid. A 1.5 ml aliquot of a TCA extract (after TCA removal) was washed into the G-25 column and eluted in two fractions. The first fraction contained the peptides that were excluded from the Sephadex (determined using a 1.5 ml aliquot of a solution of Blue Dextran). The second fraction contained the peptides that were retarded by the Sephadex. The retarded fraction was concentrated *in vacuo* to 1.5 ml and washed into the G-15 column. The G-15 column eluate likewise was collected in two similar fractions. The retarded fraction was similarly concentrated and

subjected to gel-filtration on a G-10 column to separate the peptides again into two fractions.

The peptides excluded from the columns were hydrolysed in HCl and their respective amino acid distributions determined. The fraction retarded by the G-10 column was analyzed for its amino acid distribution before and after acid hydrolysis.

Determination of Norit-A adsorable peptides.

To test for the existence of nucleotide-peptides, the acid-soluble peptides were extracted from 16 g fresh weight of cells (from the mid point of the cell cycle) with TCA. The TCA was removed from this extract with ethyl ether (as previously described); however, the pH was maintained below 4 with HCl during TCA removal to prevent hydrolysis of mixed anhydride bonds (49). The levels of the peptide-amino acids in an aliquot of the above extract was determined (after acid hydrolysis) without any further treatment. Another aliquot of the extract was adjusted to pH2 and 0.8N KCl (the reason for this adjustment will be described below), and then continuously mixed with Norit-A (1g Norit-A/15 ml of extract) for 5 min. to adsorb nucleotides and presumably any nucleotide-peptides present in the extract. The levels of the peptide-amino acids remaining in solution was determined (after acid hydrolysis) after removal of Norit-A

by centrifugation and Millipore filtration. The same procedure was performed but after treatment of aliquots of the peptide fraction with hydroxylamine (conditions of Lipmann and Tuttle (50)) or with alkali (0.5N KOH, 37° for 5 min). Both treatments rapidly hydrolyze mixed-anhydride bonds such as those in amino-acid adenylates, and alkali also hydrolyzes ester bonds. It should be noted that prior to Norit-A treatment, the sample not treated with alkali or hydroxylamine, was adjusted to the same concentration of KCl as the sample that received the alkali treatment. However, no KCl was added to the sample treated with hydroxylamine because this reagent already contained relatively high levels of other salts (NH_2OH, NaCl, $NaCOOCH_3$).

Formation and detection of hydroxymates.

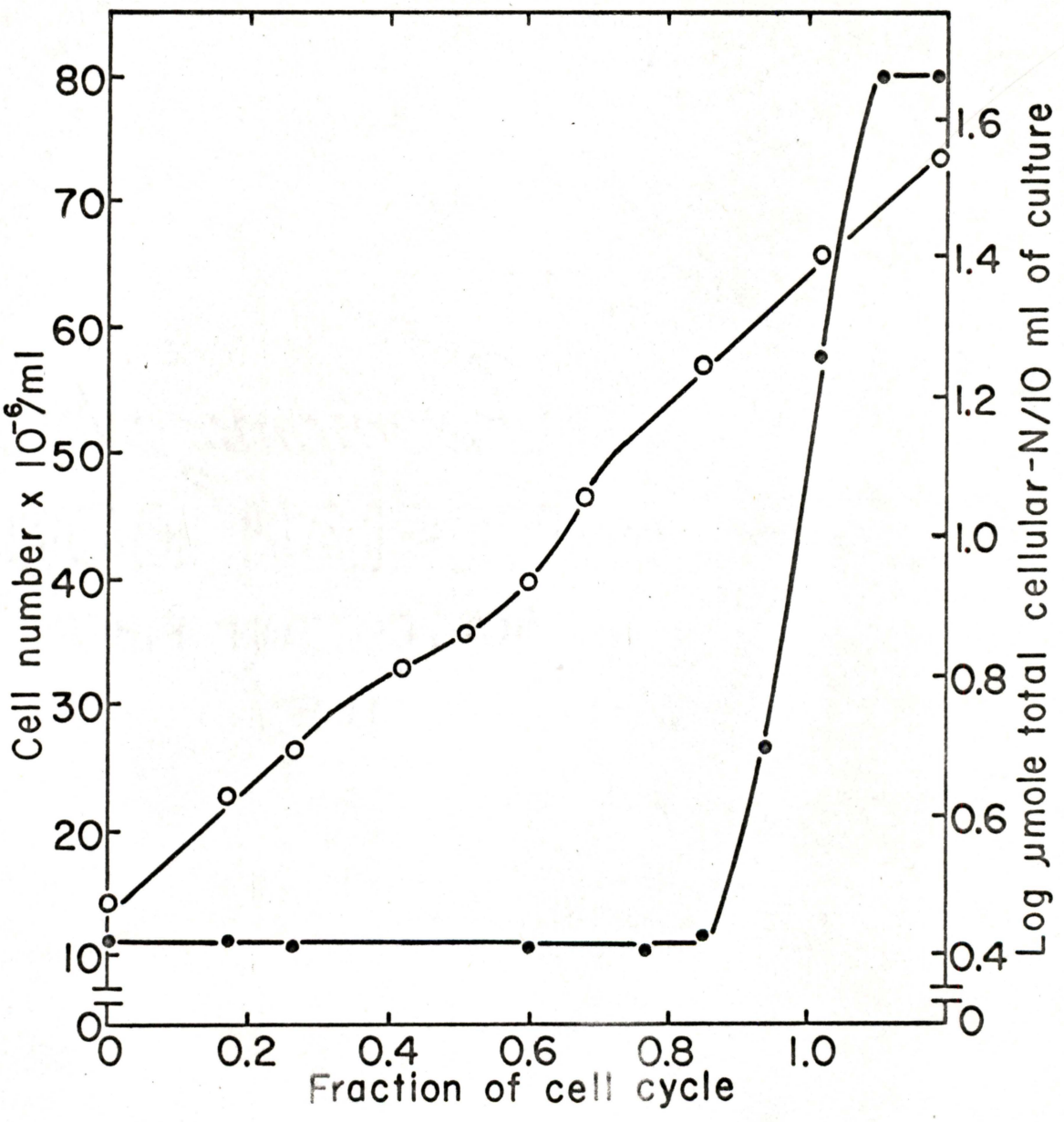
A spray procedure for detecting anhydrides, using hydroxylamine at neutral pH and room temperature, was modified from the colorimetric method of Lipmann and Tuttle (50). Samples were spotted on Whatman 3 MM chromatography paper, and sprayed with the neutralized hydroxylamine solution; then, after 10 minutes with 3N HCl, and finally with a 5% solution of $FeCl_3.6H_2O$ in 0.1 N HCl. The hydroxymates formed, by reaction between anhydrides and hydroxylamine, yield colored complexes with $FeCl_3$.

RESULTS AND DISCUSSION

Comparison of mass culturing methods.

When the cells were mass cultured by the technique of Baker and Schmidt (43), total cellular nitrogen appeared to increase exponentially during the synchronous growth cycle. However, when the data was expressed in its logarithmic form (Fig. 1), a small but reproducible deviation from log-linearity was observed during the period 0.3-0.8 fraction of the cell cycle (51). When neither nutrient concentration nor light intensity became limiting during synchronous growth, exponential increases in cellular dry weight, total cellular-P and total cellular-S were reported for this same organism (52). Total cellular-N per ml of culture and nitrate uptake also were previously observed to be exponential functions during synchronous growth of a low temperature strain (Van Neil strain, code No. Z,2.2.1) of C. pyrenoidosa (44). In the mass culturing system of Baker and Schmidt the growth parameters per ml of culture increase continuously throughout the synchronous growth cycle. However, because of the continuous dilution system employed in the culturing method described in

Fig. 1.
Cell number and total cellular-N per ml of culture during synchronous growth of C. pyrenoidosa (strain 7-11-05) with no dilution. ●——●, cell number; ○——○, total cellular-N.


Cell number x 10^{-6}/ml
80
70
60
50
40
30
20
10
0
Log µmole total cellular-N/10 ml of culture
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

this dissertation, cellular dry weight-, total cellular P-, and total cellular N-per ml of culture remained at their initial levels during cellular development (Fig. 2). In order to calculate the increase in these growth parameters had there not been a dilution, the actual amount of cellular material (cellular-P, -N, -dry weight; and cell number) per ml was multiplied by the following conversion factors; 1.2412 for the first hour, $(1.2412)^2$ for the second hour, etc., or $(1.2412)^n$ for the nth hour (each harvest contained 24.12% of the culture). A convenient method for calculating the conversion factor at n^{th} hour is : Conversion Factor=antilog (n log 1.2412).

After the dilution affect was compensated for, using the above conversion procedure, the growth functions appeared as shown in Fig. 3. Typical cell count data was obtained using the dilution procedure. Furthermore, cellular-P, -N, and -dry weight per ml of culture increased at parallel exponential rates with log-linear slopes of 0.094 units per hour.

The negative deviation from log-linearity in total cellular-N per ml of culture, observed from 0.34 to 0.77 fraction of the cell cycle during synchronous growth without dilution was not noted when the cells were diluted continuously during the cell cycle. The difference between the two growth functions may be

Fig. 2.
Cell number , total cellular-N, total cellular-P, and dry weight per ml of culture during synchronous growth of C. pyrenoidosa (strain 7-11-05) with continuous dilution. △——△ , cell number; X=,●——● , mg cellular dry weight /10 ml; ○——○ ,µg total cellular -N/0.14 ml; ▲——▲ ,µg total cellular -P/0.6 ml.

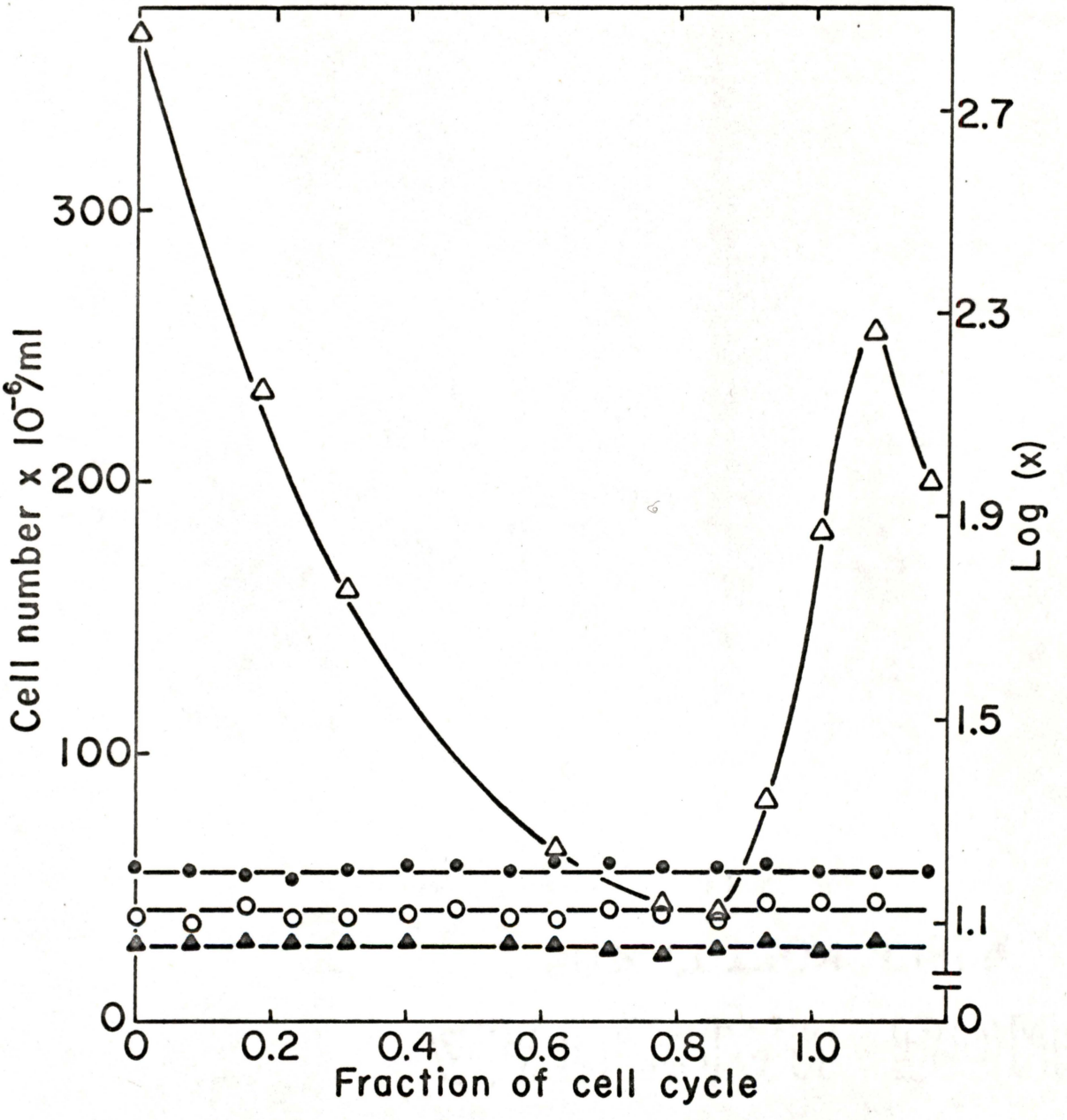
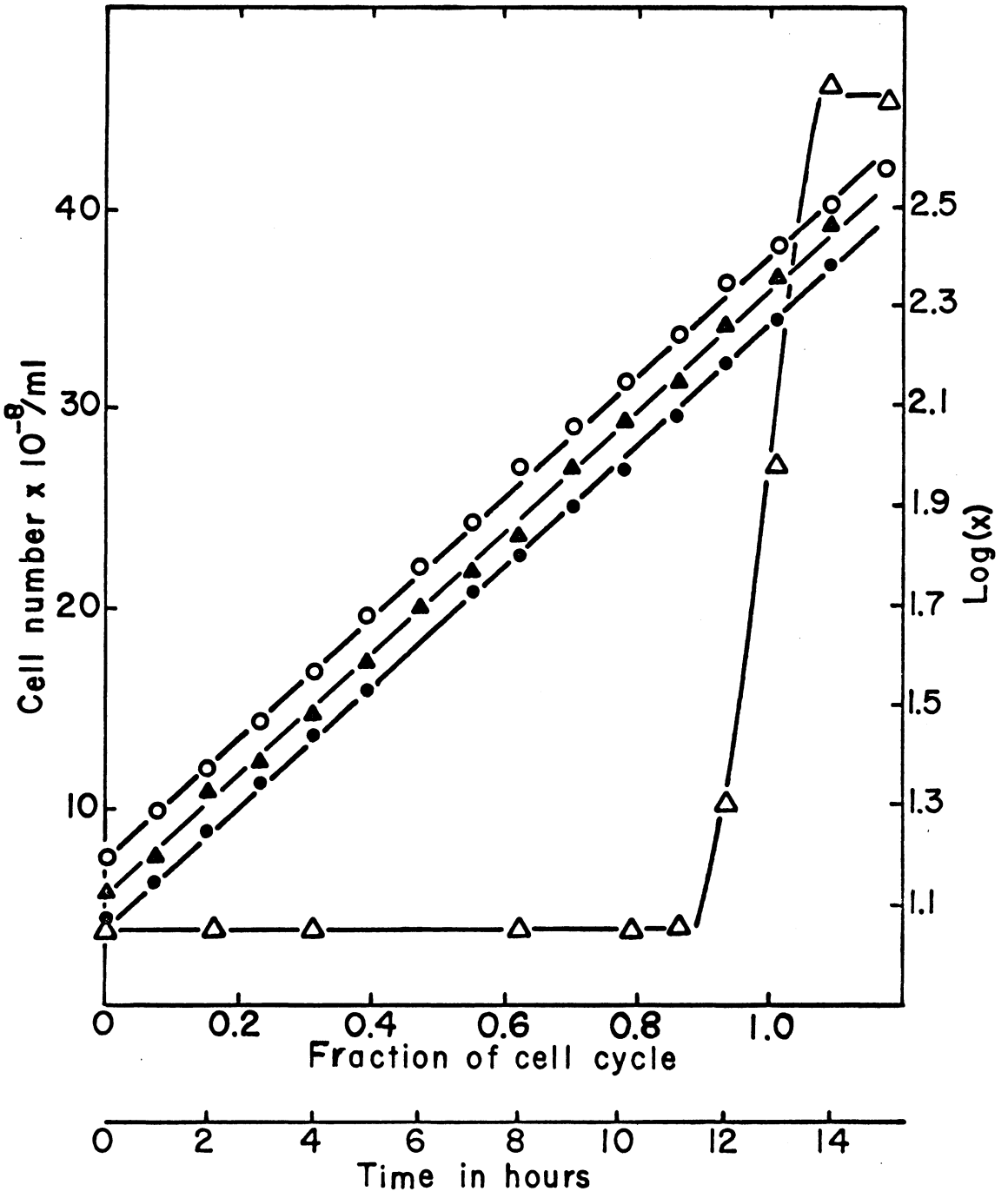

Cell number x 10^{-6}/ml
300
200
100
0
2.7
2.3
1.9
1.5
1.1
0
Log (x)
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

Fig. 3.
Cell number, total cellular-N, total cellular-P, and dry weight per ml of culture during synchronous growth of C. pyrenoidosa (strain 7-11-05) after correction for dilution. △———△ , cell number; X=,○———○ , mg cellular dry weight/ml; ▲———▲ , µg total cellular -N/0.14 ml; ●———● , µg total cellular -P/0.6ml.


Cell number x 10^{-8}/ml
40
30
20
10
Log(x)
2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle
0
2
4
6
8
10
12
14
Time in hours

due to the continually decreasing light intensity in one system and the nearly constant light intensity in the other.

Although the initial concentration of cells in the new mass culturing system was approximately 30x higher than in the previous system (43), it is evident from the exponential nature of the growth curves (Fig. 3) that light and nutrient saturation was maintained throughout the cell cycle.

The abscissa of Fig. 3 is expressed both as time in hours and as fraction of the cell cycle. The hour corresponding to the time when one-half the mother cells had divided was taken as 1 cell cycle. All other harvest hours were expressed as fraction of that time. During the division period, mother cell mass accounted for more than 50% of the total cellular mass until 1.0 cell cycles; whereas, daughter cells and cells in early development comprise the bulk of the culture after 1.0 cell cycle. Nuclear division was initiated at 0.7 fraction of the cell cycle (67); and, cell division was initiated at 0.9 fraction of the cell cycle (Fig. 3).

The nitrogen content of different cellular fractions during the cell cycle.

The nitrogen content of different cellular fractions during synchronous growth of *C. pyrenoidosa* is shown in Figs. 4-6, 8. The nitrogen content of these fractions is expressed on three

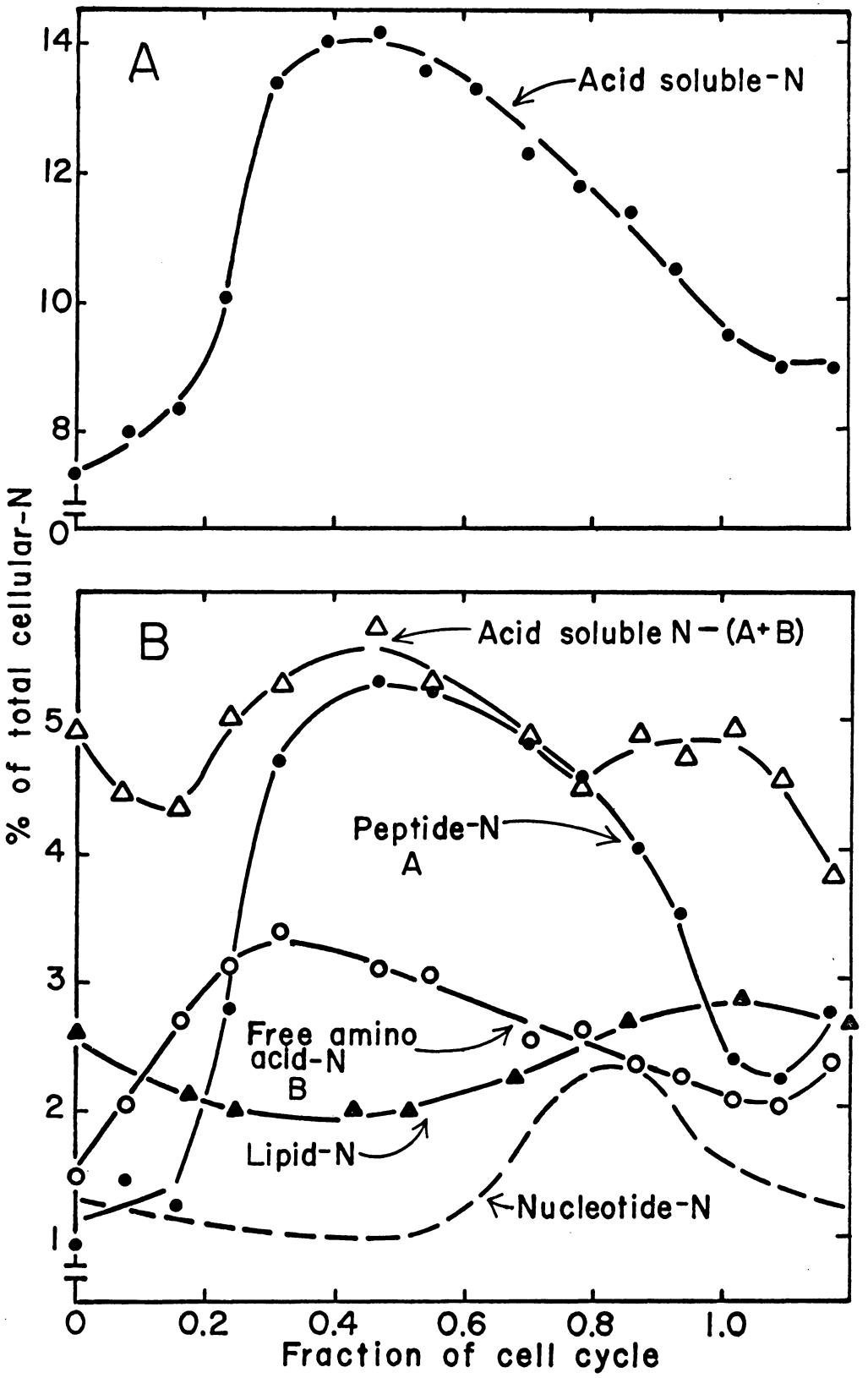
different bases. Since total cellular nitrogen increases at the same exponential rate as cellular dry weight (in the new mass culturing system), the trend obtained when a cellular component is expressed on a nitrogen basis also reveals how the component changes relative to cellular mass during the cell cycle (Figs. 4 and 6). To reveal how a component changes independently of changes in other cellular parameters, it is expressed on a per ml of culture basis (Fig. 8). It should also be noted that since the cell number per ml of culture remains constant prior to the onset of cellular division, the trend of a component on a per ml basis is the same as that on a per cell basis during this stage of development.

The acid-soluble compounds were expressed as percent of the total acid-soluble nitrogen to obtain a measure of their distribution within this fraction throughout the cell cycle (Fig. 5).

Two solubility classes are shown (as % of total cellular-N) in Fig. 4: lipid-plus chlorophyll-N (ethanol-ether soluble), and acid-soluble-N. Lipid-plus chlorophyll-N showed only slight periodism during the cell cycle. The periodism was similar to that observed for phospholipid-P (42, 43) during synchronous growth of this same organism. Acid-soluble-N, on the other hand, exhibited pronounced periodism during cellular development.

A low temperature strain of C. pyrenoidosa (Van Neil strain,

Fig. 4.
Classes of soluble nitrogen-containing compounds as percent of total cellular-N during synchronous growth of C. pyrenoidosa (Strain 7-11-05).


A
Acid soluble-N
B
Acid soluble N − (A+B)
Peptide-N
A
Free amino
acid-N
B
Lipid-N
Nucleotide-N
% of total cellular-N
Fraction of cell cycle
0
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5
8
10
12
14

de No. Z, 2. 2. 1) contained approximately 2x more acid-soluble-N (44) than the high temperature strain during synchronous growth. The lower level of acid-soluble-N in the high temperature strain is probably a manifestation of its high growth rate which is nearly double that of the low temperature strain. Because of the high growth rate, the rates of protein and nucleic acid synthesis probably more nearly keep pace with the rates of amino acid and nucleotide synthesis in the high temperature strain, thus accounting for the lower level of acid soluble-N in this organism.

The nitrogen content of three classes of nitrogen compounds (amino acid-, peptide amino acid-, and non amino acid-N) which comprise the acid soluble-N portion of the cells is also illustrated in Fig. 4. The most dramatic periodism exhibited by these three classes was shown by peptide-N which abruptly increased during the period 0.15-0.4 of the cell cycle and then gradually approached its initial level during nuclear and cellular division. The failure of peptide-N to return to its initial daughter cell level may indicate that peptides were utilized during the dark period preceding the cell cycle. The synchronization process used for this organism takes advantage of the fact that cells beyond a certain stage of cellular development can complete cell division (but cannot continue growth after division) when placed in the dark. Perhaps peptides

(and to some extent, free amino acids) are utilized for continued protein synthesis in the dark allowing completion of cellular division. Kanazawa (53) has reported that C. ellipsoidea cells, when placed in the dark, continue to accumulate protein at the expense of the peptide pool.

The free-amino-acid-N reached its maximum level during the period when peptide-N exhibited maximum rate of increase, and decreased thereafter at a constant rate.

Nitrogen compounds of the acid-soluble pool other than amino acids (free or in peptides) include inorganic-N, amide-N, nucleotide -N, etc. These compounds are grouped together and called non-amino-acid soluble-N. The nitrogen content of this group of compounds exhibited a more complex pattern of change than the other two acid-soluble classes. It is noteworthy to mention that the nitrogen content of the non-amino-acid fraction reached a minimum value during the period of nuclear division. The contribution of nucleotide-N to the non-amino-acid soluble-N pool is evident from the estimated amount of nucleotide-N (Fig. 4). Nucleotide-N was estimated from the Norit-A adsorbable acid-soluble-P data of Baker and Schmidt (54). Evidently, nucleotide-N constitutes a small percentage of the total non-amino-acid soluble-N pool except during the periods of nuclear and cellular division. In contrast to non-amino-acid soluble-N,

nucleotide-N reached its maximum level during the period of nuclear division.

Fig. 5 indicates the distribution of the acid-soluble compounds in the TCA extract during the cell cycle (expressed as % of total acid-soluble-N). Total soluble-amino-acid-N (free and in peptides) increased reaching its maximum level during nuclear division. Free amino-acid-N increased during early cellular development reaching a maximum value prior to the abrupt increase of peptide-N. The relationships between these curves suggested that amino acids, synthesized during early cellular development, may be utilized for peptide synthesis during the period of increase in peptide-N. Turnover studies will be necessary, however, to firmly establish the origin of the peptide-amino-acids during this period. The levels of free-and peptide-sulfur amino acids are discussed as they relate to turnover studies (9) in a later section of this dissertation. The non-amino-acid soluble-N fraction decreased during early cellular development, remained at a constant level during the middle of the cycle, and increased during cellular division. The estimated level of nucleotide-N is also included in Fig. 5 to point out that nucleotide-N accounts for a nearly constant portion of the non-amino-acid soluble-N except during the periods of nuclear and cellular division.

Levels of nitrogen compounds within the lipid-free cell residue are shown in Fig. 6 (as % of total cellular-N) during the cell cycle.

Fig. 5.
Classes of acid-soluble nitrogen-containing compounds as percent of total acid-soluble-N during synchronous growth of *C. pyrenoidosa* (strain 7-11-05).

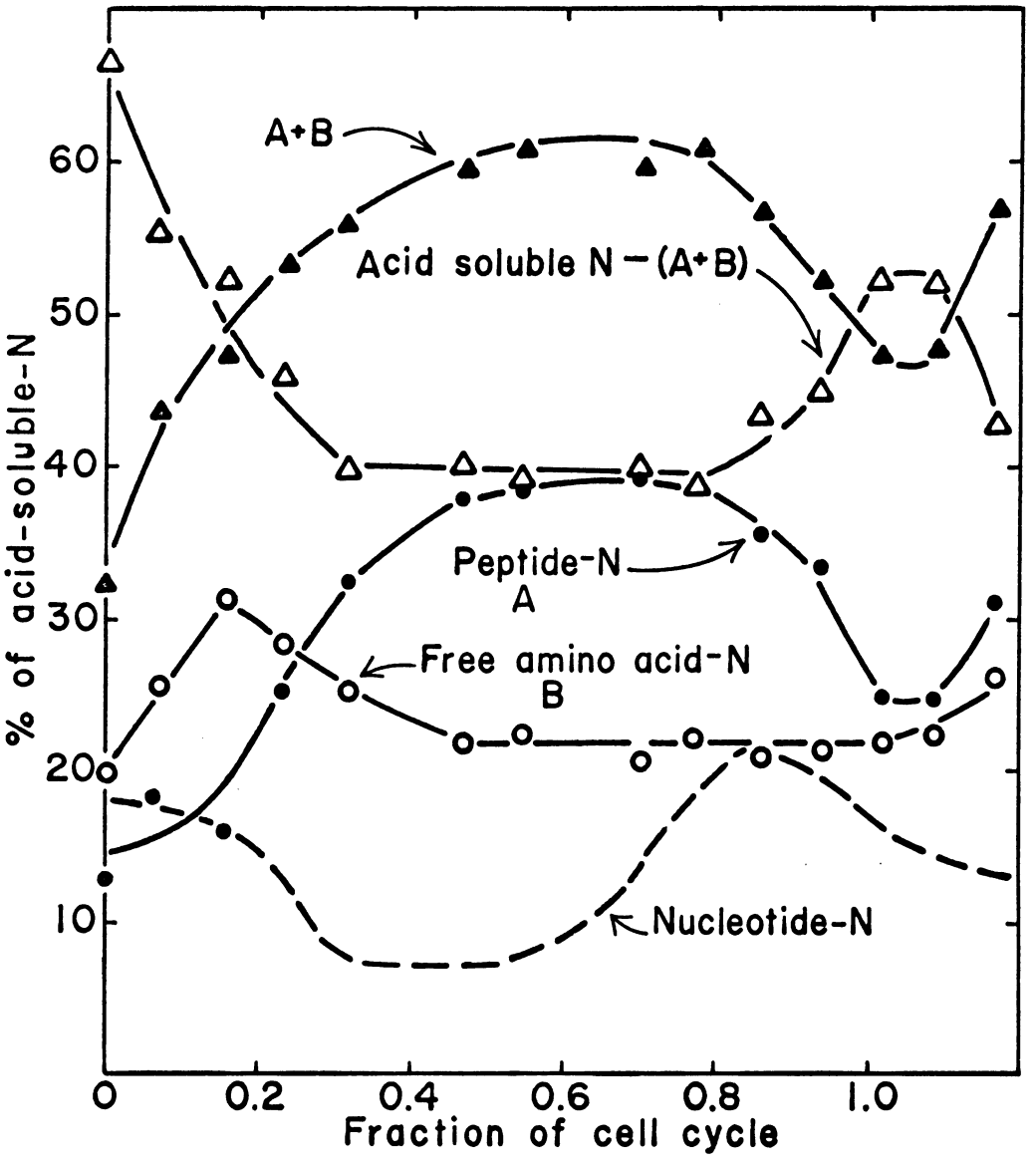
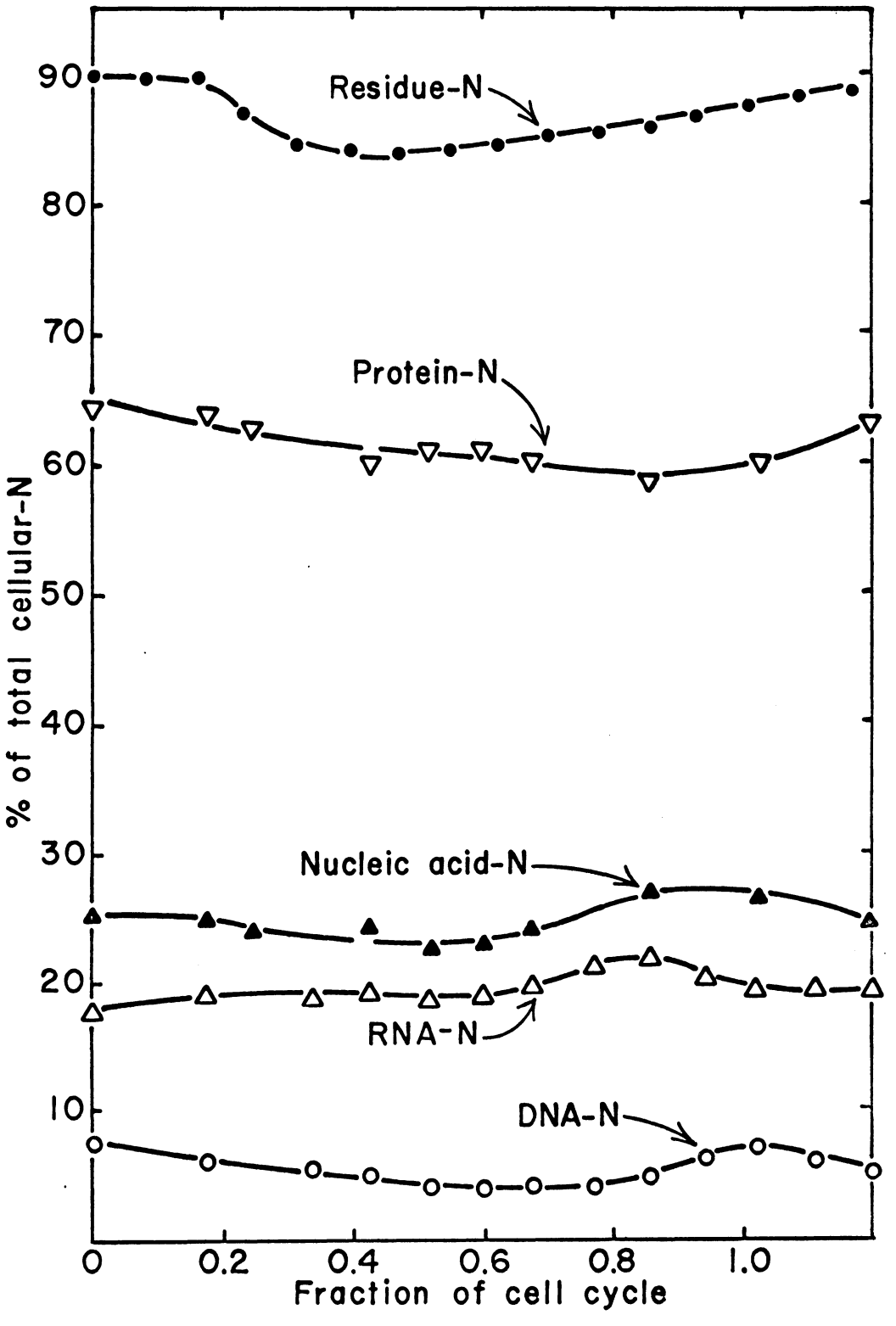

A+B
Acid soluble N − (A+B)
Peptide-N
A
Free amino acid-N
B
Nucleotide-N
% of acid-soluble-N
60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

Fig. 6.
Classes of nitrogen-containing compounds in the lipid-free cell residue as percent of total cellular-N during synchronous growth of C. pyrenoidosa (strain 7-11-05).

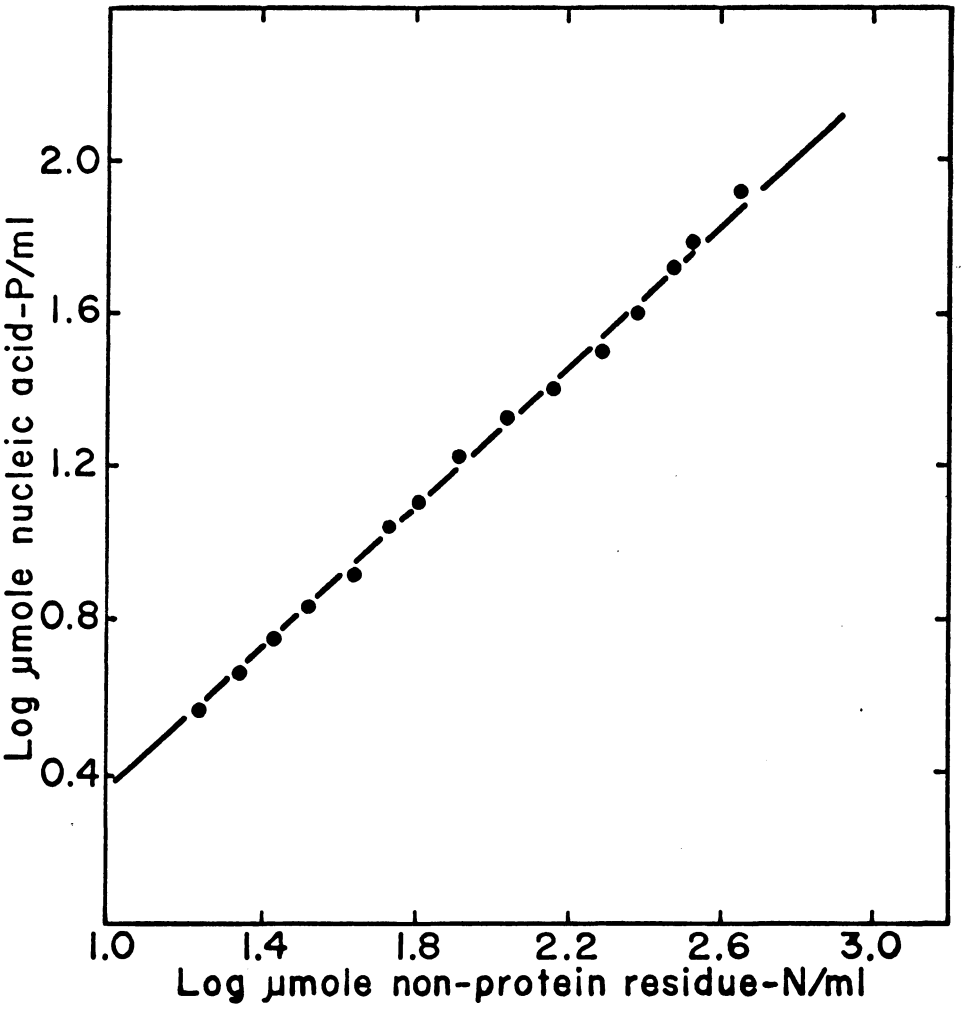

Residue-N
Protein-N
Nucleic acid-N
RNA-N
DNA-N
% of total cellular-N
90
80
70
60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

Total insoluble-N remained at its initial level during the period 0.0-0.15 fraction of the cell cycle and then decreased rapidly during the period 0.15-0.3 fraction of the cell cycle. During the remainder of the cell cycle, insoluble-N increased gradually reaching its initial daughter cell level after completion of cellular division.

Total protein-amino-acid-N decreased throughout the cell cycle until the onset of cellular division, and then returned to its initial level during cellular division. Kanazawa (53) also reported that the rate of protein accumulation lags behind the rate of accumulation of cellular mass during most of the cell cycle in synchronized C. ellipsoidea.

It seemed probable that the non protein-N of the lipid-free cell residue was primarily total nucleic acid-N. To test this possibility, the non-protein residual-N was plotted against total nucleic acid-P throughout the cell cycle (Fig. 7). Total nucleic acid-P was calculated by summing the RNA-P/ml and DNA-P/ml of culture (during synchronous growth of this organism) reported by Schmidt (55) and Herrmann and Schmidt (56) respectively. The linear relationship between these two parameters indicates that the accumulation curves of nucleic acid-P and non-protein residual-N are identical during the cell cycle. It is also important to point out that the value of the Y-intercept reveals that the ratio of non-protein residual-N

Fig. 7.
Non-protein residue-N vs. total nucleic acid-P during synchronous growth of C. pyrenoidosa (strain 7-11-05).


2.0
1.6
1.2
0.8
0.4
Log µmole nucleic acid-P/ml
1.0
1.4
1.8
2.2
2.6
3.0
Log µmole non-protein residue-N/ml

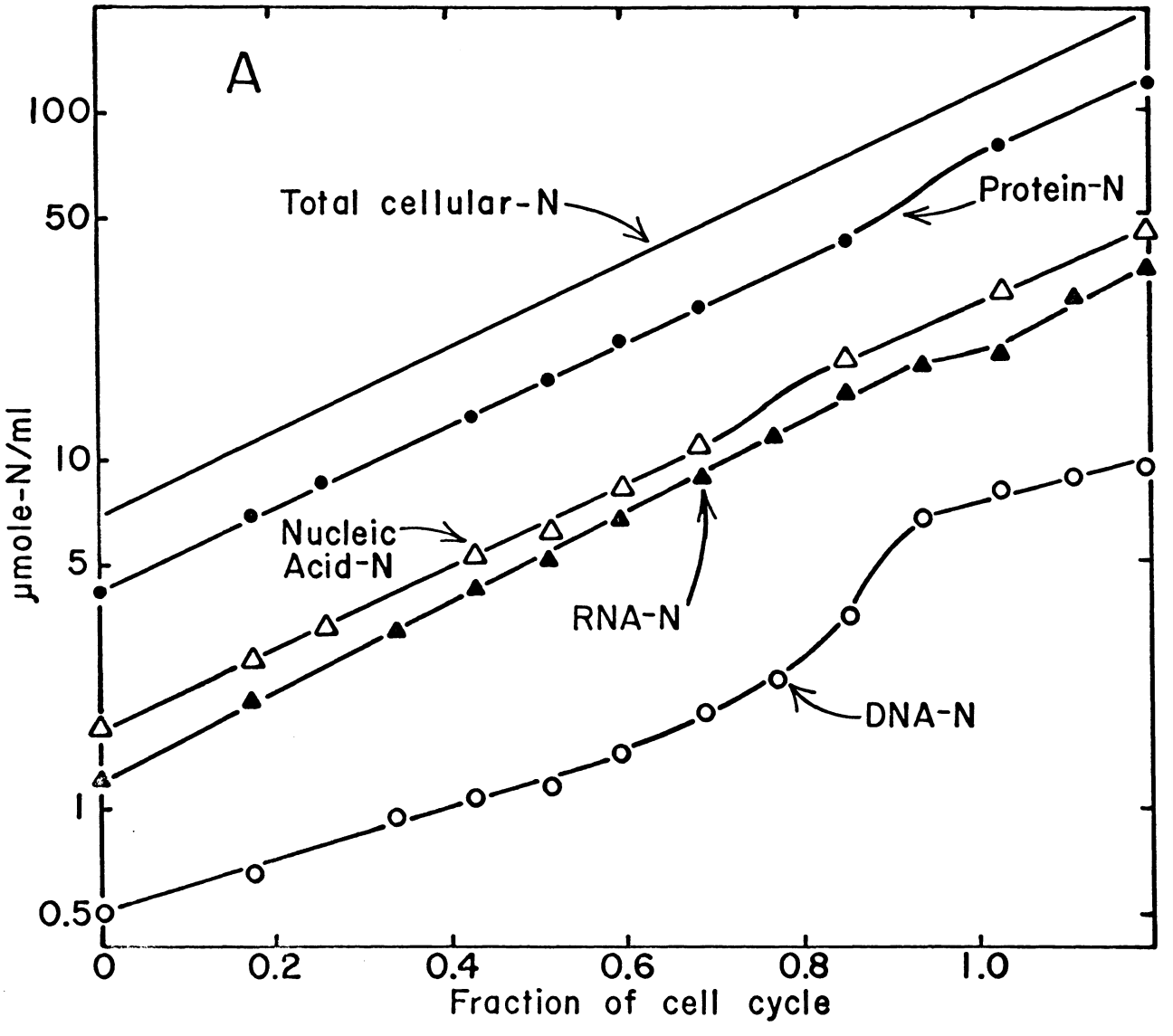
to nucleic acid-P is essentually equal to the ratio of N to P in the nucleotide moieties of nucleic acids. Thus, it is apparent that the non-protein residual-N is indeed predominantly nucleic acid.

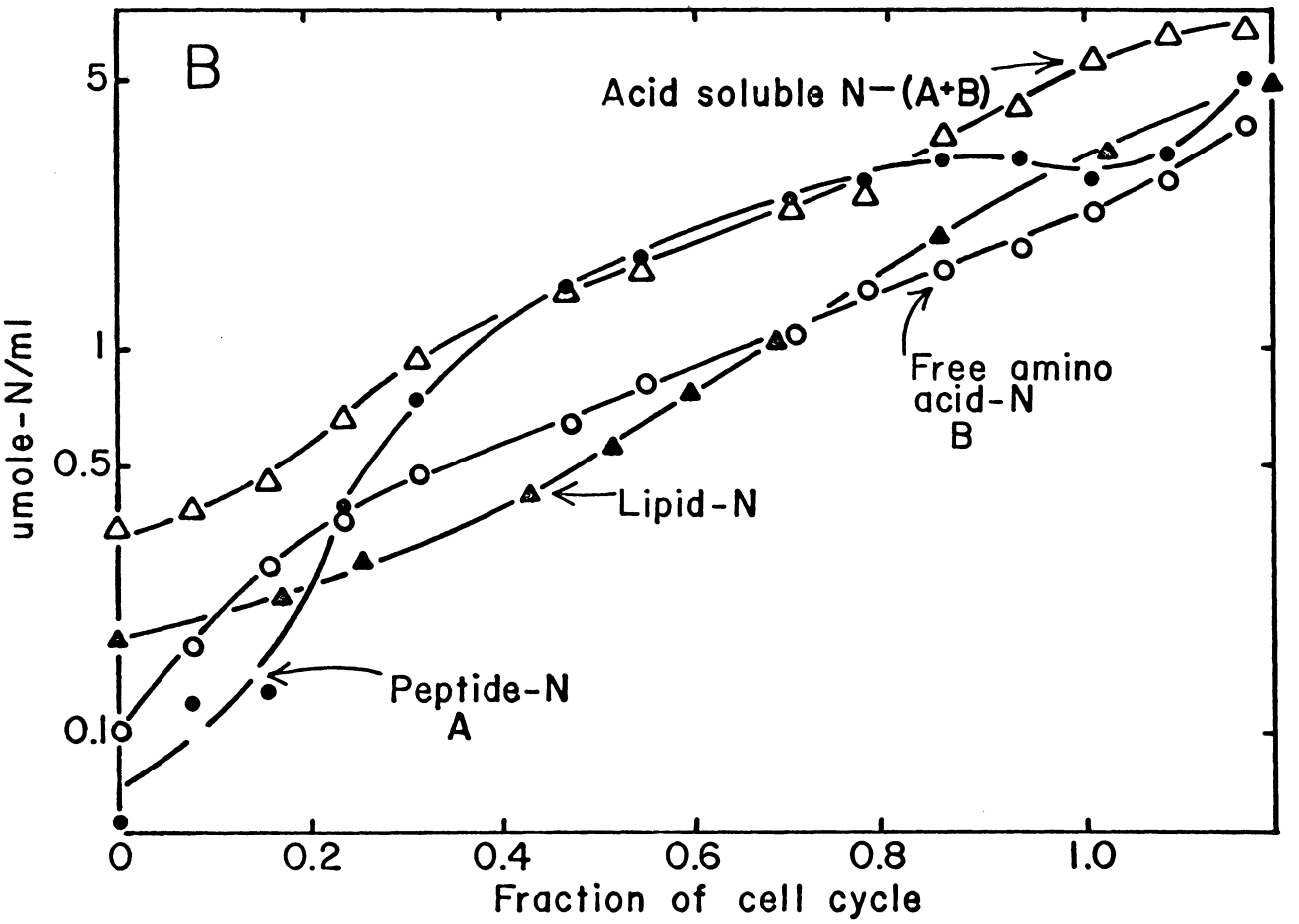
Nucleic acid-N (non-protein residual-N) exhibited a gradual decrease (% of total cellular-N) during most of the cell cycle (Fig. 6). However, during the period of nuclear division, the nucleic acid-N returned to its initial daughter cell level and then began to decrease near the end of the period of cellular division.

Since the levels of RNA-P and DNA-P had been determined(55-57) for this same organism, it was possible to calculate (from their ratios) the level of RNA-N and DNA-N during the cell cycle. The trends of these two nucleic acids (as % of total cellular-N) indicates that the rise in total nucleic acid-N during nuclear division was a result of increased levels of RNA-N (Fig. 6) while the increased level of total nucleic acid-N during cellular division resulted from an increase of DNA-N.

To reveal trends in the accumulation of different nitrogen-containing compounds independently of changes in other cellular parameters, the amount of nitrogen associated with different classes of compounds was expressed on a per ml of culture basis (Fig. 8). It is important to note that the scale of the ordinate in Fig. 8 is logarithmic; therefore, linear portions of the curves reflect exponential rates of accumulation.

Fig. 8.
Classes of nitrogen-containing compounds per ml of culture during synchronous growth of C. pyrenoidosa (strain 7-11-05).


A
100
50
10
5
1
0.5
μmole-N/ml
Total cellular-N
Protein-N
Nucleic
Acid-N
RNA-N
DNA-N
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


B
Acid soluble N−(A+B)
Free amino acid-N B
Lipid-N
Peptide-N A
umole-N/ml
5
1
0.5
0.1
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

The nitrogen content of each class of compounds (except peptide-N) increased 15 fold, as did total cellular-N during the cell cycle. However, peptide-N showed a greater fold increase, indicating that peptides started at a low initial value probably because of utilization during the dark period which preceded the cell cycle. Evidence of the utilization of peptides during cell division is illustrated in Fig. 8. Net utilization of peptide-N was observed prior to and during cellular division. Peptide-N increased rapidly during early cellular development and then exhibited exponential accumulation until the period of nuclear division.

Free amino acid-N increased rapidly during early cellular development and then increased at an exponential rate for the remainder of the cell cycle.

After initial low rates of increase, lipid-plus chlorophyll-N and non amino acid soluble-N both accumulated at nearly exponential rates. Total nucleic acid-N and protein-N both demonstrated exponential accumulation at rates less than the rate of increase of total cellular-N during most of the cell cycle, but the rates of accumulation increased at the onset of nuclear and cellular division respectively. On the other hand, RNA-N increased exponentially at a rate greater than that of total cellular-N except for a lower rate of accumulation at the onset of cellular division. In contrast to the trend

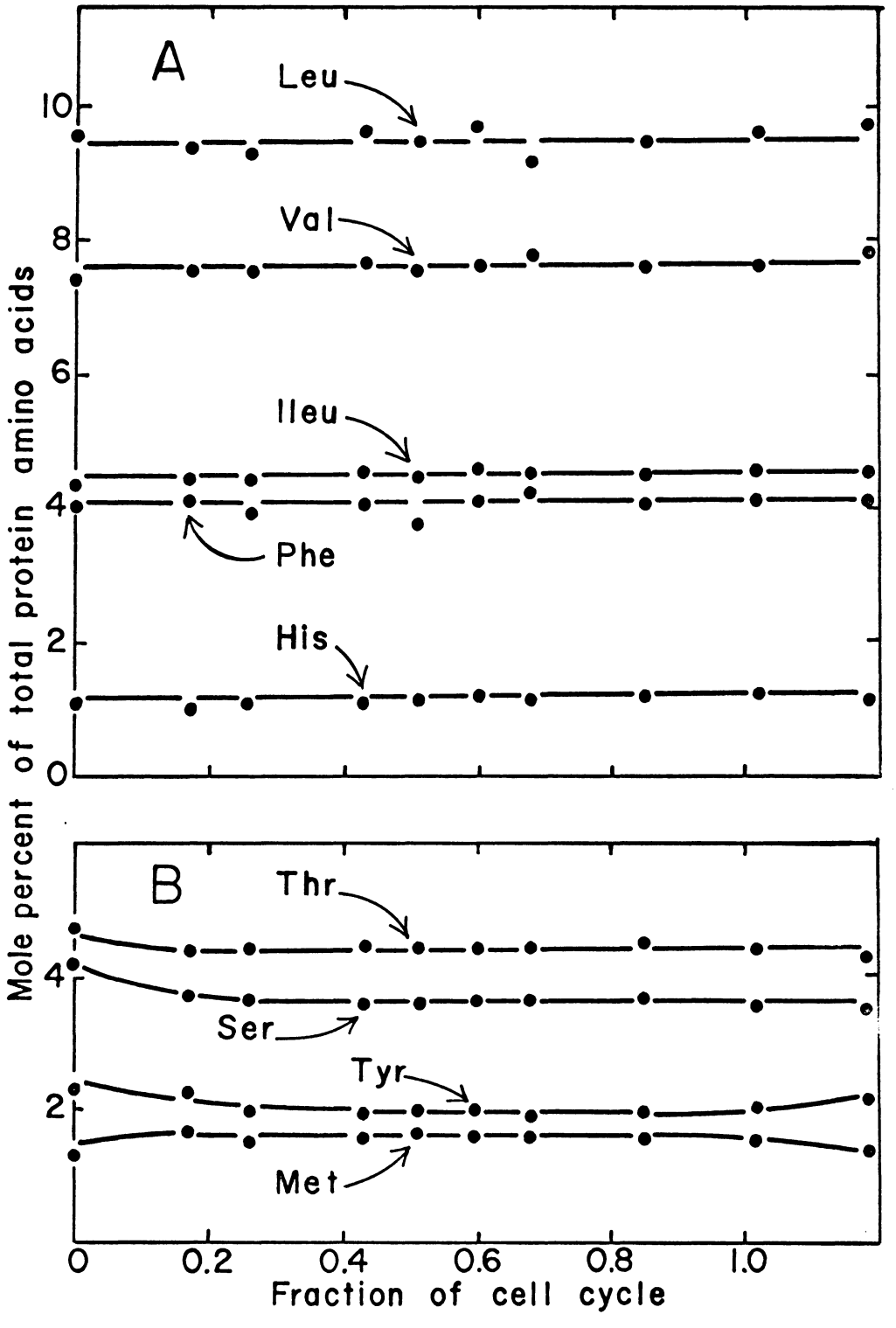
of RNA-N, DNA-N showed exponential accumulation at a rate very much lower than that of total cellular-N until the period of nuclear division. During this cellular event, DNA-N increased at an accelerated rate and then returned to its original exponential rate prior to cellular division.

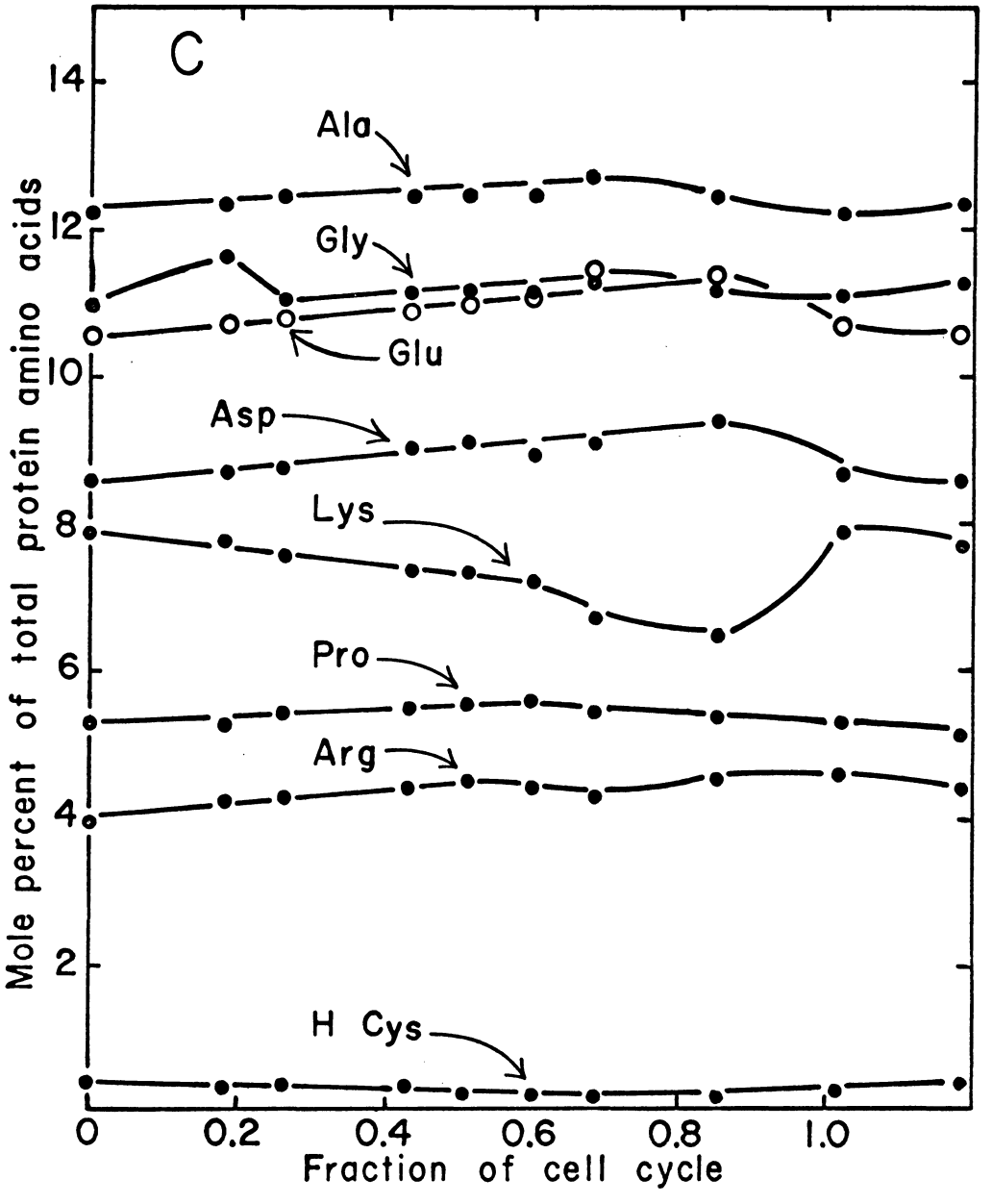
The exponential rate of synthesis of DNA prior to nuclear division is difficult to reconcile with current concepts of the mode of DNA replication (68) because exponential synthesis implies that the point of replication transverses the chromosome at an increasing rate or that the number of chromosomes being replicated at any given time increases exponentially. However, since certain sub-cellular organelles (mitochondria, chloroplasts) replicate their own DNA (69), the trend observed in the present study may be the net result of stepwise increases of mitochondrial, chloroplastic, and nuclear DNA.

Protein-amino acid distribution.

The distributions of individual protein amino acids during the cell cycle are expressed in Fig. 9, on a mole-percentage basis (μ mole amino acid X100/μ mole total protein-amino acid). The levels expressed in these Figures show that the protein amino acid composition remained nearly constant throughout cellular development. The protein amino acids which did not comprise a constant percentage

Fig. 9.
Protein-amino acids as mole percent during synchronous growth of C. pyrenoidosa (strain 7-11-05).


A
Leu
Val
Ileu
Phe
His
B
Thr
Ser
Tyr
Met
Mole percent of total protein amino acids
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

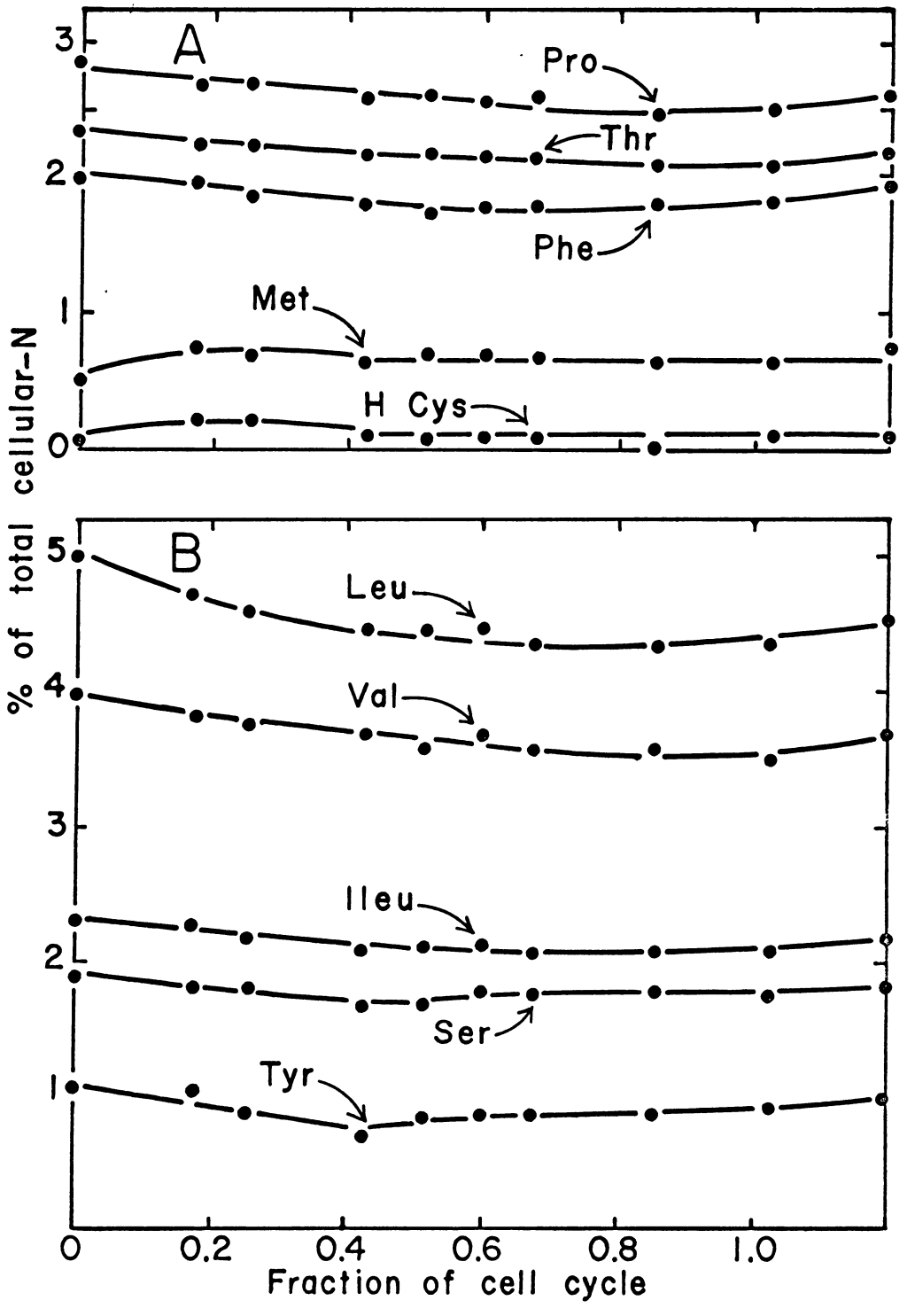

C
Ala
Gly
Glu
Asp
Lys
Pro
Arg
H Cys
14
12
10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
Mole percent of total protein amino acids
Fraction of cell cycle

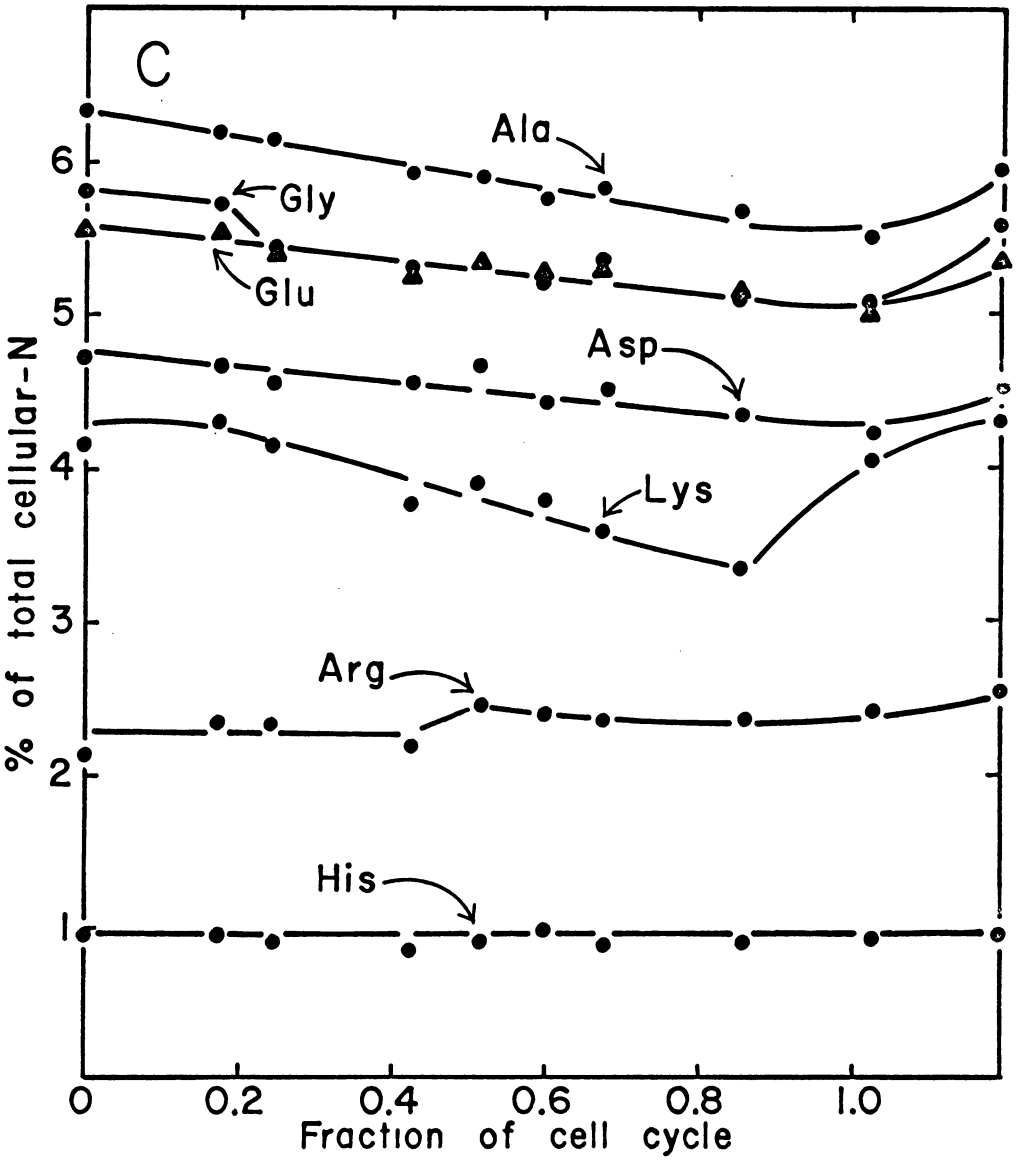
of total cellular protein during the cell cycle were glycine, glutamate, aspartate, lysine, arginine, proline and half-cystine.

The protein amino acid distribution is expressed as percent of total cellular-N in Fig. 10. The levels of many of the amino acids decreased gradually (relative to cellular mass) until the onset of either nuclear or cellular division and then returned to their initial daughter cell values. Each of the basic protein amino acids exhibited independent changes in level during the cell cycle. Histidine was a constant percentage of total cellular-N throughout the entire cell cycle. Arginine remained at a constant level during early cellular development but increased to a higher level prior to the mid-point of the cell cycle. On the other hand, lysine decreased during most of the cell cycle, reaching its minimum level prior to the onset of cellular division. It then increased rapidly to its initial daughter cell level during this cellular event. Protein methionine and half-cystine each comprised a nearly constant percentage of the total cellular-N throughout the cell cycle.

Although most of the protein-amino acids did not exhibit dramatic changes in level during the cell cycle, their turnover may vary widely during cellular development. This possibility is supported by the observation that although little change in level was exhibited by protein methionine and half-cystine during the cell cycle,

Fig. 10.
Protein amino acids as percent of total cellular-N during synchronous growth of C. pyrenoidosa (strain 7-11-05).


A
Pro
Thr
Phe
Met
H Cys
B
Leu
Val
Ileu
Ser
Tyr
% of total cellular-N
Fraction of cell cycle
0
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5


C
Ala
Gly
Glu
Asp
Lys
Arg
His
% of total cellular-N
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

Johnson and Schmidt (9) observed dramatic and opposite shifts in the incorporation of ^{35}S into these same protein-amino acids during the period 0.26-0.60 fraction of the cell cycle. Thus, it can be concluded that the protein-sulfur-amino acids exhibit significant shifts in turnover rate, even though their levels remain nearly constant during cellular development. It will be necessary, therefore, to obtain turnover rate data for all of the protein-amino acids before relationships between the metabolism of the protein-amino acids and periodic cellular events can be elucidated. It is tempting, nevertheless, to speculate on a possible relationship between the changes in level of protein-lysine (Fig. 9-C and Fig. 10-C) and the synthesis of DNA during cellular development. The change in level of protein-lysine showed an inverse relationship to the pattern of change observed for DNA (56, 57) during synchronous growth of Chlorella. Furthermore, protein lysine reached its lowest level (as % of total cellular-N, Fig. 10-C), prior to cellular division, when DNA-N exhibited an abrupt increase in its rate of accumulation (Fig. 8). These observations lend some physiological significance to the *in vitro* observation of Gurley *et al.* (58) that lysine-rich histones inhibit (80% at a histone/DNA ratio of 2) the activity of DNA polymerase. These workers suggested that lysine-rich histones may control DNA synthesis *in vivo* by acting as inhibitors of the polymerase at certain

periods of the cell cycle.

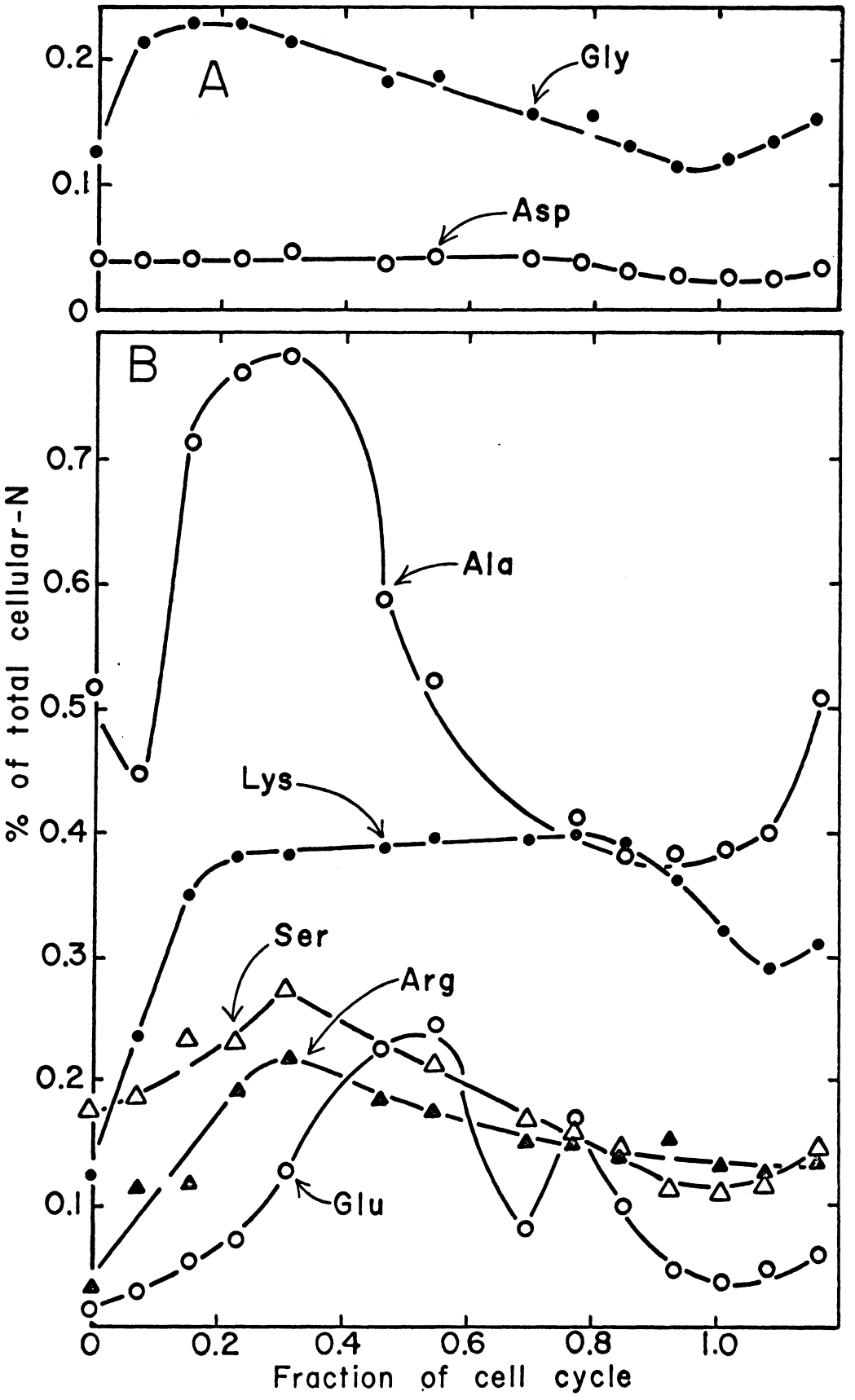
Specific enzymes have been observed to fluctuate in apparant level during cell cycles of a variety of microorganisms (6). However, the present studies show that the cellular capacity for protein synthesis remains nearly constant during cellular development. Evidently, therefore, the rates of synthesis of specific proteins are individually regulated during the cell cycle at a biochemical level not common to the synthesis of all proteins.

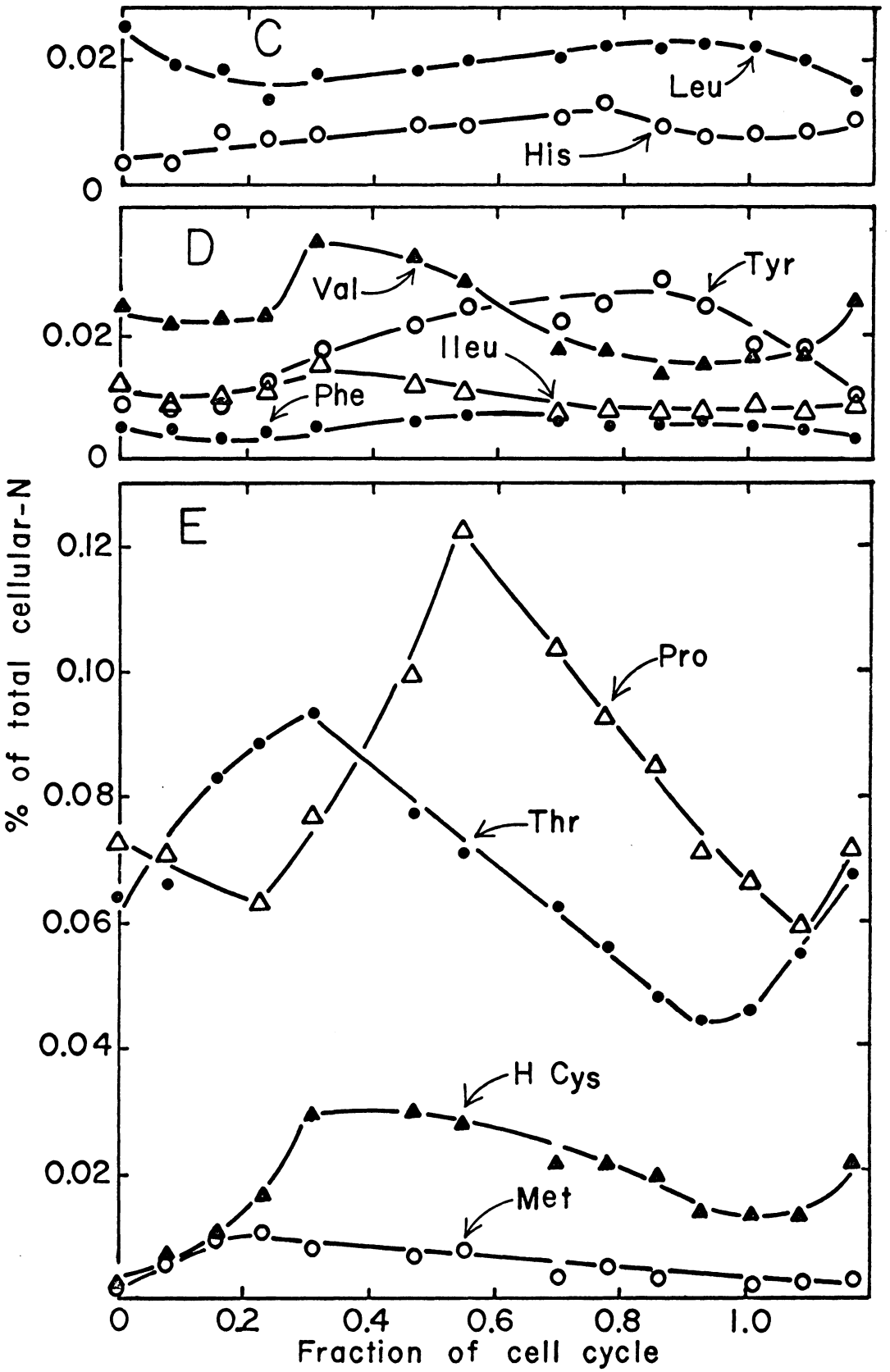
Free-amino acid distribution.

The levels of free amino acids during synchronous growth are expressed as percent of total cellular-N in Fig. 11. The amino acids present at relatively high concentrations (0.05-0.8%) are shown in Fig. 11-A and -B. These amino acids demonstrated a variety of changes in level during the cell cycle; however, alanine, lysine, glutamate, serine, glysine, and arginine all increased to relatively high levels (0.78, 0.38, 0.29, 0.28, 0.23, and 0.22%, respectively) during early cellular development. Together the two predominant amino acids (alanine and lysine) accounted for 50% of the free amino acid content of the cells throughout the cell cycle.

The amino acids at relatively low levels (0.001-0.12%) are presented in Fig. 11-C, -D, and -E (the ordinates are expanded relative to those of Figs. 11-A, and -B). It is evident that the amino

Fig. 11.
Free amino acids as percent of total cellular-N during synchronous growth of C. pyrenoidosa (strain 7-11-05)


A
B
Gly
Asp
Ala
Lys
Ser
Arg
Glu
0.2
0.1
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
% of total cellular-N
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


C
0.02
0
Leu
His
D
Val
Tyr
Ileu
Phe
0.02
0
E
0.12
0.10
0.08
0.06
0.04
0.02
Pro
Thr
H Cys
Met
% of total cellular-N
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

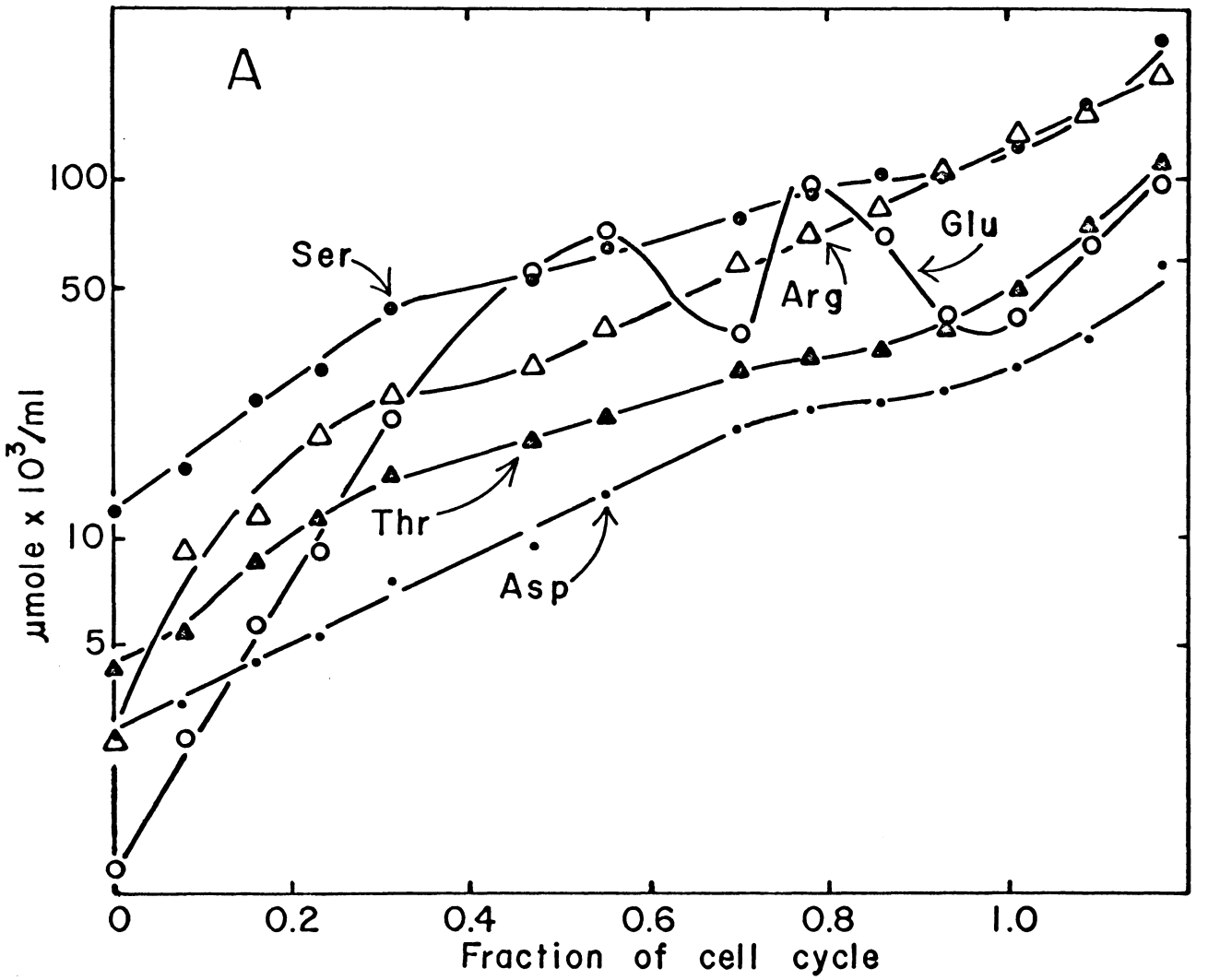
acids in lower concentration also exhibited a variety of quite different changes in level during cellular development.

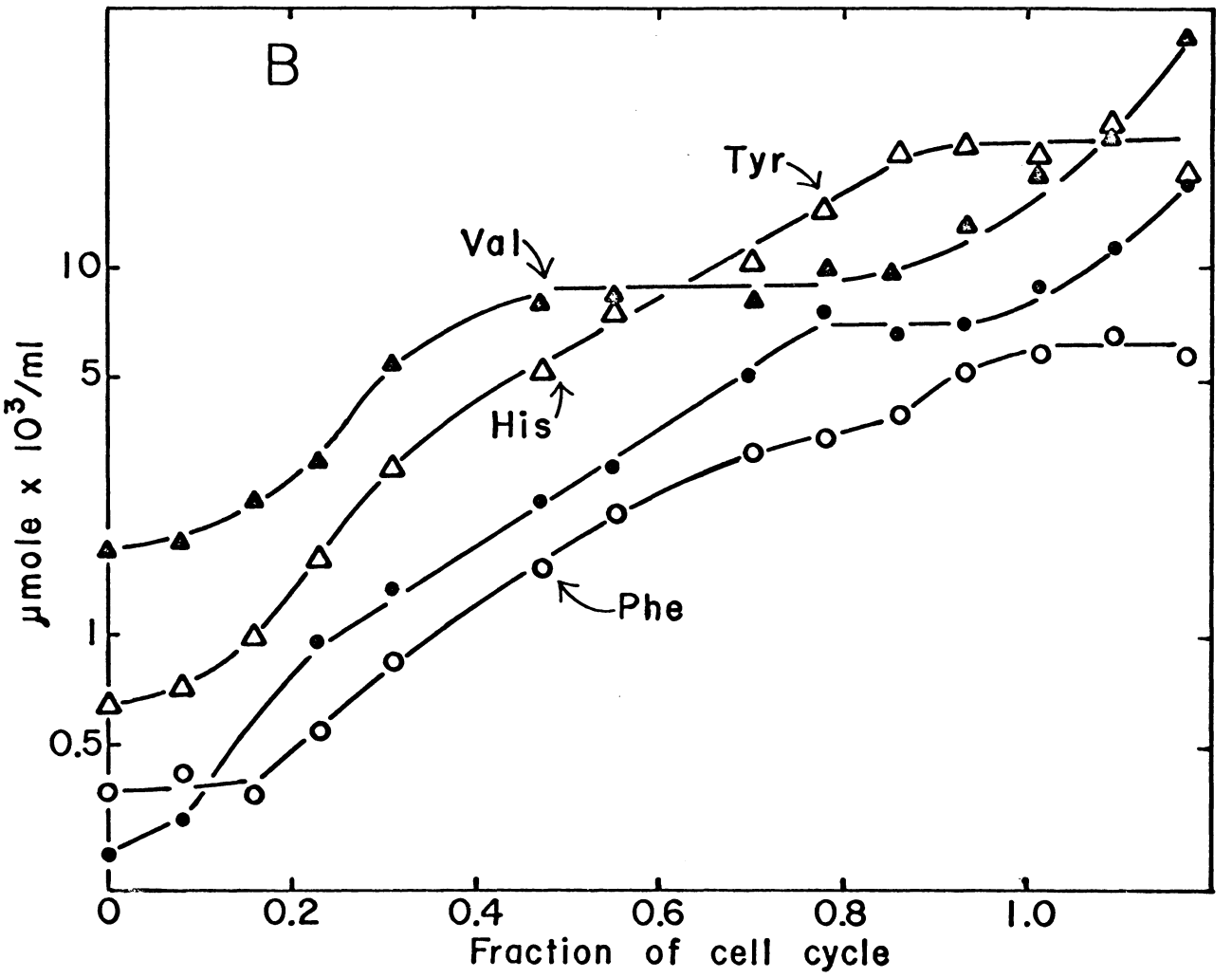
The free amino acids expressed on a per ml of culture basis are illustrated in Fig. 12. A number of the amino acids exhibited little or no accumulation during different periods of cellular development, e.g. valine, proline, and alanine during the middle of the cell cycle. However, glutamate was the only free-amino acid which exhibited net loss at certain periods during the cell cycle.

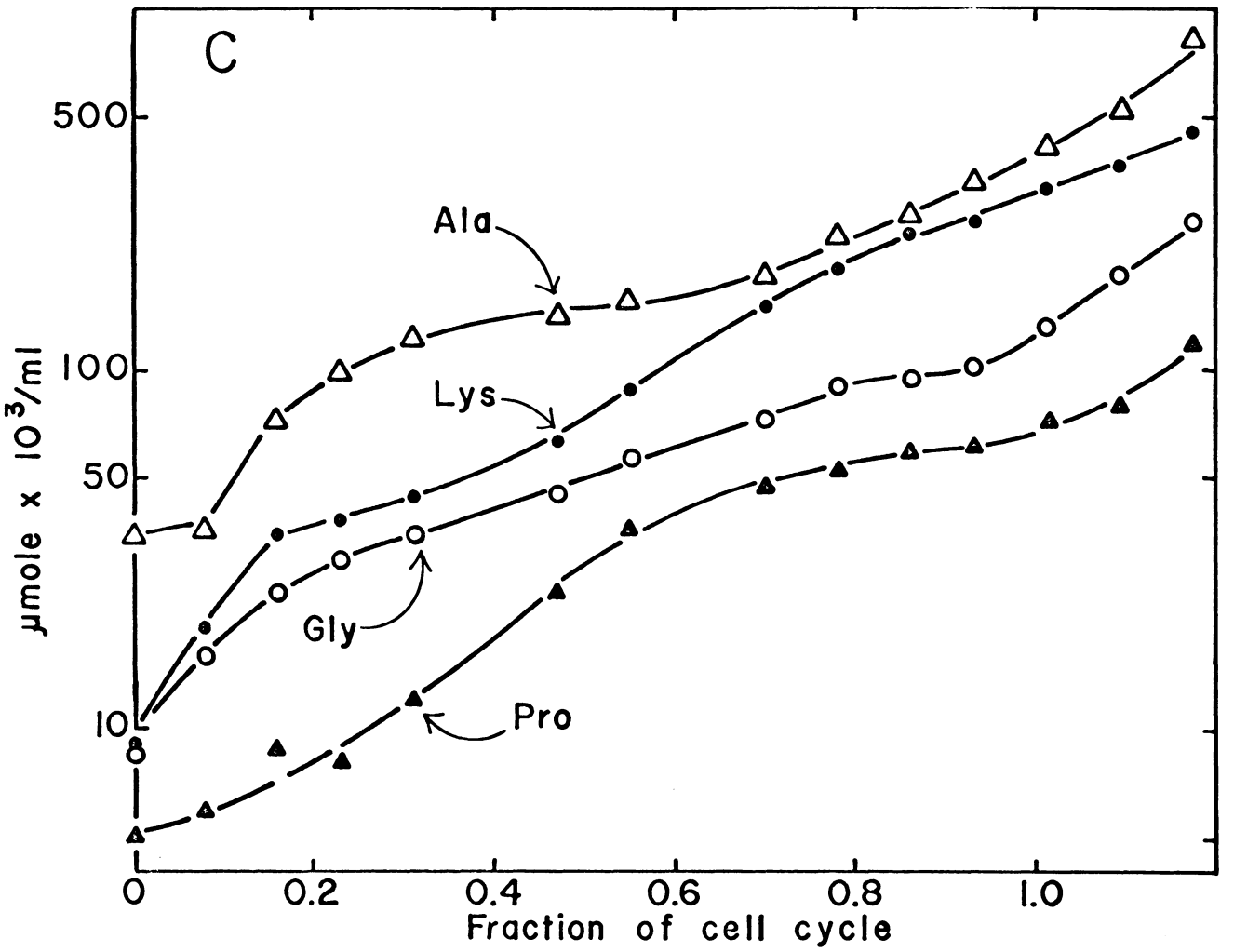
Since the individual free amino acids showed little similarity to each other in fluctuations in levels, it is probable that a number of different mechanisms regulate the levels of the different amino acids during the cell cycle. Furthermore, it is unlikely that the fluctuations in free amino acid levels resulted from changes in utilization of free amino acids for net protein synthesis since the composition of total cellular protein remained nearly unchanged throughout cellular development (Fig. 9).

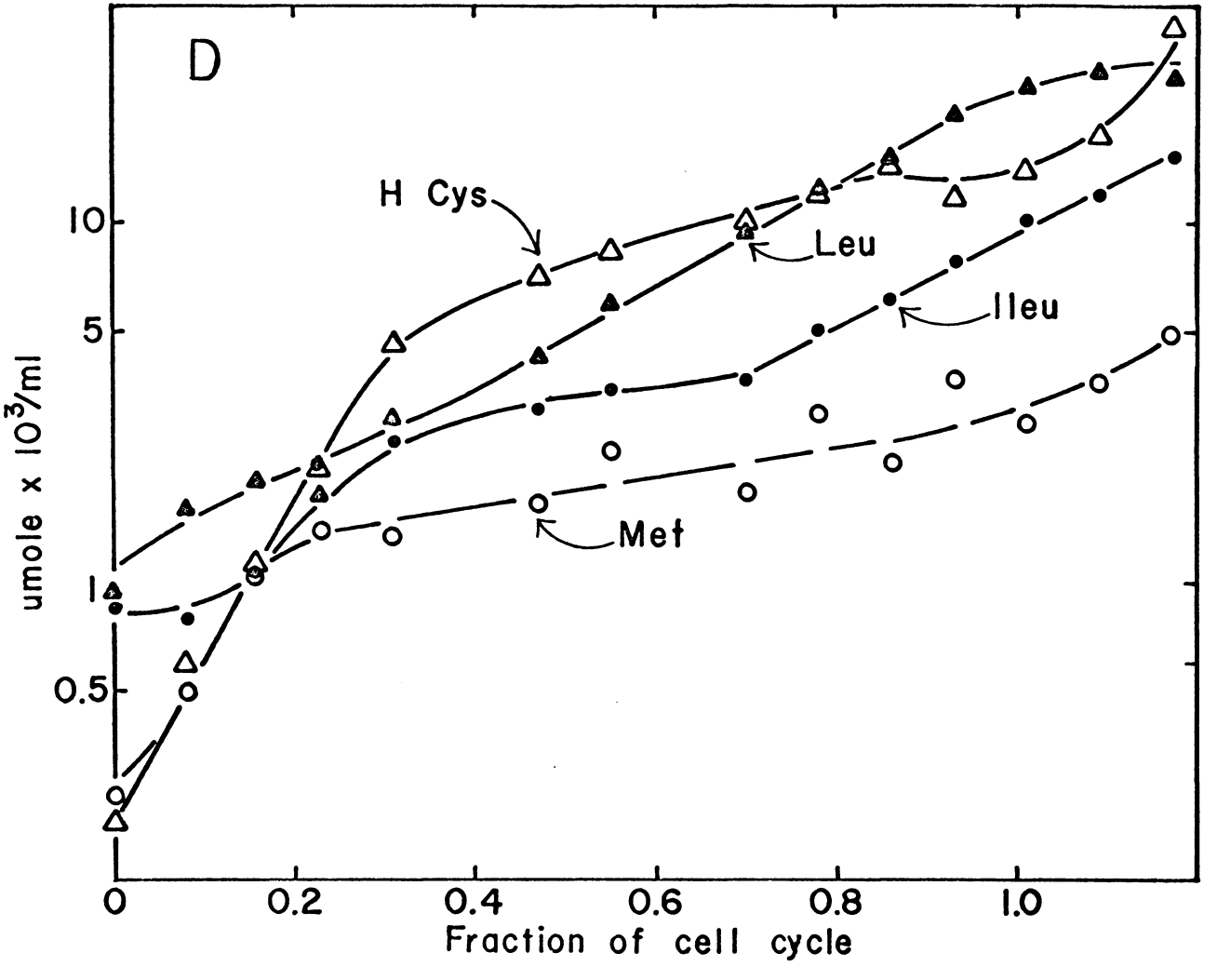
A number of unknown ninhydrin-positive compounds were separated by the amino acid analyzer. The levels of the unknowns that could be measured during the cell cycle are illustrated in Fig. 13 as relative units/ umole of total cellular-N (equivalent to % total cellular-N), and in Fig. 14 as relative units/ml of culture. Two of these compounds, X_1 and X_2, were eluted from the basic amino acid column before

Fig. 12.
Free amino acids per ml of culture during synchronous growth of *C. pyrenoidosa* (strain 7-11-05).


A
100
50
10
5
μmole x 10³/ml
Ser
Thr
Asp
Arg
Glu
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


B
Tyr
Val
His
Phe
10
5
1
0.5
μmole x 10³/ml
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


C
500
100
50
10
μmole x 10³/ml
Ala
Lys
Gly
Pro
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


D
H Cys
Leu
Ileu
Met
umole x 10³/ml
10
5
1
0.5
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

ammonia and after arginine, respectively. Unknown X_1 decreased in level (as % of total cellular-N) from the beginning of the cycle until just prior to cellular division; then, it increased abruptly and remained nearly constant during this cellular event. The elution position of this unknown, relative to the basic amino acids, suggested that it was ethanolamine (59). Likewise, its change in level is consistent with that reported for ethanolamine during synchronous growth of C. ellipsoidea (53). It is noteworthy to mention that the abrupt increase in level exhibited by this compound (Fig. 13) prior to cellular division, as well as the trend of accumulation of this compound (Fig. 14), suggests that it may serve a primary role in the cellular division process. The only other compounds (or class of compounds) which showed similar accumulation trends were DNA-N (Fig. 8) and protein-lysine (possibly associated with DNA in lysine-rich histones--see previous discussion).

Five unknown compounds, separated on the acidic and neutral amino acid column, are also illustrated in Fig. 13 and Fig. 14. Unknowns X_a, X_b, and X_c were eluted, in that order, prior to aspartate. Unknowns X_d and X_e were eluted after aspartate and methionine, respectively. Two of the seven unknown compounds, X_b and X_e, probably were peptides since they were destroyed during acid hydrolysis and exhibited trends of accumulation (Fig. 14) similar to

Fig. 13.
Unknown ninhydrin positive compounds as relative units per μ mole total cellular-N during synchronous growth of C. pyrenoidosa (strain 7-11-05).

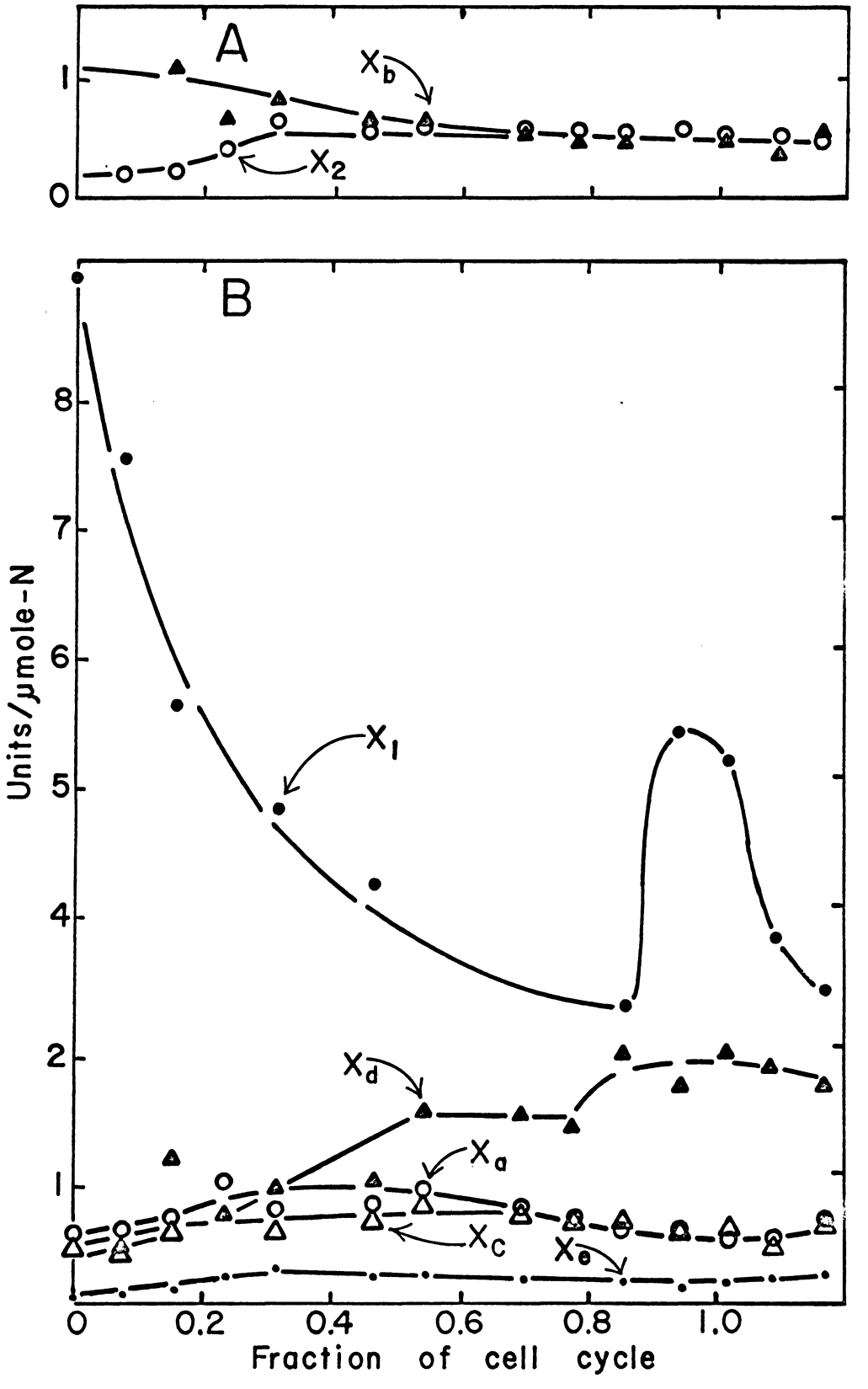
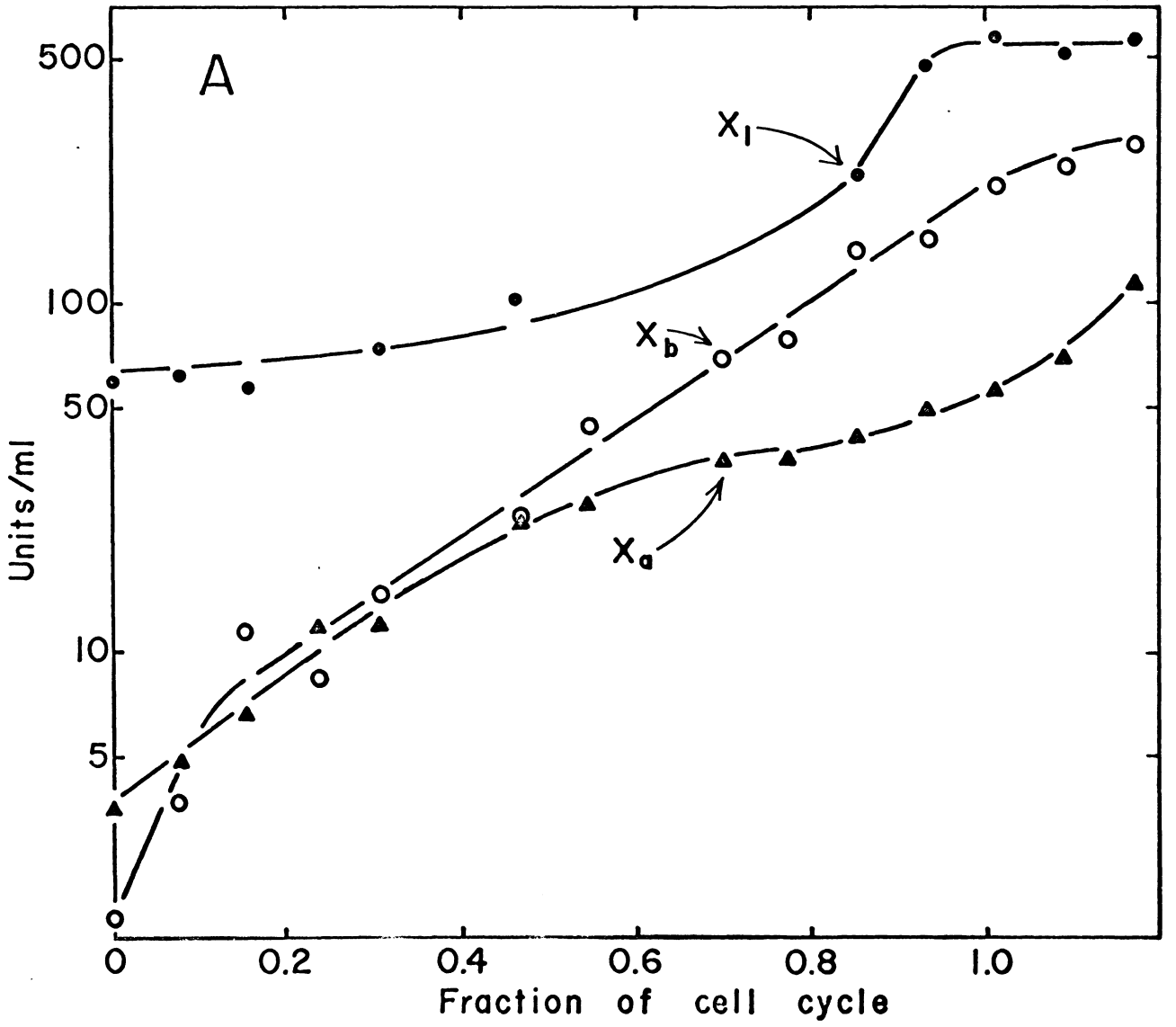
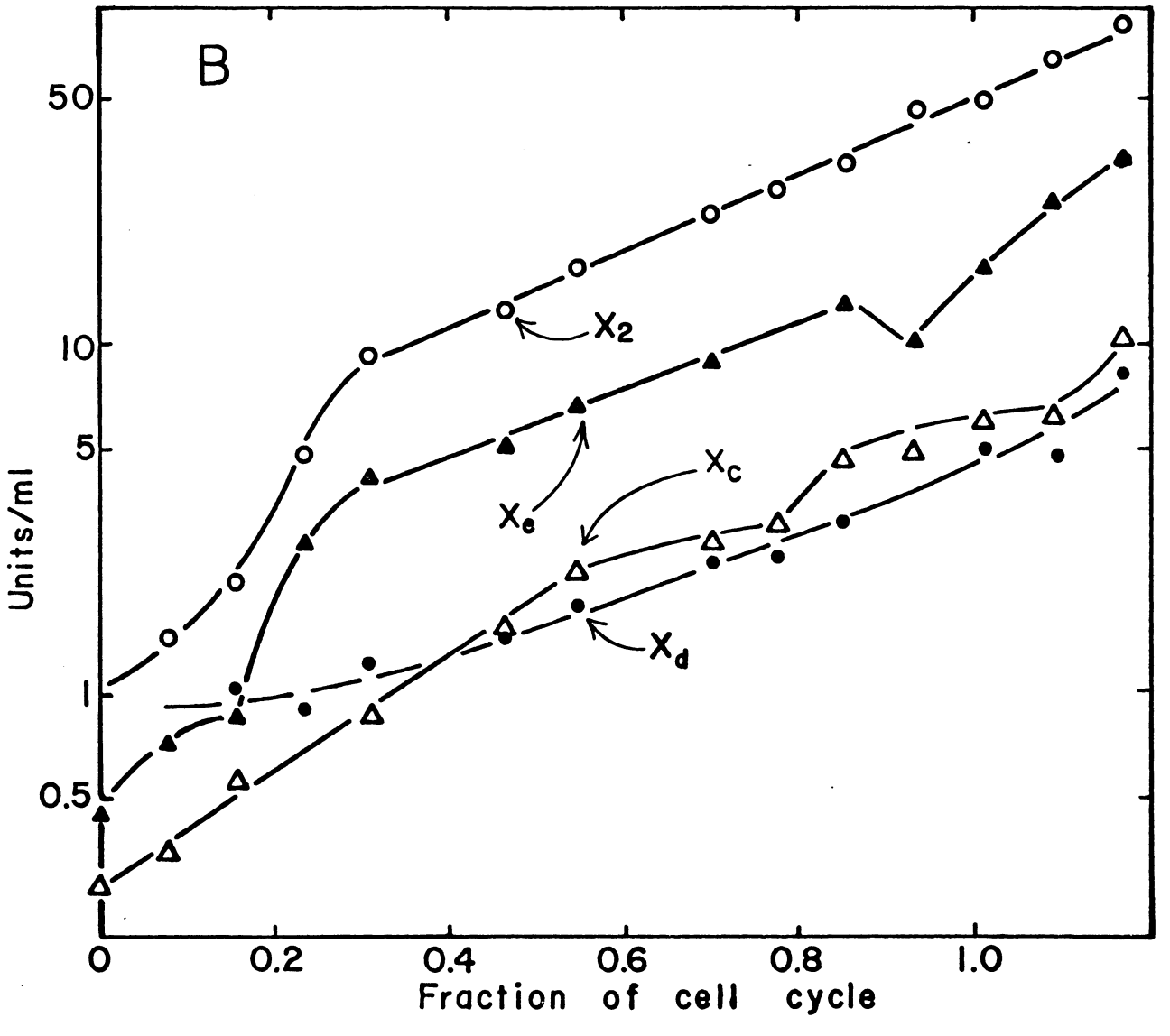

A
B
X_b
X_2
X_1
X_d
X_a
X_c
X_e
Units/μmole-N
Fraction of cell cycle
0
0.2
0.4
0.6
0.8
1.0
1
2
4
5
6
7
8

Fig. 14.
Unknown ninhydrin positive compounds as relative units per ml of culture during synchronous growth of C. pyrenoidosa (strain 7-11-05).


A
500
100
50
10
5
Units/ml
X_l
X_b
X_a
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


B
50
10
5
1
0.5
Units/ml
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle
X_2
X_e
X_c
X_d

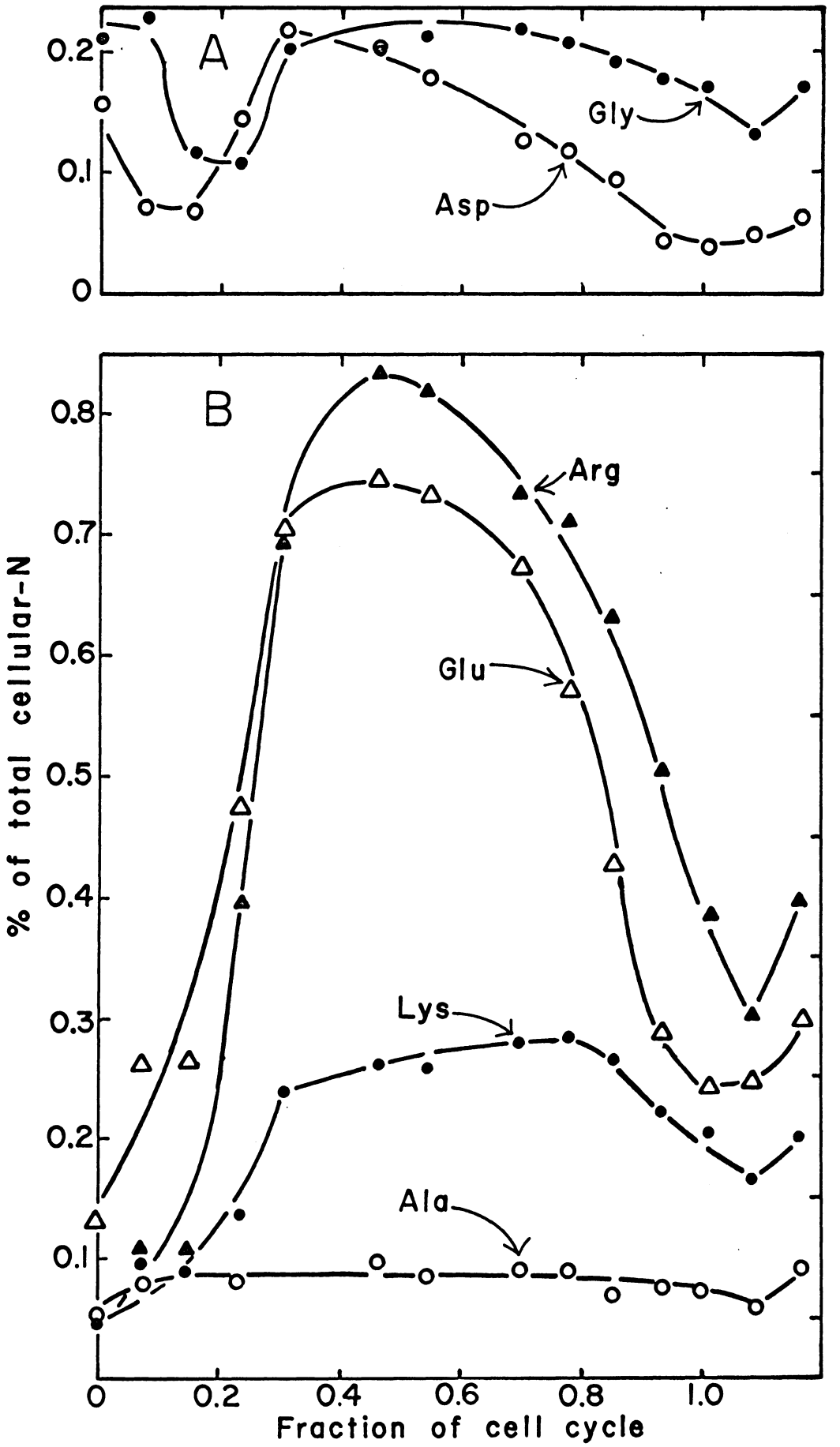
that of peptide-N (Fig. 8).

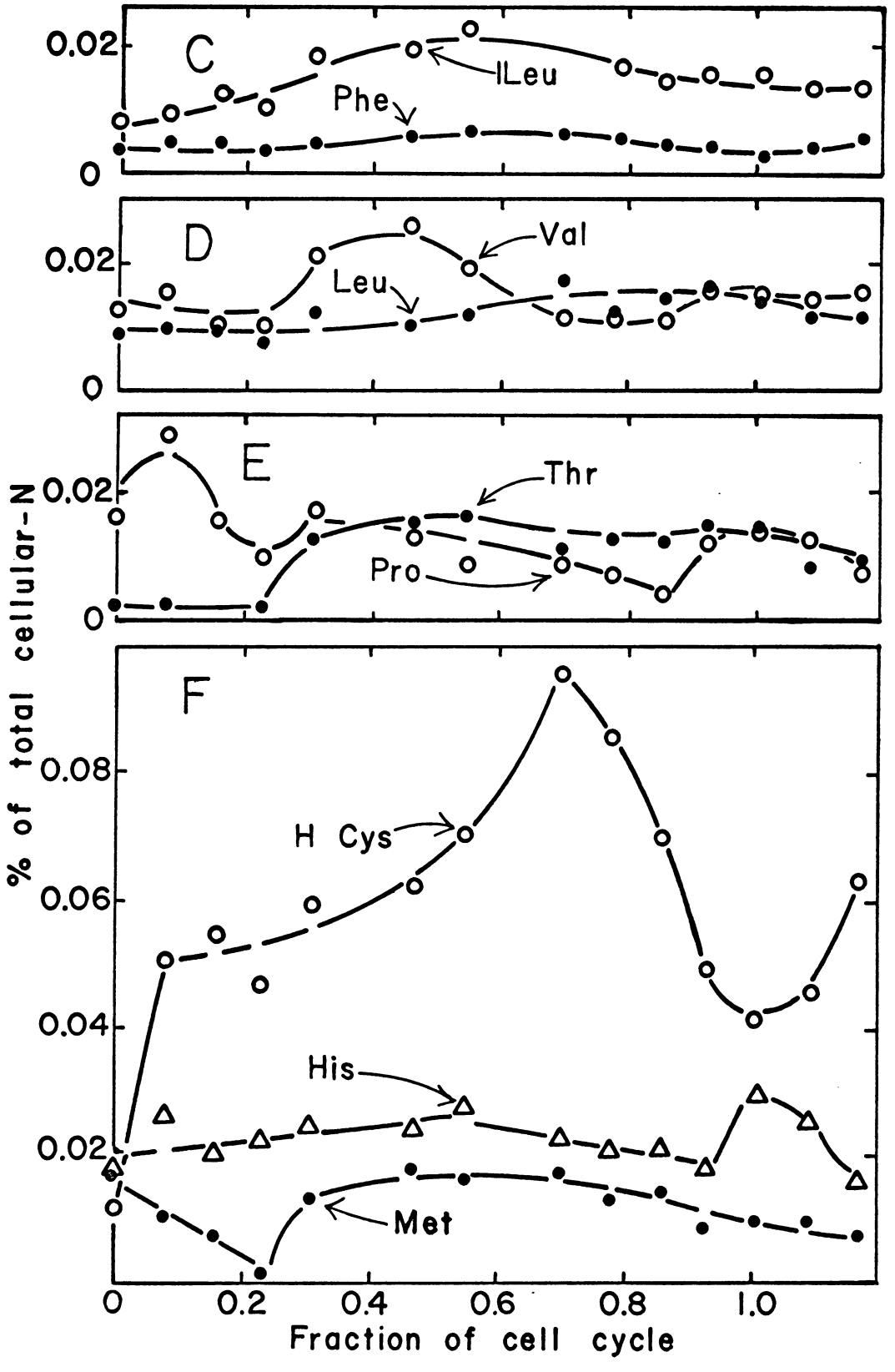
Peptide-amino acid distribution.

The levels of peptide-amino-acids during cellular development are expressed as percent of total cellular-N in Fig. 15. Peptide-amino-acids present at relatively high levels (0.5-0.85%) are shown in Fig. 15-A, and -B. Peptide alanine was present at nearly a constant level from 10% to 20% of the level of free-alanine during the cell cycle. Kanazawa *et al.* (60) reported three classes of peptides, containing either arginine, arginine and glulamate, or arginine, glutamate, and aspartate, in *C. ellipsoidea*. In this organism these peptides increased during early cellular development and reached maximum levels at different periods during the cell cycle. The present studies also indicate that arginine, glutamate, and aspartate are linked together in a number of peptides which reach maximum levels during different stages of cellular development. Peptide-aspartate, -glutamate, and -arginine showed similar, although not parallel, trends of change in level during the cell cycle, indicating that they are probably linked together in a number of peptides.

The peptide amino acids present at relatively low levels (0.001-0.09) are shown in Figs. 15-C, -D, -E, and F (ordinates are expanded relative to those in Figs. 15-A, and-B). Peptide phenylalanine was similar in level to free phenylalanine throughout the cell cycle.

Fig. 15.
Peptide amino acids as percent of total cellular-N during synchronous growth of C. pyrenoidosa (strain 7-11-05).


A
B
Gly
Asp
Arg
Glu
Lys
Ala
% of total cellular-N
Fraction of cell cycle
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1.0


C
0.02
0
ILeu
Phe
D
0.02
0
Val
Leu
E
0.02
0
Thr
Pro
F
0.08
0.06
0.04
0.02
H Cys
His
Met
% of total cellular-N
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

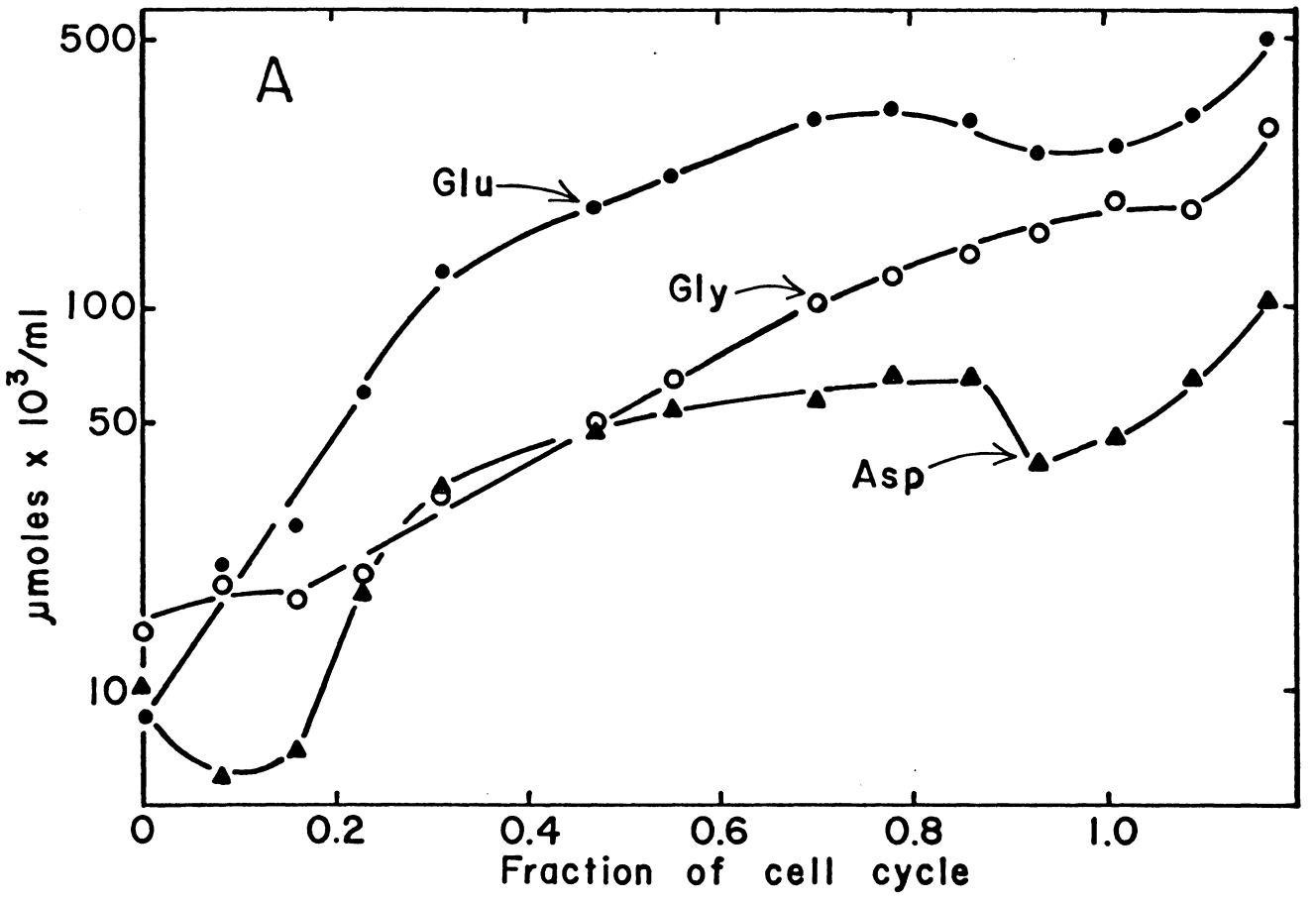
Likewise, peptide-valine and-leucine each showed trends (at lower levels) similar to the trends of their respective free amino acids. Peptide histidine remained at a constant level during most of the cell cycle but increased abruptly during cellular division.

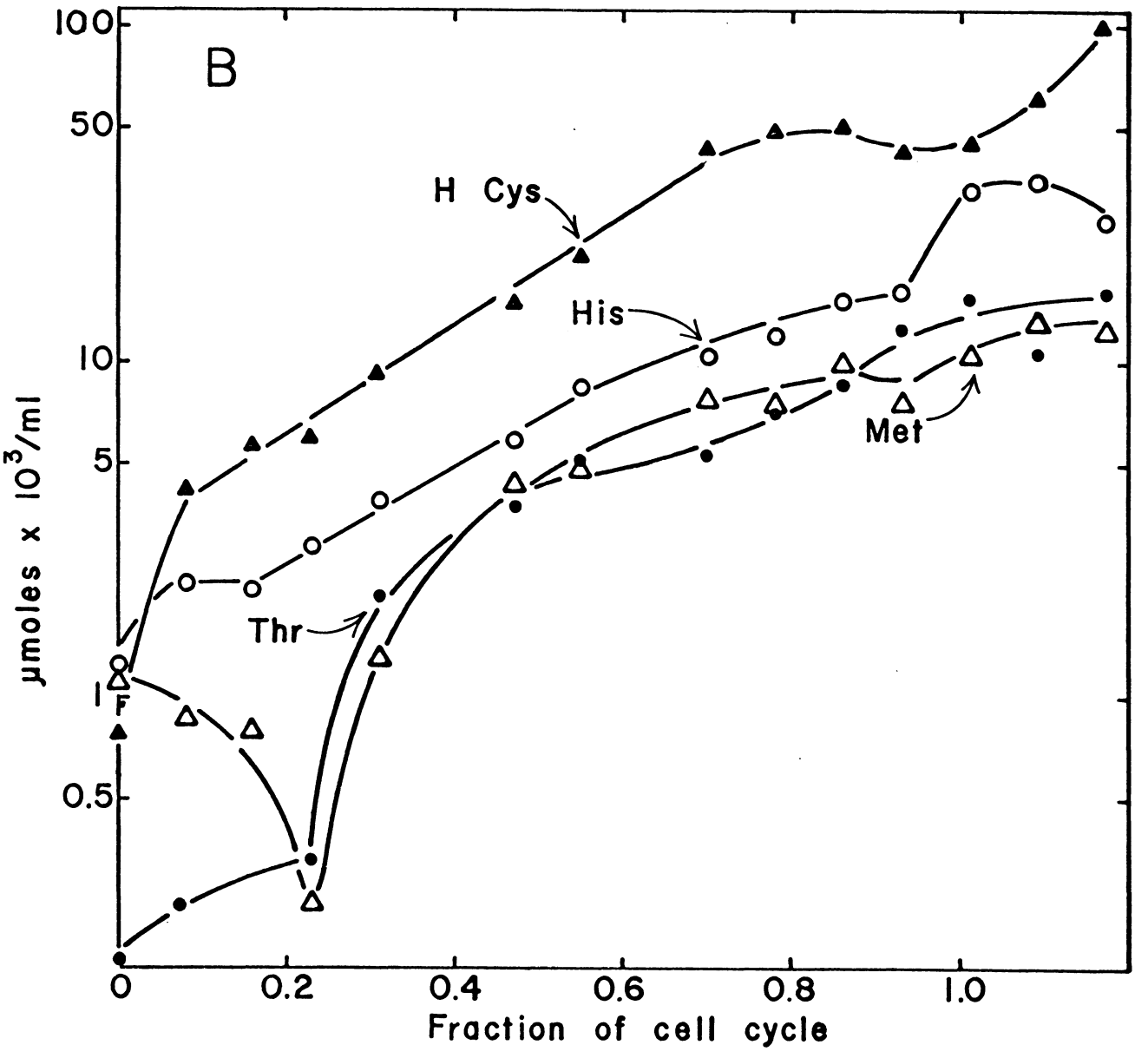
In contrast to the gradual changes in level of the free sulfur-amino-acids the peptide sulfur-amino-acids showed abrupt changes in level during the cell cycle. Peptide methionine decreased in level during early cellular development approaching zero at 0.23 fraction of the cell cycle. However, it then increased to a maximum level prior to the middle of the cell cycle. Peptide half-cystine, on the other hand, reached its maximum level during nuclear division.

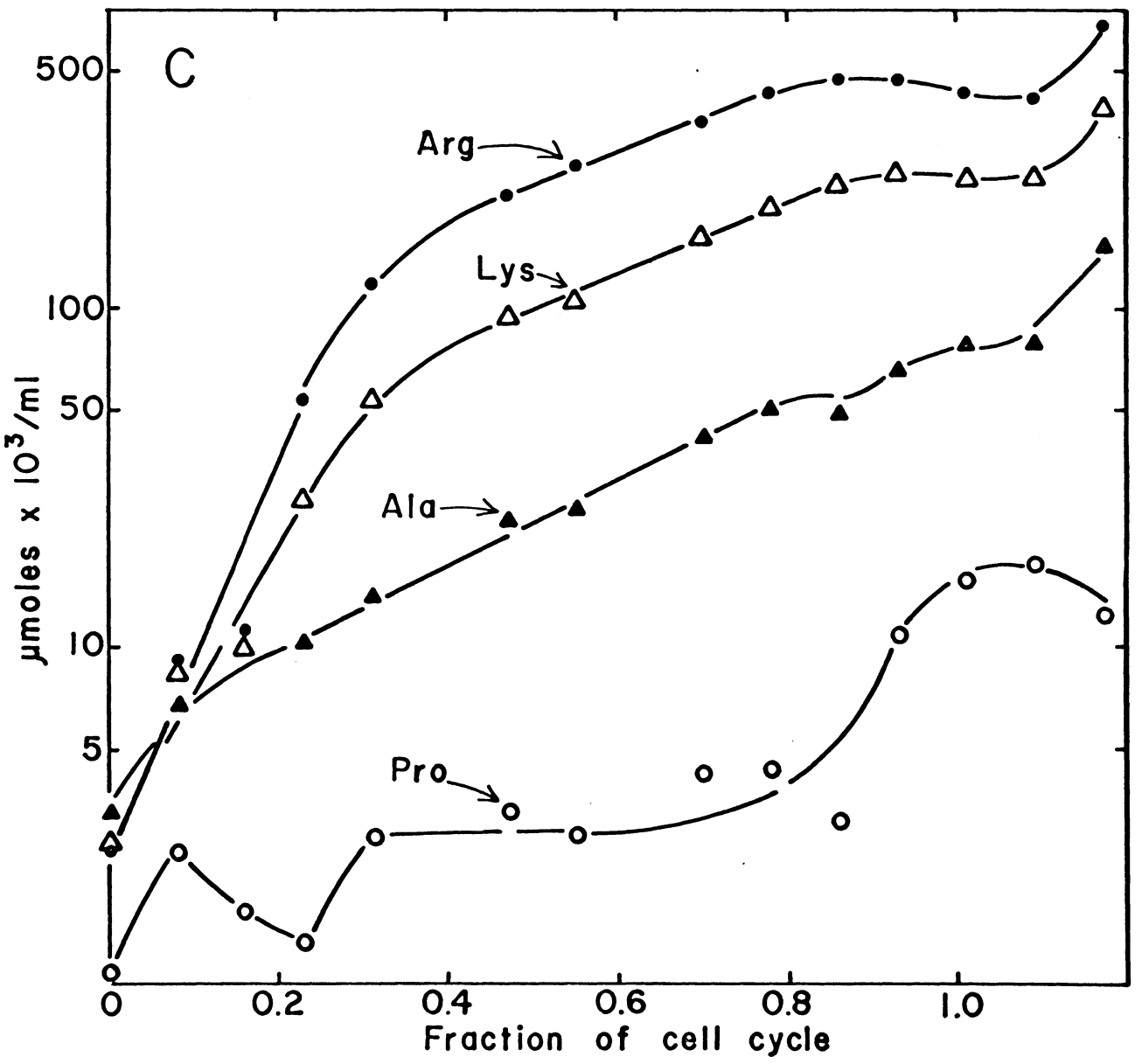
The levels of the peptide amino acids are shown on a per ml of culture basis in Fig.16-A and -B. In contrast to the accumulation patterns exhibited by the free amino acids (see Fig. 11), many of the peptide amino acids exhibited net loss during different periods of cellular development. For example, peptide methionine decreased dramatically during early cellular development. Others of particular interest are those which showed net loss prior to and during cellular division. These peptides may possibly be utilized for assembly of the mitotic apparatus or for formation of nuclear or cytoplasmic membranes during the division events.

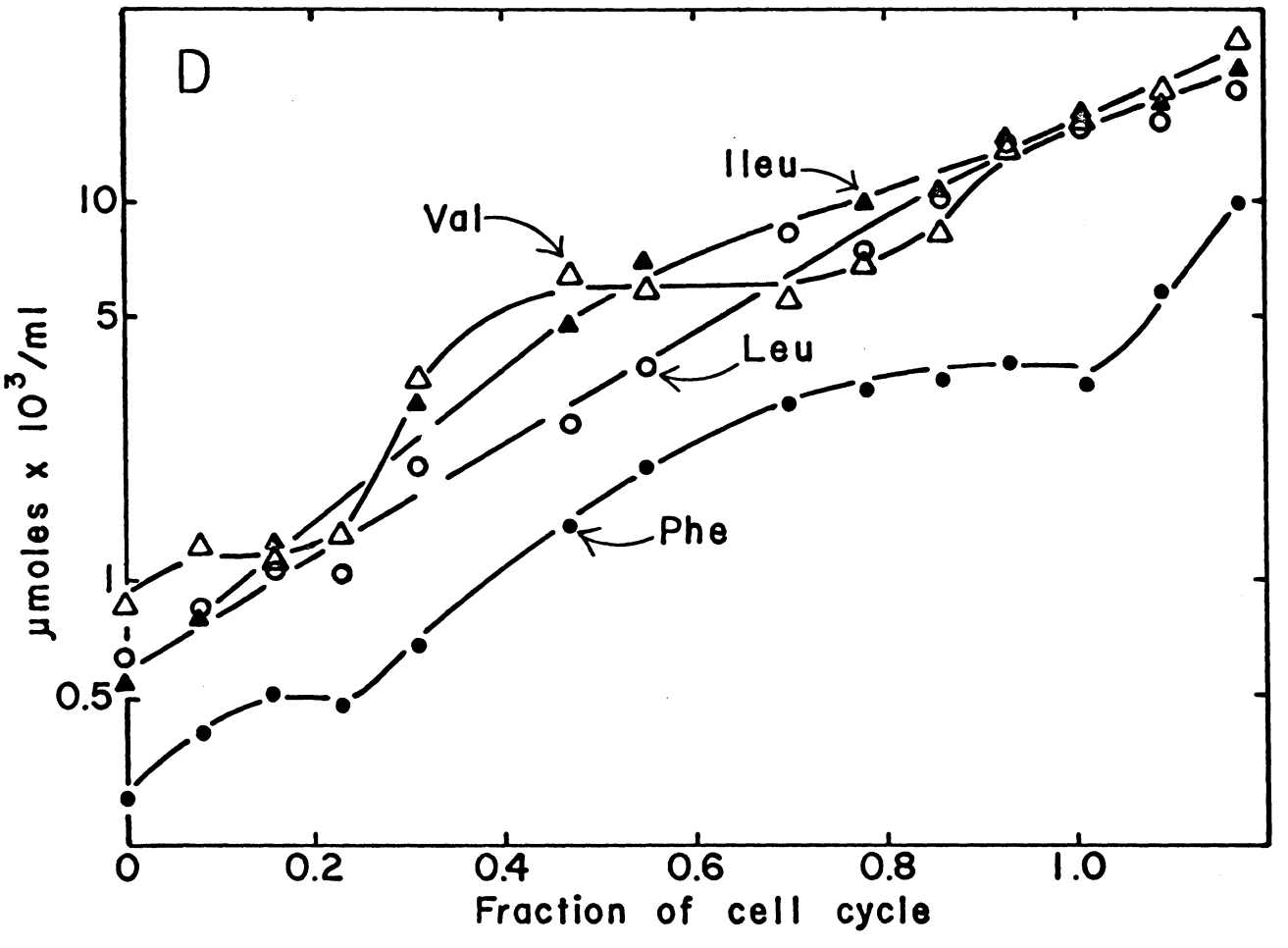
The distribution of amino acid residues was determined in peptides

Fig. 16.
Peptide amino acids per ml of culture during synchronous growth of *C. pyrenoidosa* (strain 7-11-05).


A
Glu
Gly
Asp
500
100
50
10
μmoles x 10³/ml
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


B
H Cys
His
Met
Thr
100
50
10
5
1
0.5
μmoles x 10³/ml
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


C
500
100
50
10
5
μmoles x 10³/ml
Arg
Lys
Ala
Pro
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle


D
Ileu
Val
Leu
Phe
10
5
1
0.5
μmoles x 10³/ml
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

of different sizes separated using Sephadex gel-filtration fractionation. Approximately 75% of the amino acids were components of peptides having molecular weights less than 700 (Table I); while, an average of only 2.8% of the peptide amino acids were associated with peptides having molecular weights greater than 5000 (excluded by Sephadex G-25).

Characteristics of acid-soluble-peptide.

While measuring the uptake and intracellular distribution of sulfate-^{35}S in synchronized cultures of C. pyrenoidosa, Johnson and Schmidt (9) observed that the level of acid-soluble-^{35}S was maximum (as % of total cellular-^{35}S) at 0.4 fraction of the cell cycle. These investigators also found that nearly all of the acid-soluble-^{35}S (at 0.4 fraction of the cell cycle) was contained in peptides or peptide derivatives. The present study has shown that peptide sulfur-amino-acids reached a maximum level at 0.7 fraction of the cell cycle (the onset of nuclear division).

An investigation was undertaken to characterize the predominant sulfur-containing peptides in the TCA extract of C. pyrenoidosa in cells harvested at 0.4 and at 0.7 fraction of the cell cycle. Approximately 95% of the ^{35}S in a TCA extract (from uniformly-labled cells harvested at 0.4 fraction of the cell cycle) was eluted in the first 400 fractions from an anion exchange column (Fig. 17). All major radioactive peaks yielded free amino acids after hydrolysis indicating the peptide

TABLE I

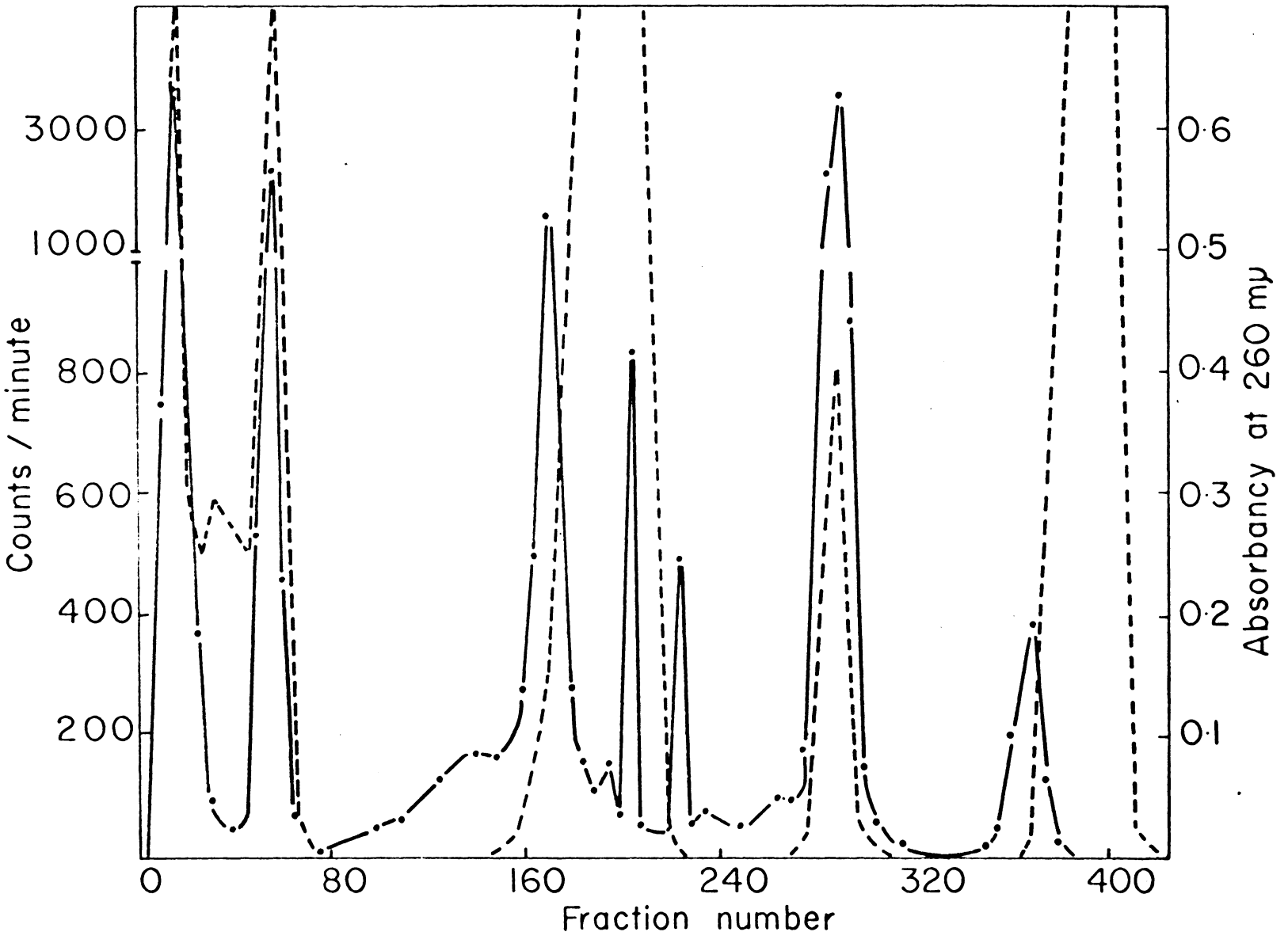
FRACTIONATION OF PEPTIDES ON THE BASIS OF MOLECULAR WEIGHT USING SEPHADEX GEL-FILTRATION

Amino Acid	Approximate Molecular Weight Range >5000	1500-5000	700-1500	<700
Lys	0.5	1.5	14	85
His	1.2	---	---	<99
Arg	0.2	0.6	13	86
Asp	1.4	3.6	5.6	89
Thr	5.3	6.9	17	71
Glu	0.3	0.9	6.9	92
Pro	11	45	35	9
Gly	0.5	2.4	13	84
Ala	3.6	7.3	11	78
H Cys	0.4	---	---	<99
Val	2.5	5.6	7.9	84
Ileu	1.4	---	---	<99
Leu	8.3	11	13	68
Tyr	2.1	---	---	<98
Phe	3.6	---	---	<96
Ave	2.8	8.5	13	75

Data expressed as % of respective peptide-amino acid content of the extract

Extract obtained from cells harvested at 0.5 fraction of the cell cycle

Fig. 17.
Elution pattern of ^{35}S-containing and ultraviolet-absorbing acid-soluble compounds from a Dowex-1-formate column. o——o , radioactivity; -----, absorbancy. Resin: Dowex-1-x4 (200-400 mesh). Linear gradient elution: 0.005N ⟶ 0.3N formic acid in 400 fractions. Fraction size: 60 drops (4-5ml).


3000
1000
800
600
400
200
Counts / minute
0·6
0·5
0·4
0·3
0·2
0·1
Absorbancy at 260 mμ
0
80
160
240
320
400
Fraction number

nature of these compounds. Some of these radioactive peptides appeared to have an ultraviolet-light-absorbing component associated with them (for example fractions 45-65 and 275-300) while others seemed to exist as free peptides (see asymmetry between absorbancy at 260 mu and radioactivity for fractions 155-185, 220-230, and 355-380). Hase and co-workers (22-26) have tentatively identified ultraviolet-absorbing peptides, obtained from the TCA extract of C. ellipsoidea, as sulfur-containing nucleotide-peptides.

When fractions 155-185 (asymmetry between ultraviolet absorption and radioactivity) were subjected to further purification by cation exchange column chromatography, the ultraviolet compounds were separated from the radioactive peptide (Fig. 18). Similar cation exchange chromatography (Fig. 19) of fractions 275-300 (from the anion exchange column) resulted in simultaneous elution of the radioactive and ultraviolet-absorbing components (note symmetry of radioactivity and absorbancy at 260 mu), indicating that these fractions contain an ultraviolet-absorbing peptide or peptide derivative. The ultraviolet-absorbing peptide exhibited a typical nucleotide absorption spectrum (A_{s-max} 272 mu) and contained half-cystine, glycine, and glutamate in an approximate ratio of 1:1:1. Furthermore, the ultraviolet-absorbing peptide readily gave a colored product after treatment with neutral hydroxylamine and $FeCl_3$, a characteristic also exhibited by carboxyl-activated amino acids (49). (Reaction with

Fig. 18.
Separation of ultraviolet-absorbing compounds from a ^{35}S-peptide on a Dowex-50 H^+ column. ●———● , radioactivity; ▲----▲ ,absorbancy. (Peptide from fractions 155-185 of Dowex-1-x4 column, see Fig. 17). Resin: Dowex-50-x8 (200-400 mesh). Linear gradient elution: 0.005 N ⟶ 1.5 N HCl in 400 fractions. Fraction size: 60 drops (4-5 ml).

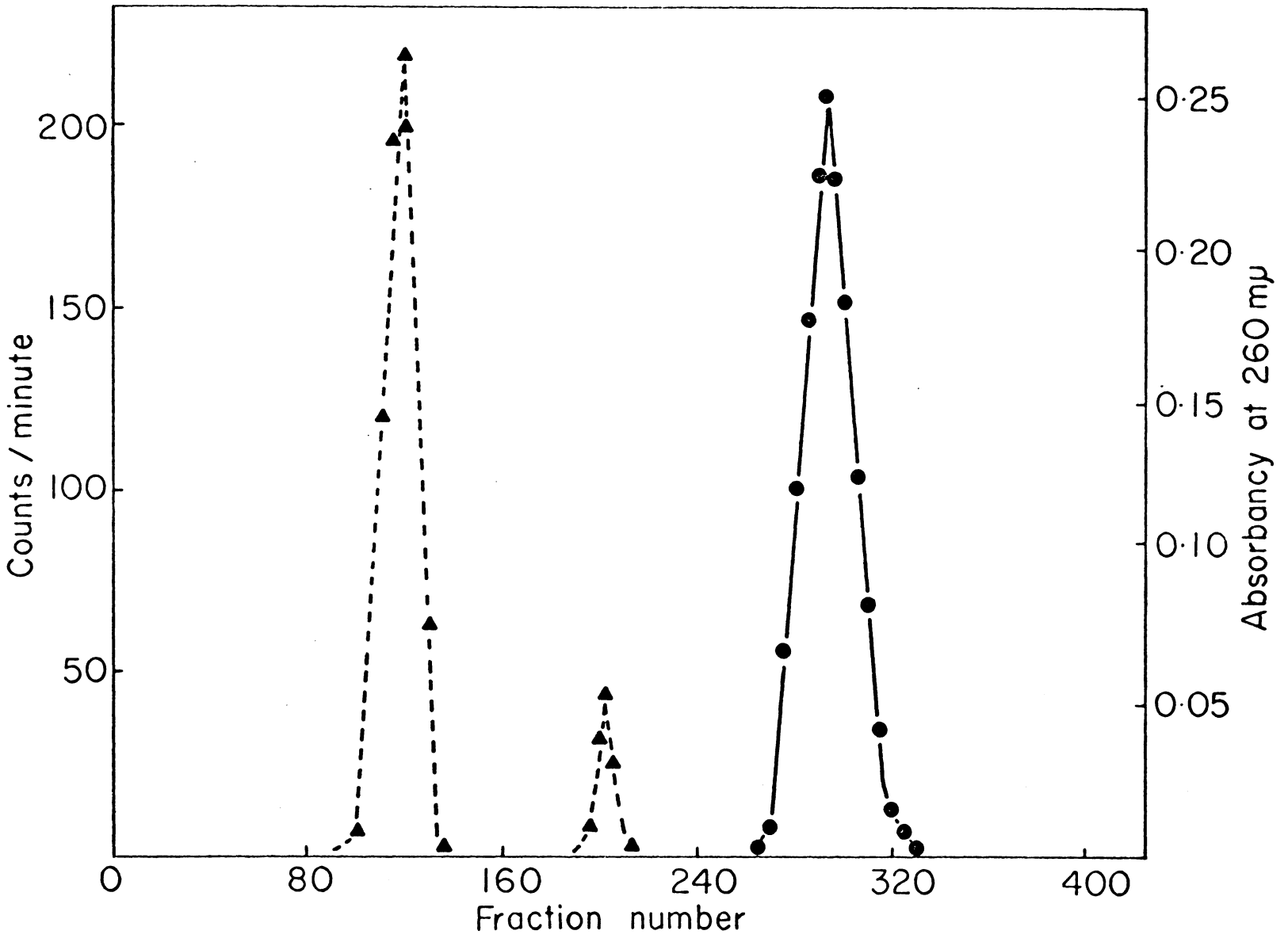
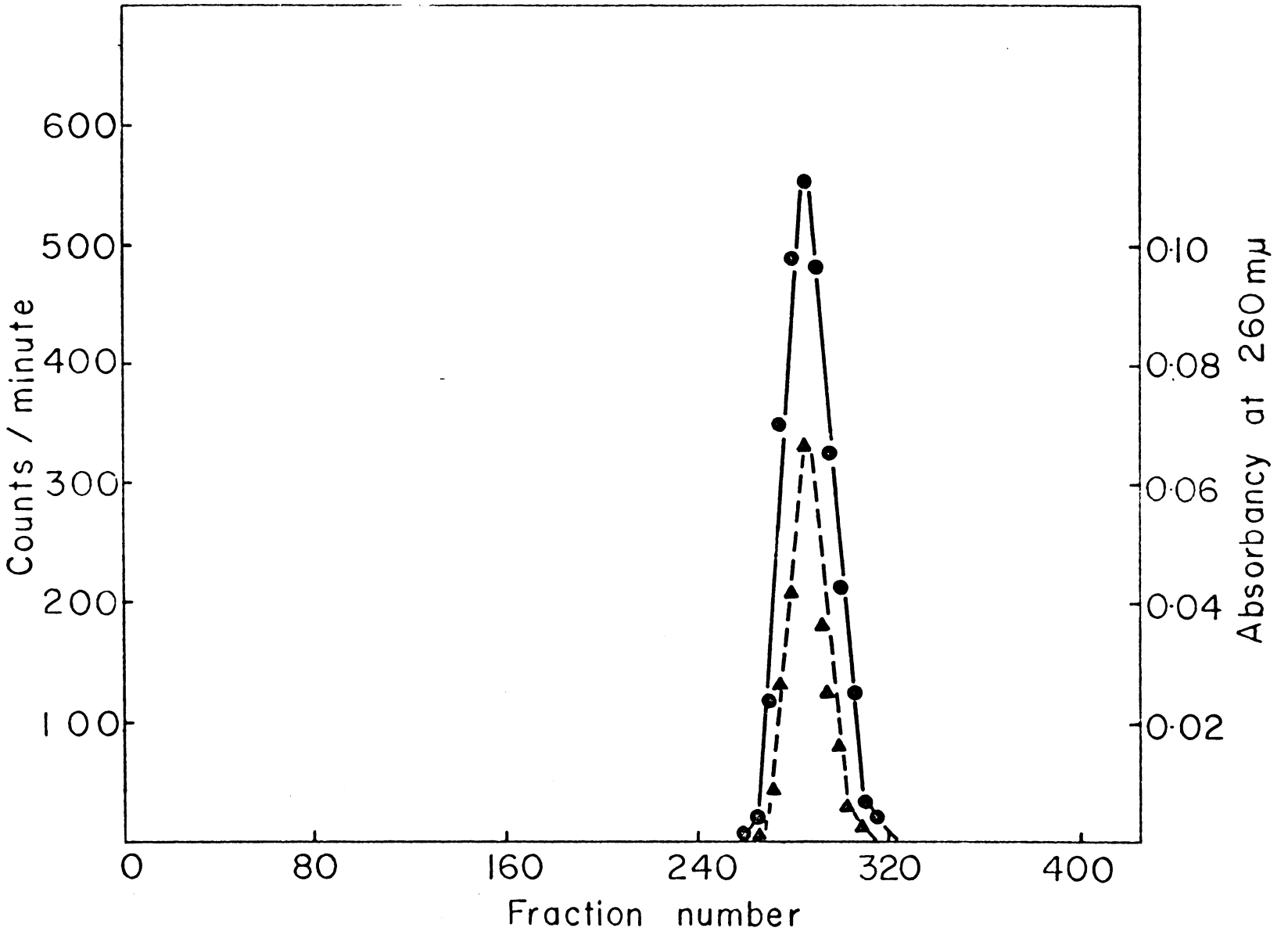

Counts / minute
200
150
100
50
0
80
160
240
320
400
Fraction number
0·25
0·20
0·15
0·10
0·05
Absorbancy at 260 mμ

Fig. 19.
Elution pattern of an ultraviolet-absorbing ^{35}S-peptide from a Dowex-50 H^+ column. o——o, radioactivity; △----△, absorbancy. (Peptide from fractions 280-300 of Dowex-1-x4 column, see Fig. 17). Resin: Dowex – 50 – x8 (200-400 mesh). Linear gradient elution: 0.005 N ⟶ 1.5 N HCl in 400 fractions. Fraction size: 60 drops (4-5 ml).


600
500
400
300
200
100
Counts / minute
0·10
0·08
0·06
0·04
0·02
Absorbancy at 260 mμ
0
80
160
240
320
400
Fraction number

hydroxylamine to form hydroxymates was reported (50) to be specific, under the conditions employed, for anhydride linkages. These hydroxymates when complexed with $FeCl_3$ yield colored reaction products, the formation of which indicates the prior existence of anhydride bonds.)

These preliminary data suggested that the ^{35}S-peptide was linked to a nucleotide through a mixed-anhydride linkage possibly between the C-terminal carboxyl group of the peptide and the phosphate of the nucleotide as reported for peptidyl-nucleotidates in yeast (19-21, 61). Also the ultraviolet-absorbing peptide appeared to have properties similar to the sulfur-containing nucleotide-peptides reported in the TCA extract of C. ellipsoidea(22-26) and the sulfur-containing nucleotides reported in the perchloric acid extract of Euglena gracilis (62).

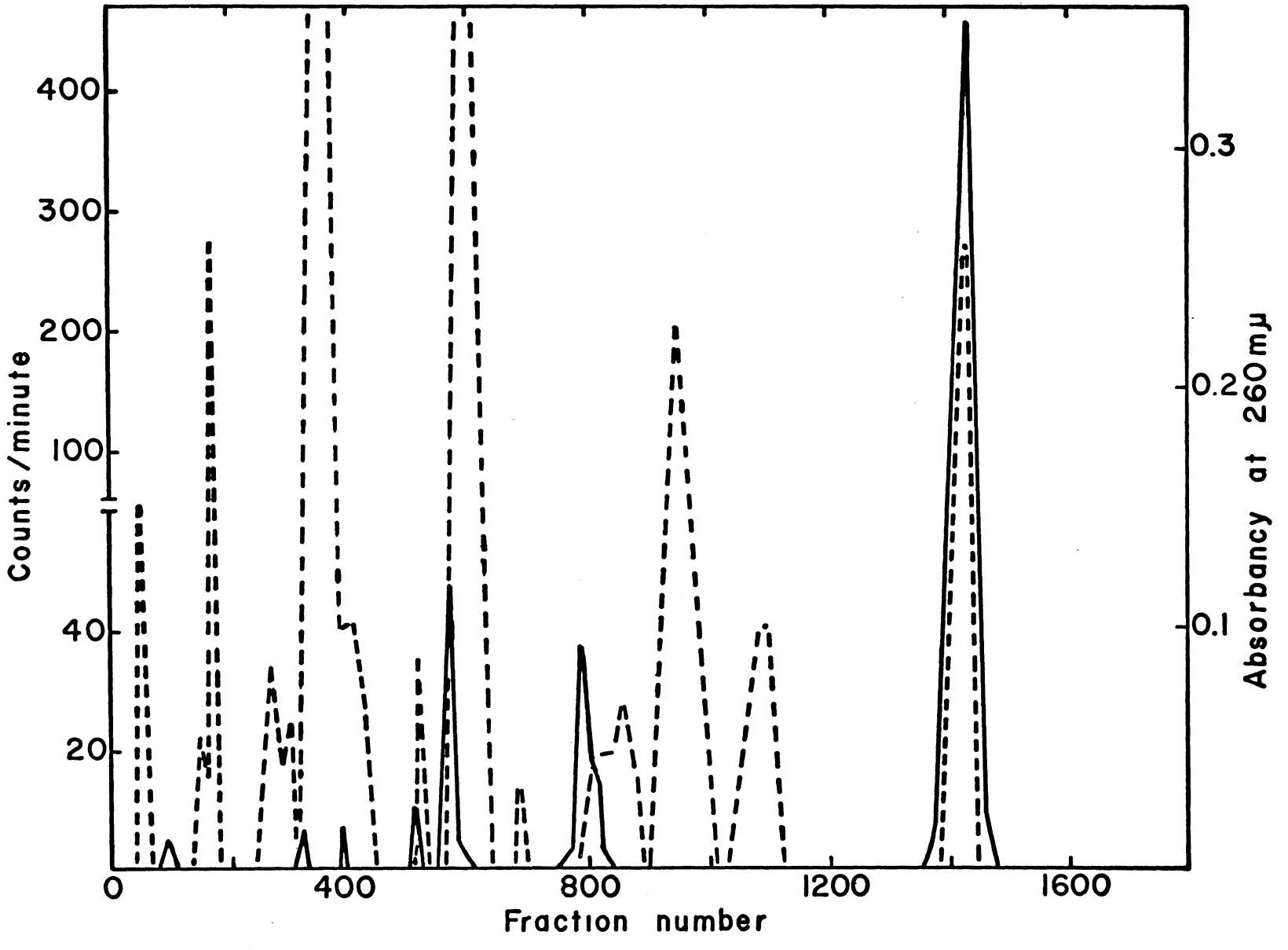
To obtain enough ultraviolet-absorbing peptide for further characterization, and to investigate the possibility of there being other ultraviolet-absorbing ^{35}S-peptides, a more concentrated (5x) extract (from uniformly labled cells harvested at both 0.4 and at 0.7 fraction of the cell cycle) was subjected to similar anion-exchange column chromatography. Those compounds significantly retarded by the column were collected as a group (corresponding to fractions 120-400 of Fig. 17) and rechromatographed under the same

conditions using more gradual gradient elution. Of the peptides eluted from the column (using the slower elution gradient),only the major ^{35}S-peptide showed symmetry between ^{35}S and ultraviolet absorption (Fig. 20). The major ^{35}S-peptide was again found to absorb ultraviolet light maximally at 272 mu and to contain glulamate, glycine, and half-cystine at relative levels of 1:1:1.

When this peptide was passed through the amino acid analyzer, it moved as a single component corresponding in elution position to reduced glutathione (GSH). Co-chromatography of the peptide and known GSH on the amino acid analyzer revealed that these peptides moved as a single component. It was subsequently found that GSH absorbs ultraviolet light maximally at 272 mu (70) and exhibits an absorption spectra identical to that of the peptide. When known GSH was subjected to the neutral hydroxylamine test, a positive reaction was noted. However, it was established that this apparantly positive test resulted from the formation of a colored complex between GSH and $FeCl_3$, rather than from a reaction between GSH and hydroxylamine.

Thus, the peptide isolated in the present study, which exhibited characteristics of a nucleotide-peptide, appeared to be GSH. To firmly establish its identity, an aliquot of the radioactive-peptide was co-recrystalized with known GSH in 50% ethanol. The ^{35}S and

Fig. 20.
Purification of an ultraviolet-absorbing ^{35}S-peptide on a Dowex-1 formate column. ————, radioactivity; -----, absorbancy. Resin: Dowex-1-x4 (200-400 mesh). Linear gradient elution: 0.005 N ⟶ 0.3 N formic acid in 1600 fractions. Fraction size: 120 drops (8-10 ml).


Counts/minute
400
300
200
100
40
20
0
400
800
1200
1600
Fraction number
0.3
0.2
0.1
Absorbancy at 260mμ

GSH content of the resulting crystals was measured (Table II). These data further establish the identity of the peptide with GSH.

Since the ion-exchange studies did not reveal the existence of nucleotide-peptides, the following experiments were undertaken to estimate the contribution (if any) of nucleotide-peptides to the pool of TCA-soluble peptides in C. pyrenoidosa. The peptide amino acid distribution, in aliquots from a TCA extract, was determined after the aliquots were treated in the following manner: A_1 received no treatment, A_2 was treated with Norit-A, B_1 was exposed to alkali but was not treated with Norit-A, B_2 received both the alkaline and the Norit-A treatment, C was treated with hydroxylamine and Norit-A.

Both ester and anhydride linkages, reported to exist between the nucleotide and the peptide moieties in nucleotide-peptides, are labile to alkaline pH (49). Likewise, hydroxylamine is reported to specifically cleave anhydride bonds at neutral pH and room temperature (50). Thus, a decrease of Norit-A adsorbable peptides after exposure to alkali or hydroxylamine should indicate the presence of nucleotide-peptides. It is apparent (Table III) that peptides were not released from Norit-A adsorbable compounds during the alkaline treatment, i. e. no difference was observed in the ratio B_1 / A_1 (before Norit-A) and the ratio B_2/A_2 (after Norit-A). The presence of nucleotide-peptides would have been indicated by values greater than 1 in column 3 of Table III.

TABLE II

CO-RECRYSTALIZATION OF AN ^{35}S-CONTAINING PEPTIDE AND REDUCED GLUTATHIONE

	A μ Moles GSH	B CPM	Relative A/B
1 st crystals	1.21	424	1.00
2 nd "	1.12	371	1.05
3 rd "	1.97	706	0.98

TABLE III

TEST FOR THE ADSORPTION OF ACID-SOLUBLE PEPTIDES TO NORIT-A BEFORE AND AFTER TREATMENT WITH ALKALI AND HYDROXYLAMINE

Amino acid	$\frac{B_1}{A_1}$	$\frac{B_2}{A_2}$	$\frac{B_2/A_2}{B_1/A_1}$	$\frac{C}{A_2}$
Lys	1.00	0.93	0.93	----
Arg	0.87	----	----	1.06
Asp	0.93	0.90	0.97	1.05
Thr	0.94	0.93	0.99	0.99
Ser	0.96	0.94	0.98	0.97
Glu	0.96	0.97	1.01	1.02
Pro	0.90	0.93	1.03	0.94
Gly	0.98	0.91	0.93	1.30
Ala	0.97	1.00	1.03	1.06
Val	0.99	0.90	0.91	1.04
Ileu	0.87	0.84	0.97	----
Leu	0.93	0.94	1.01	----
Ave			0.98	1.05

Treatments:
A_1 - no treatment
A_2 - Norit-A
B_1 - alkali
B_2 - alkali & Norit-A
C - hydroxylamine (rm. temp. & neutral pH) & Norit-A

Likewise, when the TCA extract was treated with hydroxylamine, Norit-A adsorbable compounds did not yield significant quantities of peptides (column 4, Table III). Unfortunately, the peptides in A_2 were in a KCl solution during the Norit-A treatment; whereas, those in C were in a buffered acetate, NaCl, hydroxylamine solution. Because of this difference in ionic environment, peptides in the hydroxylamine treated sample may have been adsorbed differently to Norit-A than those in the control (A_2). Furthermore, since hydroxylamine cleaves only anhydride bonds while alkali attacks both ester and anhydride linkages, it would seem that the alkaline treated samples should give a more reliable index of the nucleotide-peptide content of the extract. In any event, it appears to be unlikely that nucleotide-peptides contribute significantly to the pool of acid-extractable peptides in C. pyrenoidosa.

Interrelationship among protein-, peptide-, and free-sulfur amino acids.

GSH was the predominant ^{35}S -containing acid-soluble compound, accounting for approximately one-third of the total acid-soluble-^{35}S, in cells from 0.4 fraction of the cell cycle (Fig. 17). However, in the extract obtained from 0.4 plus 0.7 fraction of the cell cycle (50% from each period), GSH accounted for approximately two-thirds of the total acid-soluble-^{35}S. Apparently, therefore, the level of GSH at the onset of nuclear division was higher than that at 0.4 fraction of

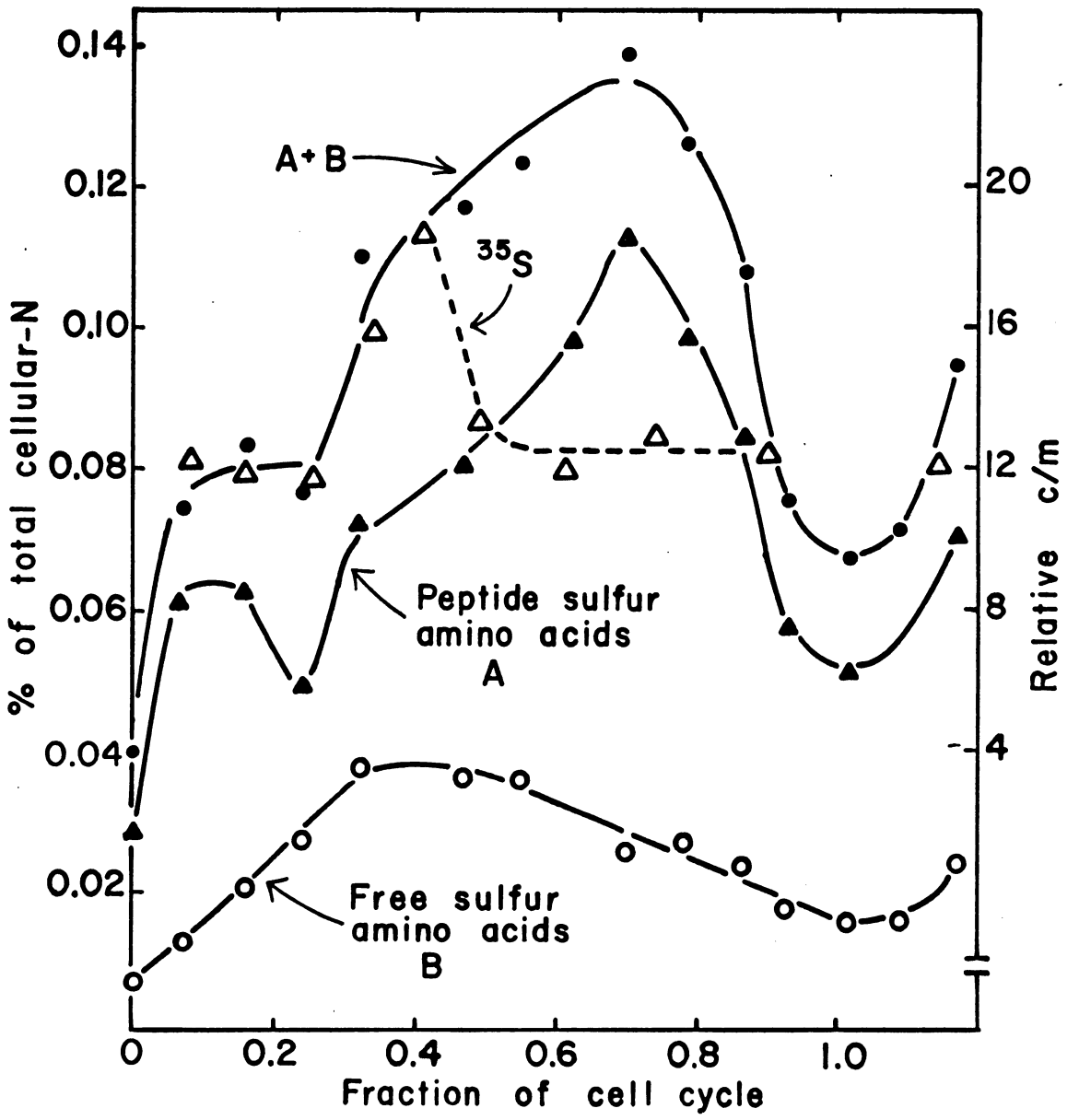
the cell cycle. In addition, since GSH was the predominant acid-soluble ^{35}S-containing compound, and also was the predominant sulfur-peptide, at both periods of cellular development, it is probable that GSH accounted, in large part, for the changes in level of peptide half-cystine (Fig. 15-F).

Dan (63) and Sakai (64) attributed the fluctuations of TCA-soluble sulfhydryl groups in synchronized sea urchin eggs, observed initially by Rapkine (7), to non-dialyzable sulfydryl protein(s). Furthermore, they reported that both reduced and oxidized glutathione remained at constant levels during the growth and division cycle of those cells. Sparkes and Walker (65), on the other hand, found that reduced and oxidized glutathione comprized 90% or more of the sulfhydryl plus disulfide content of acid extracts of Earle's L-strain of mouse fibroblast cells. Likewise, GSH has been implicated as an essential component for mitosis in plant cells. Stern (66) observed a marked increased of GSH in lily anther microspores during mitosis. Kanazawa (53) measured glutathione during synchronous growth of C. ellipsoidea and found that its level increased during early cellular development and reached a maximum prior to cellular division. The trends observed for glutathione in lily anther miscrospores and in sychronized C. ellipsoidea were similar to that observed in the present study for peptide half-cystine in C. pyrenoidosa (Fig. 15-F).

While studying the uptake of sulfate-^{35}S by C. pyrenoidosa Johnson and Schmidt (9) noted that the level of acid-soluble-^{35}S increased markedly and then returned to its initial level (as % of total cellular-^{35}S) prior to the onset of nuclear division. Their data is reproduced in Fig. 21 along with the data from the present study for total soluble sulfur amino acid (free plus peptide) as percent of total cellular-N. The above two curves coincide except during the period 0.4-0.9 fraction of the cell cycle (which includes nuclear division). The levels of total soluble-^{35}S and total soluble-sulfur-amino-acids changed similarly during early cellular development (0.0-0.4 fraction of the cell cycle), reflecting de novo synthesis of total soluble-sulfur-amino-acids from sulfate during that period. However, the rate of de novo synthesis was reduced during the period 0.4-0.5 fraction of the cell cycle, i. e. total soluble -^{35}S decreased. During the same period, the actual level of total soluble-sulfur-amino-acids continued to increase, indicating that the increase in total soluble-sulfur-amino-acids resulted from breakdown of non-labled compounds, i. e. proteins.

The levels of both total peptide sulfur amino acids and total free sulfur amino acids are also expressed in Fig. 21. During nuclear division, when the level of total soluble-^{35}S and the level of total soluble-sulfur-amino-acids show their maximum difference, total peptide-sulfur-amino-acids reached its maximum level. Apparently,

Fig. 21.
Interrelationships of sulfur-proteins, -peptides, and -free amino acids during synchronous growth of C. pyrenoidosa (strain 7-11-05).


0.14
0.12
0.10
0.08
0.06
0.04
0.02
% of total cellular-N
20
16
12
8
4
Relative c/m
A+B
35S
Peptide sulfur
amino acids
A
Free sulfur
amino acids
B
0
0.2
0.4
0.6
0.8
1.0
Fraction of cell cycle

therefore, the sulfur peptides (mostly GSH), present at high level during nuclear division, were products largely from breakdown of sulfur protein(s).

If the sulfur peptides present at the onset of nuclear division were synthesized from unlabled free-amino-acids (which in turn resulted from unlabled proteins), it is likely that the level of free sulfur amino acids would have exhibited an increase prior to nuclear division. The level of free sulfur amino acids, however, continuously decreased prior to and during the period of nuclear division. Therefore, it is probable that the increased level of sulfur peptides at the onset of nuclear division resulted directly from the breakdown of sulfur proteins. However, additional turnover studies will be required to firmly establish whether or not the unlabled sulfur proteins were first degraded to free amino acids which were then assembled into sulfur peptides.

Mazia has indicated that acid soluble sulfhydryl-disulfide compounds may serve as oxidants and reductants during formation and disassembly of the mitotic apparatus (8), respectively. Evidence from the present study together with that of Johnson and Schmidt (9) indicates that acid-soluble GSH is released from the protein fraction prior to the onset of nuclear division in C. pyrenoidosa. Since GSH appeared to be released in the form of an assembled peptide, it may have been associated with protein

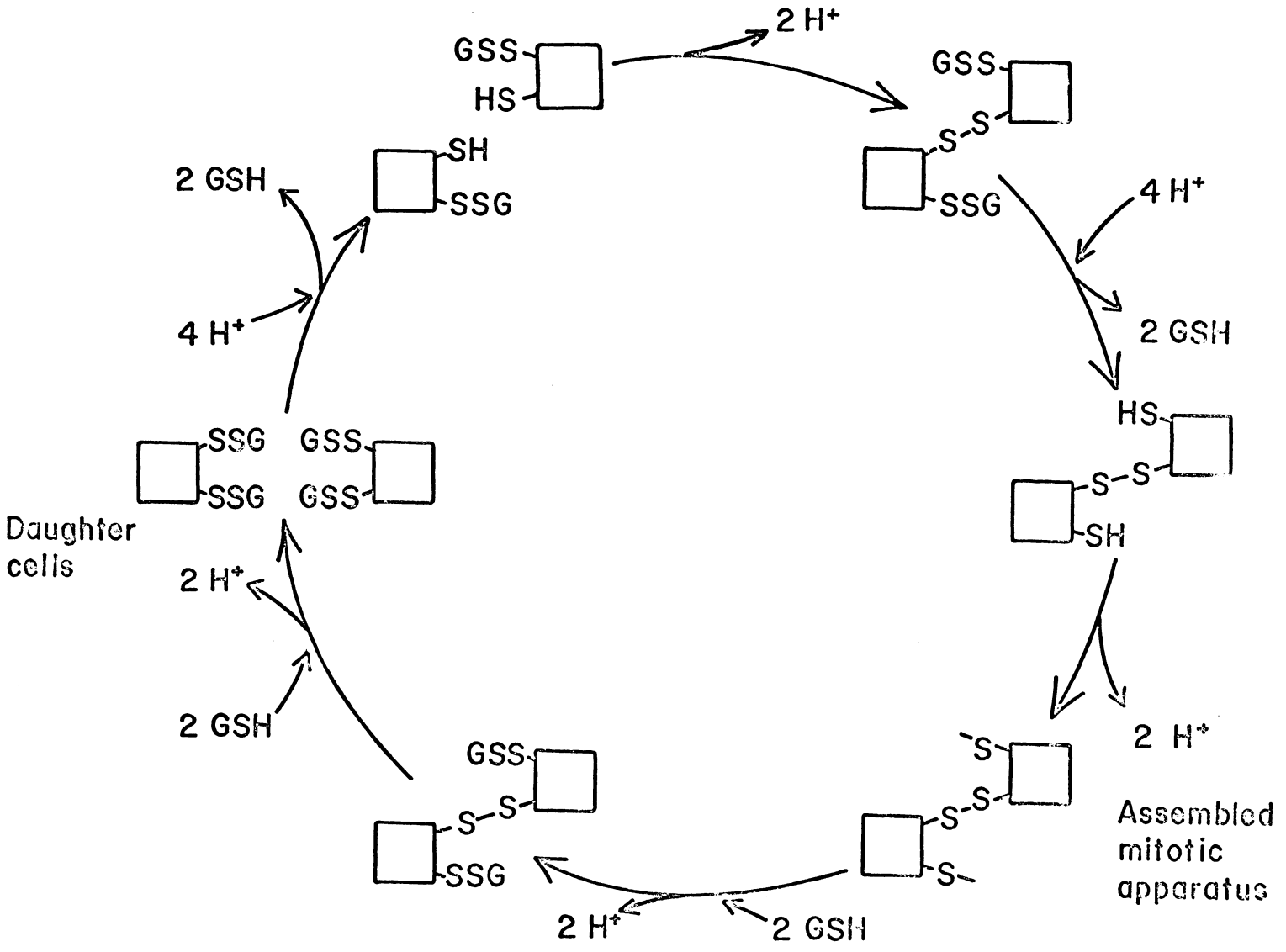
through a disulfide linkage rather than through a peptide bond.

Fig. 22 illustrates a hypothetical scheme which may account for both the origin of GSH prior to nuclear division and the proposed regulation of mitotic apparatus assembly through acid-soluble sulfhydryl compounds.

At the beginning of the cell cycle, the high molecular weight mitotic protein units are pictured as being associated with glutathione through disulfide linkages. Conversion of the protein-glutathione disulfide bonds to interprotein disulfide bonds could result in polymerization of the protein units to form the assembled mitotic apparatus (the conversion is illustrated in the figure as alternating reduction-oxidation steps). Reversal of the formation procedure would then result in the original daughter cell protein-glutathione combination.

This hypothesis is consistent with the data of the present study and that of Johnson and Schmidt (9). If ^{35}S were added to the culture at the beginning of a cell cycle, the GSH that would be released during mitotic apparatus assembly would be unlabled GSH.

Fig. 22.
A hypothetical scheme accounting for both the origin of GSH prior to nuclear division and the proposed regulation of the mitotic apparatus through acid-soluble sulfhydryl compounds.


GSS
HS
2 H+
GSS
S-S
SSG
4 H+
2 GSH
HS
S-S
SH
2 H+
Assembled mitotic apparatus
S
S-S
S
2 GSH
2 H+
GSS
S-S
SSG
SH
SSG
2 GSH
4 H+
SSG
GSS
SSG
GSS
Daughter cells
2 H+
2 GSH

SUMMARY

A continuous dilution method for the mass culture of synchronized microorganisms was developed. The new mass-culturing technique yielded approximately 3X as much cellular material as previous mass-culturing methods for *C. cyrenoidosa* in the same time period and in less than one-tenth the culture volume. Total cellular dry weight-, phosphorus-, and nitrogen-, per ml of culture increased exponentially throughout the cell cycle when the new mass culturing technique was utilized.

The levels of nitrogen in different cellular fractions were estimated during the cell cycle (i.e. lipid-plus chlorophyll-N; TCA soluble-N; individual free-, peptide-, and protein-amino acid residues; nucleotide-N; DNA-N; and RNA-N).

Protein-N accounted for approximately 60% of the total cellular-N throughout the cell cycle. In addition the amino acid distribution within the cellular protein remained nearly constant during cellular development.

The free amino acids exhibited a variety of trends in change of level during the cell cycle. The free amino acids present at relatively

high levels were alanine, lysine, serine, glycine, arginine, and glutamate.

Peptide-N increased in level (as % of total cellular-N), during early cellular development, largely because of increase in peptide-arginine, -glutamate, and -lysine. The other peptide amino acids demonstrated a variety of different trends. Peptide-N exhibited net loss (per ml of culture) during the periods of nuclear and cellular division.

RNA-N demonstrated exponential accumulation throughout the cell cycle with a reduced rate of accumulation during the period of cellular division. DNA-N on the other hand, increased during most of the cell cycle at an essentially exponential rate which was lower than the rate of increase of RNA-N. However, the rate of accumulation of DNA-N increased abruptly at the onset of nuclear division.

The present study indicates that GSH is the predominent acid-soluble sulfhydryl-containing compound throughout the cell cycle of *C. pyrenoidosa.* Its trend (as % of total cellular-N) was similar to that previously reported for total TCA-soluble sulfhydryl compounds in sea urchin eggs. Furthermore, there appeared to be *de novo* synthesis of GSH from the beginning to 0.4 fraction of the cell cycle. The continued synthesis of GSH, after this period until the onset of nuclear division, was at the expense of cellular protein. GSH also exhibited properties similar to certain compounds tentatively identified

by previous workers as sulfur-containing nucleotide-peptides.

Norit-A adsorption studies indicated that nucleotide-peptides were not present in the TCA extracts of *C. pyrenoidosa.*

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INRACELLULAR DISTRIBUTION OF NITROGEN DURING SYNCHRONOUS GROWTH OF

CHLORELLA PYRENOIDOSA

ABSTRACT

by: Theodore Arthur Hare

A continuous dilution method for the mass culture of micro-organisms was developed which yielded 3x as much cellular material as previous culturing methods for C. pyrenoidosa in the same time period and in less than one-tenth the culture volume. Cellular growth parameters increased exponentially throughout the cell cycle of C. pyrenoidosa when the new mass culturing technique was utilized.

The levels of nitrogen in different cellular fractions were estimated during the cell cycle (i.e. lipid-plus chlorophyll-N; TCA soluble-N; individual free-, peptide-, and protein-amino acid residues; nucleotide-N; DNA-N; RNA-N).

The amino acid distribution within the cellular protein remained nearly constant during cellular development. Protein-N accounted for approximately 60% of the total cellular-N throughout the cell cycle.

The free amino acids exhibited a variety of trends in change of level during the cell cycle. Free-alanine, -lysine, -serine, -glycine, -arginine, and -glutamate were present at relatively high levels.

Increase in level of peptide-N during early cellular development resulted largely from increase in levels of peptide-arginine, -glutamate, and -lysine. The other peptide amino acids exhibited a variety of different trends.

RNA-N demonstrated exponential accumulation throughout cellular development with a reduced rate of accumulation during cellular division. DNA-N increased during most of the cell cycle at a lower exponential rate; however, the rate of DNA-N accumulation increased abruptly at the onset of nuclear division.

Reduced glutathione (GSH) was found to be the predominent acid-soluble sulfhydryl-containing compound throughout the cell cycle. Its trend (as % of total cellular-N) was similar to that of TCA-soluble sulfhydryl-containing compounds in synchronized sea urchin eggs.

GSH exhibited properties similar to certain compounds tentatively identified as sulfur-containing nucleotide-peptides by previous workers. Norit-A adsorption studies indicated that nucleotide-peptides were not present in the TCA extracts of C. pyrenoidosa.

During the period 0.4-0.9 fraction of the cell cycle GSH was synthesized at the expense of cellular protein. A hypothetical scheme was presented to account for both the origin of GSH prior to nuclear division and the proposed regulation of mitotic apparatus formation through acid-soluble sulfhydryl compounds.