The Rate of Mitochondrial Protein Synthesis during Synchronized Division of *Astasia**

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ABSTRACT: Cultures of *Astasia longa* were synchronized by a repetitive temperature cycle. At various times of the cycle, cells were pulse labeled with $^{35}SO_4$ for 30 min, then rapidly chilled, and collected. A portion of the cells was used for determining the specific activity of the sulfur amino acids in the trichloroacetic acid soluble pool. About 85% of the radioactivity in the trichloroacetic acid soluble pool was in cysteine, the remainder being mostly in methionine. The specific activity of the cysteine pool at the end of a 30-min pulse increased throughout the warm period, reaching a level of about three times that obtained during a pulse in the cold period. Mitochondria were prepared from the remainder of the cells and purified by isopycnic sucrose gradient sedimentation. The purified mitochondrial fraction was extracted with trichloroacetic acid. Over 95% of the radioactivity in the trichloroacetic acid insoluble pellet was present as protein. Amino acid analysis of this pellet showed no significant change in the sulfur amino acid composition of mitochondrial protein during the warm period of the cell cycle.

From the measured specific activities of the mitochondrial protein and of the sulfur amino acid pool, the amount of newly synthesized mitochondrial protein per unit mitochondrial protein was computed for each 30-min pulse period. The results showed that the rate of mitochondrial protein synthesis was highest early in the warm period (*i.e.*, before cell division), decreased as the warm period proceeded, and continues throughout the cold period. The data, however, do not indicate any significant change in the ratio of mitochondrial protein synthesis rate to cell protein synthesis rate at any time of the cell cycle.

Recent studies have strengthened the view (Gibor and Granick, 1964; Reich and Luck, 1966) that mitochondria are self-replicating organelles. The capacity of mitochondria for growth and, in some cases at least, for division (Manton, 1959; Luck, 1963) raises many questions concerning the time and location of biosynthesis of mitochondrial components. In the case of *Micromonas*, the single mitochondrion must double in size by growth before it divides during cytokinesis (Manton, 1959). In cells containing many mitochondria, it is not known whether the rate of biosynthesis of mitochondrial protein is uniform throughout the cell cycle (regardless of whether it is synthesized intra- or extramitochondrially) or if mitochondrial protein is synthesized more rapidly at certain stages of the cell cycle. To our knowledge, no work has been reported on the rate of biosynthesis of mitochondrial protein relative to the cell cycle.

Since synchronized populations provide a source of large numbers of cells at approximately the same stage of their cell cycle, suitable labeling techniques would permit the study of the rate of biosynthesis of mitochondrial protein. *Astasia longa* can be synchronized in mass cultures by means of a repetitive temperature cycle using temperatures within the physiological growth range (Blum and Padilla, 1962). The organisms do not appreciably incorporate exogenous amino acids, but since the cells grow in a chemically defined medium with sulfate as the sole source of sulfur (except for 10^{-4} M thioglycolate), ^{35}S-labeled sulfate was chosen as a convenient marker of newly synthesized protein. In this study we have exposed synchronized cultures of *Astasia* to pulses of $^{35}SO_4$ at various times in the cell cycle and, from specific activity measurements of the mitochondrial protein, whole cell protein, and TCA[1]- soluble pool of amino acids, have computed the rate of biosynthesis of mitochondrial protein and whole cell protein at various times of the cell cycle. A preliminary account of this work has appeared (Kahn and Blum, 1966).

Experimental Section

Culture Conditions. A. longa (Jahn strain) were grown axenically in the dark in a modified Cramer–Meyer medium containing 10^{-4} M thioglycolic acid in specially designed culture vessels (Blum and Padilla, 1962), and gassed with 95% air–5% CO_2 (Van Dreal and Padilla, 1965). Cells were counted with a Coulter

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[1] Abbreviations used: TCA, trichloroacetic acid; DPNH, reduced diphosphopyridine nucleotide; ADP, adenosine diphosphate.

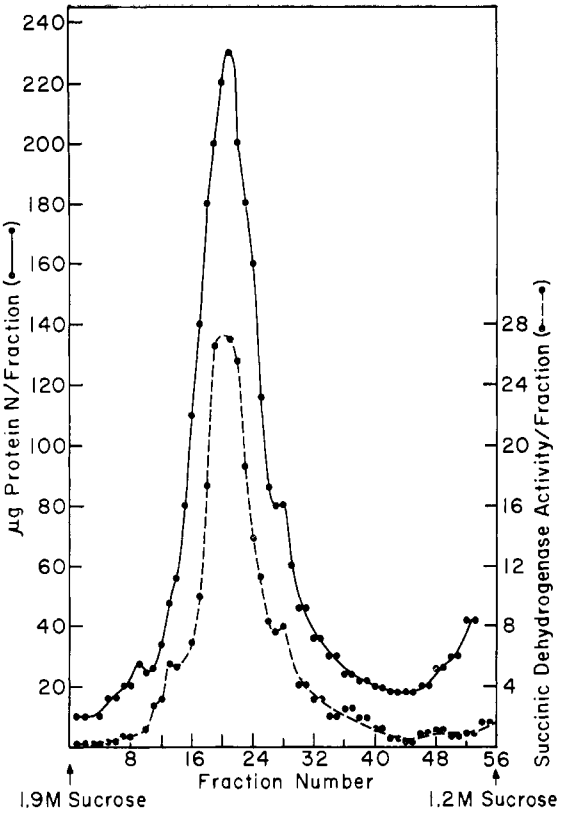

FIGURE 1: Sucrose gradient profiles of mitochondria isolated from *Astasia*. Crude mitochondria were layered on 24 ml of a linear sucrose gradient and brought to density equilibrium by centrifugation for 2 hr at 24,500 rpm. Fractions of about 0.4 ml were collected for analysis. Succinic dehydrogenase activity was assayed at 30° in a reaction mixture of 3 ml that contained: 375 μmoles of Tris, pH 7.4, 0.9 mg of neotetrazolium, 60 μmoles of succinate, and a suitable aliquot of each fraction (10–150 μg of protein nitrogen). Activities, computed from the initial rates, are shown in arbitrary units in the right-hand ordinate. The left-hand ordinate shows the amount of protein per fraction.

counter. Synchronization of cell division was achieved by use of a temperature cycle consisting of 17.5 hr at 13.0° (cold period, cycle time 0–17.5 hr) and 6.5 hr at 28.5° (warm period, cycle time 17.5–24 hr).

Growth of Cells in Radioactive Media. In experiments designed to study $^{35}SO_4$ incorporation into *Astasia*, the cells were grown in a medium containing 7.37×10^{-5} M unlabeled sulfate instead of the normal amount of 8.11×10^{-4} M, after it had been established that the degree of synchrony was not adversely affected by this reduction in sulfate concentration. At the appropriate physiological stage of growth, $Na_2{}^{35}SO_4$ (purchased from the Volk Radiochemical Co.) was added to the medium to give a specific activity of approximately 10^6 cpm/μmole (1 μc = 1.14×10^6 cpm), and incubated for 30 min. At the end of the pulse period, several operations were performed almost simultaneously. (1) Four samples containing about 10^6 cells were collected, chilled briefly, and centrifuged, and the radioactivity of the supernatant was determined as described below. The packed cells were washed three times in 5 ml of 0.02 M $MgSO_4$ and collected on 25-mm diameter Millipore filters (pore size 1.2 μ) and the filters were assayed for radioactivity as described below. (2) At the end of the 30-min pulse and immediately after the samples described above were taken, excess $MgSO_4$ and ice cubes of frozen unlabeled medium were added to the culture vessel, and water at about 4° was circulated through the stainless steel coils of the vessel. In this way the specific activity of the sulfate was reduced to 2% of its original value, thus defining the end of the pulse, and, within 5 min, the temperature of the 8–16 l. of cells was reduced below 15°. Collection of the cells by continuous flow centrifugation began immediately after the $MgSO_4$ and ice cubes were added. The packed cells were washed in 0.02 M $MgSO_4$ and about 20% of the cells were saved for extraction of the TCA-soluble pool (see below). The remaining 80% of the cells were washed in sucrose medium (0.25 M sucrose–0.024 M Tris–10^{-4} M EDTA, pH 7.4) and the mitochondrial fraction was isolated and layered onto a sucrose gradient (see below).

Isolation of Mitochondria. Cells were harvested by continuous flow centrifugation at 3000*g* in a refrigerated Servall RC2 centrifuge. All subsequent operations were also carried out in the cold. The mitochondrial fraction was isolated essentially as described by Buetow and Buchanan (1964) for *Euglena*. The packed cells were washed once in sucrose medium, 20 g of washed glass beads (Superbrite, type 100-5005, Minnesota Mining and Manufacturing Co.) were added/10^9 packed cells, and the cells were ground in a mortar for 1 min. About 50 ml of sucrose medium was then added, the resulting slurry was mixed thoroughly in a cylinder, and the glass beads were allowed to settle. The crude homogenate was centrifuged at 1000*g* for 5 min and the resulting supernatant was centrifuged at 3000*g* for 10 min to remove all cell debris, paramylon granules, etc. The supernatant was then centrifuged for 15 min at 21,000*g* and the resulting pellet was washed twice by centrifugation at 21,000*g*, using about 5 ml of sucrose medium for resuspension. The pellet was finally resuspended in 1 ml of sucrose medium with the aid of a glass homogenizer and is subsequently referred to as the crude mitochondrial fraction.

Purification of the Crude Mitochondrial Fraction. A linear gradient from 1.2 to 1.9 M sucrose in 0.024 M Tris–10^{-4} M EDTA, at pH 7.4, was prepared and 0.5–0.8 ml of the crude mitochondrial fraction layered on top of the gradient. The samples were centrifuged at 24,500 rpm in an SW25 rotor of the Spinco Model L centrifuge with the temperature set to maintain the rotor near 0°. Control experiments established that isopycnic equilibrium was essentially achieved after 1 hr of centrifugation. Prolonging the time of centrifugation to 3 hr, or centrifuging for 2 hr at 35,000 rpm in the SW39 rotor, did not affect the position of the mitochondrial band. Centrifugation for 2 hr at 24,500

rpm in the SW25 rotor was chosen for all experiments reported in this paper. It was also established that the position and shape of the mitochondrial band was constant when aliquots of the crude mitochondrial fraction containing up to 1.1 mg of protein nitrogen were layered on top of the gradient. Following centrifugation, the bottom of each tube was perforated, and 30-drop fractions were collected. After isopycnic sedimentation, fractions containing the purified mitochondrial layer (see Figure 1) were pooled, the sucrose concentration was brought to about 0.25 M by the addition of water, and the sample was centrifuged for 15 min at 21,000*g*. The pellet thus obtained (purified mitochondrial fraction) was used for further analysis as described below.

Assay Procedures. Succinic dehydrogenase activity was determined spectrophotometrically using neotetrazolium as electron acceptor as described in the legend to Figure 2. The activity was completely inhibited by malonate. Because of its simplicity and applicability to the assay of many fractions, this assay was used for the routine characterization of mitochondria collected from isopycnic gradients. Oxygen consumption was measured manometrically. Inorganic phosphate was assayed by the procedure of Taussky and Shorr (1953). Unless otherwise indicated, protein was determined by the method of Lowry *et al.* (1951), but allowing overnight alkaline digestion of the samples at room temperature before addition of the phenol reagent. When the protein concentration of the TCA-insoluble whole cell pellet (see below) was determined, the assay mixture was briefly centrifuged before the absorbance was measured in order to remove turbidity probably owing to paramylon granules.

Radioactive Measurements. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer. Aqueous samples (usually 0.05 ml) were counted in glass vials containing 10 ml of a scintillation mixture whose composition has been specified elsewhere (Kahn and Blum, 1965) except that 0.5 ml of Hyamine hydroxide was added in order to avoid apparent loss of radioactivity with time, probably owing to absorption of sulfate on the surface of the glass vials. Radioactivity of samples collected onto Millipore filters was determined in the same scintillation mixture but without Hyamine. Counts were corrected for radioactive decay when necessary. No corrections were made for any different efficiencies of counting of aqueous samples as compared to samples counted on Millipore filters.

Distribution of Counts in Whole Cells. After a pulse was terminated by the addition of $MgSO_4$ and chilling, about 20% of the cells collected was washed in $MgSO_4$ as described above. These cells were then extracted three times for 30 min with cold 5% TCA with stirring. The pooled supernatant was centrifuged at 5000*g* and the pellet (TCA-insoluble whole cell pellet) was saved for further analysis.

Amino Acid Analysis of the TCA-Soluble Pool of Whole Cells. TCA was removed by ether extraction and the ether was removed by flushing with nitrogen.

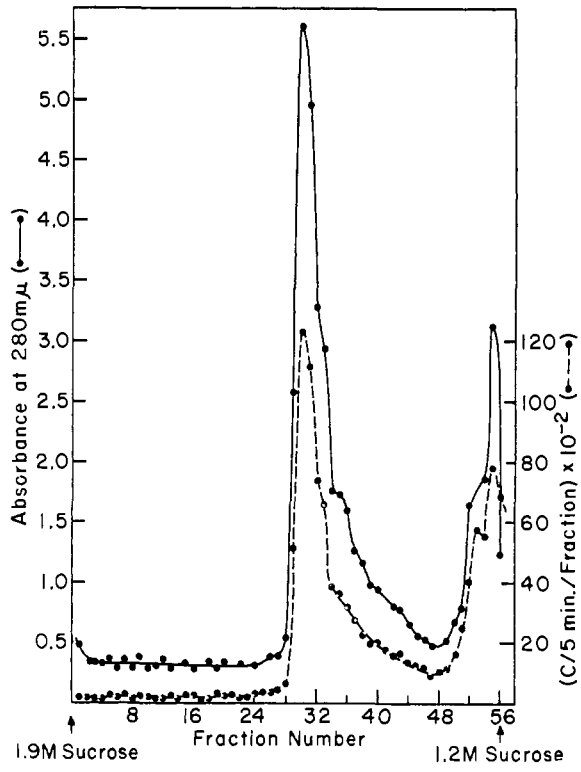

FIGURE 2: Distribution of radioactivity in mitochondria obtained from *Astasia* exposed to $^{35}SO_4$. Cells were pulse labeled with $^{35}SO_4$ (sp act. 2.44 × 10^6 cpm/μmole) for 30 min and the crude mitochondria fraction purified on a sucrose gradient as described in the legend to Figure 1. The right-hand ordinate shows the counts per fraction. The left-hand ordinate shows the absorbancy at 280 mμ/fraction, obtained from absorbancy measurements of suitable dilutions.

The pH was adjusted to about 3 and the sample was applied on a Dowex-1-formate (Bio-Rad, AG1 × 4, 200–400 mesh) resin column (1 × 50 cm). The column was washed with water and the eluent (about 150–200 ml), containing the sulfur amino acids, was lyophilized and then oxidized with performic acid (Moore, 1963). The oxidized sample was hydrolysed *in vacuo* for 24 hr in 6 N HCl at 100°. The hydrolysate was evaporated to dryness, dissolved in 0.2 N citrate buffer, pH 3.25, applied onto the long column of a Beckman amino acid analyzer, and eluted with citrate buffer (Spackman *et al.*, 1958). The eluate was not led into the coil for reaction with ninhydrin, but instead was collected in a fraction collector. Aliquots of 0.1 ml were taken from those fractions known to contain methionine sulfone and cysteic acid and their radioactivity was measured. The tubes containing the bulk of the radioactivity were pooled and further purified by ascending chromatography on Whatman No. 3 paper developed with a solvent consisting of *t*-butyl alcohol–88% (w/v) formic acid–water (14:3:3, v/v). Following development, a narrow longitudinal test

strip was cut from the paper and sprayed with ninhydrin, and the radioactivity was measured. The amount of methionine sulfone was found to be too small for further analysis. About 10–20% of the cysteic acid fraction consisted of a ninhydrin-positive contaminant which was separated by the paper chromatography step. Only the area corresponding to cysteic acid (R_F 0.1) was radioactive. This area was cut out and eluted with water, and the recovered material was concentrated *in vacuo* to a small volume. One portion of the purified cysteic acid sample was then assayed with ninhydrin in the amino acid analyzer and a second portion was assayed for radioactivity. The specific activity of cysteic acid could then be computed. Although the measured quantities were cysteic acid and methionine sulfone, we shall frequently refer to these quantities as cysteine and methionine.

Specific Activity of the TCA-Insoluble Whole Cell Pellet. The TCA-insoluble whole cell pellet was washed with water and homogenized. To determine the specific activity (counts per minute per microgram of protein nitrogen), aliquots were used for protein determination and other aliquots put onto Millipore filters for measurement of radioactivity. To determine the distribution of ^{35}S in the TCA-insoluble whole cell pellet, it was further fractionated. The pellet was first hydrolyzed in 3 ml of 1 N NaOH at 36° for 90 min and then reprecipitated with 5% TCA. DNA in the resulting precipitate was then hydrolyzed by heating at 90° for 15 min in 10% TCA. After centrifugation, the resulting residue was washed in 5% TCA. The lipids were removed by extraction with 50% ethanol in water at 60° followed by two extractions with ethyl ether–ethanol (7:3, v/v). The remaining residue was washed once with water and its specific activity was determined by measuring the protein content and radioactivity of suitable aliquots.

Analysis of the Purified Mitochondrial Fraction. The purified mitochondrial pellet obtained after sucrose gradient sedimentation was extracted three times with cold 5% TCA, washed in water, and homogenized by hand in a glass homogenizer. An aliquot of the TCA-insoluble mitochondrial suspension was oxidized with performic acid and hydrolyzed *in vacuo* in 6 N HCl, and cysteic acid and methionine sulfone were assayed on an amino acid analyzer as described above for the TCA-soluble pool of whole cells.

To determine the distribution of ^{35}S in the TCA-insoluble mitochondrial pellet, an aliquot was hydrolyzed in 1 N NaOH at 40° for 2 hr, and the protein was then reprecipitated in 5% TCA. Lipids were extracted first with acetone and then with ethanol–ethyl ether (2:1, v/v). Radioactivity and protein measurements were made at each step.

In a separate experiment, the distribution of labeled sulfur in various classes of proteins of the purified mitochondrial fraction was determined as follows. The purified mitochondrial fraction was first extracted with 0.12 M KCl for 5 min at 30° and then centrifuged at 21,000*g* for 15 min. The supernatant was saved and the pellet was extracted with 0.6 M KCl at 30° for 5 min and then centrifuged as above. The proteins in each KCl supernatant and in each pellet were precipitated with 5% TCA, washed three times with 5% TCA, and then once with water. The washed protein pellets were homogenized and aliquots taken for protein determination and put onto Millipore filters for radioactivity assay.

Results

Properties of Astasia Mitochondria. Some properties of mitochondria isolated from *Euglena gracilis* and from the closely related organism *A. longa* have recently been reported (Buetow and Buchanan, 1965; Webster and Hackett, 1965). As shown in Table I, the crude mitochondrial fraction of *Astasia* isolated by us can oxidize succinate, malate, α-ketoglutarate, and DPNH, with succinate the most rapidly utilized substrate. The magnitudes of Q_{O_2} reported in Table I are comparable to those reported for the mitochondria of *Euglena* (Buetow and Buchanan, 1965) and of yeast (Heick and Stewart, 1965). No oxygen consumption was detected in the absence of added substrate. Higher rates of respiration were found in the crude mitochondrial pellet obtained by centrifugation at 21,000*g* for 15 min than by centrifugation

TABLE I: Oxygen Consumption by Mitochondria Isolated from *Astasia*.[a]

Prepn of Crude Mitochondria	Substrate	μl of O_2/hr mg of Protein N
(A) 10 min at 10,000*g*	Succinate	220
	α-Ketoglutarate	40
	L-Malate	10
(B) 15 min at 21,000*g*	Succinate	300
	α-Ketoglutarate	150
	L-Malate	150
	DPNH	120

[a] Crude mitochondria were obtained as described in the Experimental Section, except for the last centrifugation step. In expt A, the mitochondria were assayed in a total volume of 2.2 ml containing 20 μmoles of sodium phosphate (pH 7.2), 10 μmoles of $MgCl_2$, 10 μmoles of NaF, 2 μmoles of ADP, and 20 μmoles of the indicated substrate. In expt B, the mitochondria were assayed in a total volume of 3 ml containing 20 μmoles of sodium phosphate (pH 7.2), 20 μmoles of $MgCl_2$, 40 μmoles of NaF, 6 μmoles of ATP, 0.5 mg of yeast hexokinase, 30 μmoles of glucose, and 20 μmoles of the indicated substrate. Incubation was at 30° for 20–40 min. Each vessel contained from 100 to 400 μg of protein N.

TABLE II: Distribution of Radioactivity in Mitochondria.[a]

Material	Treatment	% ^{35}S Counts	Sp Act. (cpm/μg of protein N)
(A) Purified mitochondrial fraction	None	100	55.6
	0.12 M KCl		
	(1) Soluble protein	8	43.8
	(2) Insoluble protein	92	55.2
	0.60 M KCl		
	(2a) Soluble protein	7	66.0
	(2b) Insoluble protein	85	41.4
(B) TCA-insoluble pellet of purified mitochondria	None	100	8.84
	(1) Hydrolysis in NaOH	1	—
	(2) Lipid extraction	3	—
	(3) Remaining TCA-insoluble pellet	96	9.70

[a] Cells were pulse labeled with $^{35}SO_4$ for 30 min at specific activities of 2.7×10^6 and 0.65×10^6 cpm/μmole for expt A and B, respectively. Total sulfate accumulated at the end of 30-min pulse was 2.4 μmoles of $SO_4^{2-}/1 \times 10^9$ cells and 0.77 μmole of $SO_4^{2-}/1 \times 10^9$ cells for expt A and B, respectively.

at 10,000*g* for 10 min (Table I); thus, the former conditions were used routinely (see Methods). Electron microscopic examination of the crude mitochondrial fraction (courtesy of Dr. J. R. Sommer) showed the presence of apparently intact mitochondria similar in appearance to those observed in electron micrographs of whole *Astasia* cells. No disappearance of inorganic phosphate was observed with either the 10,000*g* or the 21,000*g* pellet under the experimental conditions described in Table I or with several modifications thereof.

When the crude mitochondrial fraction was subjected to sedimentation in a sucrose gradient until density equilibrium was attained, the pattern of distribution of protein and of succinic dehydrogenase activity showed that the majority of the enzymatic activity occurred in a sharp band corresponding to the majority of the protein (Figure 1). Comparison of the position of the peak observed in the experiment shown in Figure 1 with that shown in Figure 2 shows that the absolute position of the peak was not the same in all experiments. The variations are probably owing to variations in the preparation and collection of the gradients. Since there was no correlation between the position of the peak and the physiological state of the culture (*i.e.*, log growth, warm or cold period of synchronized cultures), the causes of these small fluctuations were not investigated. In all of the experiments reported in Tables III and IV, the patterns were like that of Figure 2. The purified mitochondrial fraction consisted of, *e.g.*, tubes 29–32, but because of slight variations in the amount of impurities on the low sucrose-density side of the mitochondrial peak, the cut-off point was somewhat arbitrary.

Cellular Distribution of Radioactivity. The rate of incorporation of labeled sulfate into cells grown at 25° in 7.37×10^{-5} M sulfate was about 6 μmoles/10^9 cells per hr and was linear for at least 3 hr. Following a 30-min pulse with labeled sulfate, about 70–80% of the radioactivity that had accumulated in the cells could be extracted into the TCA-soluble pool and one-half of this radioactivity could be accounted for by sulfur amino acids.

Removal of the RNA, DNA, and lipid fractions from the whole cell TCA-insoluble pellet resulted in 15–20% loss of activity, and this decrease was the same at all stages of the cell cycle. Since over 80% of the radioactivity was in protein, all subsequent analyses were done on the TCA-insoluble pellet directly, without the extraction of lipids and nucleic acids.

Mitochondria isolated from cells that had grown in the presence of labeled sulfate for 30 min were purified on a sucrose gradient and the radioactivity per fraction was determined (Figure 2). About 1% of the total radioactivity of the cell was recovered in the mitochondria. Fractions 29–32 of this preparation were pooled, the sucrose concentration was adjusted to about 0.25 M, and the purified mitochondrial fraction was recovered by centrifugation. Analysis of the purified mitochondria (Table II) showed that about 8% of the radioactivity in the mitochondria was soluble in 0.12 M KCl and an additional 7% in 0.60 M KCl. For *Astasia* mitochondria, the data (Table II) indicate that the specific activities of the mitochondrial proteins soluble in KCl was not significantly different from that of the whole mitochondrial protein, suggesting that during a 30-min pulse with $^{35}SO_4$ the various proteins had been synthesized at comparable rates. There is some evidence suggesting that mitochondrial proteins may not all be synthesized at the same sites

TABLE III: Sulfur Amino Acid Analysis and Composition of the Mitochondrial TCA-Insoluble Pellet during Synchronized Growth of *Astasia*.[b]

	Cycle Time, hr (min)			
	18 (10)–18 (40)	21–21 (30)	23 (30)–24 (00)	
A. Sulfur Amino Acid Analysis				
Cpm/μg of protein N	23.9	28.0	33.7	
^{35}S-Cysteine:^{35}S-methionine	1:1.94	1:1.88	1:1.55	
Cpm/μmole of cysteine	10,955	14,650	20,960	
Cpm/μmole of methionine	11,910	16,310	16,160	
Av cpm/μmole of *S*-AA[a]	11,430	15,480	18,560	
B. Computed Composition				
				Av
mμmoles of *S*-AA/μg of protein N	2.09	1.81	1.82	1.91
mμmoles of cysteine/μg of protein N	0.71	0.63	0.71	0.69
mμmoles of methionine/μg of protein N	1.38	1.18	1.11	1.22

[a] In Table III and Table IV, *S*-AA stands for sulfur amino acids. [b] At the times indicated during the synchrony cycle, cells were pulse labeled with $^{35}SO_4$ (sp act. 1.7×10^6 cpm/μmole) for 30 min, and harvested, and mitochondria were collected and analyzed as described in the Experimental Section.

(Beattie *et al.*, 1966; Sherman *et al.*, 1964, 1966; Truman, 1964), but since we were not concerned with this question, a situation where the different protein fractions of the mitochondria had approximately equal specific activities was actually an advantage for the study of rates of mitochondrial protein synthesis at various times of the cell cycle.

About one-half of the radioactivity present in the purified mitochondrial fraction could be extracted with 5% TCA. Analysis of the remaining TCA-insoluble pellet (Table II) showed over 95% of the remaining label was present in protein. All analyses in the following experiments were made on the insoluble pellet obtained after TCA extraction.

Sulfate Incorporation at Various Times of the Cell Cycle. The data so far presented show that most of the radioactivity in the TCA-extracted pellet of the purified mitochondria was in protein, that the bulk of the radioactivity was in KCl-insoluble protein, and that the relatively small amount of KCl-soluble protein had approximately the same specific activity after a 30-min pulse as the KCl-insoluble (structural?) protein. Thus the rate of labeled sulfate incorporation into mitochondrial protein was a satisfactory criterion for the rate of increase of total mitochondrial protein. Similar considerations indicated that the incorporation of labeled sulfate into whole cell protein was an accurate reflection of the rate of protein synthesis of the cell. Measurements of sulfur amino acid incorporation into mitochondrial protein during pulses at different times of the cell cycle could be used to compute the rate of mitochondrial protein synthesis in each pulse period provided that: (a) the sulfur amino acid composition of the mitochondrial protein does not change throughout the cell cycle; and (b) the specific activity of the sulfur amino acid pool could be measured. Analyses of the sulfur amino acid composition were performed on TCA-extracted purified mitochondria isolated from cells pulsed at three times during the warm period (Table III). The ratio of counts in cysteine to counts in methionine was 1:1.94 at the beginning of the warm period and decreased to 1:1.55 at the end of the warm period (Table III, part A). A similar ratio (1:1.5) of cysteine to methionine was reported for the whole cell protein of *Euglena* (Kempner and Miller, 1965). The specific activities of the two sulfur amino acids were the same at each time of the cycle, but, on the average, increased with increased time into the warm period. Dividing the specific activity associated with the TCA pellet of the mitochondria (counts per minute per microgram of protein nitrogen) by the average specific activity of the sulfur amino acids yields the total amount of sulfur amino acids per microgram of mitochondrial protein nitrogen. Knowing the total amount of each sulfur amino acid present, and the ratio of cysteine to methionine, then gives the amount of each sulfur amino acid present per unit weight of protein. In this way one finds average values of about 0.69 mμmole of cysteine and 1.22 mμmoles of methionine/μg of mitochondrial protein nitrogen (Table III, part B). It can be concluded that there was no significant change in the sulfur amino acid composition of mitochondrial protein during the warm period. It is worth noting that although the ratio of cysteine to methionine in the mitochondrial protein was about 1:1.8, their specific activities were the same. This observation suggests that these two amino acids had the same specific activity in the TCA-soluble pool of the cell.

Further experiments were performed in which

TABLE IV: Incorporation of Sulfur Amino Acids into Mitochondria and Whole Cells during the Cell Cycle.[a]

Expt	Cycle Time, hr (min)	Total Sulfate Accumulated (mμmoles of SO_4^{2-}/10^6 Cells	Sp Act. of TCA Pellet (cpm/μg of protein N)		Sp Act. of Cysteic Acid in TCA Pool (cpm/mμmole)	Newly Synthesized *S*-Amino Acid (mμmoles of *S*-AA/μg of protein N)		Rel. Rates of Biosynthesis	
			Mitochondria	Whole Cells		Mitochondria	Whole Cells	Mitochondra	Whole Cells
I	16 (00)–16 (30)	0.56	2.78	3.24	13.0	0.214	0.249	0.46	0.58
	18 (00)–18 (30)	3.06	22.2	20.6	48.1	0.461	0.429	1.00	1.00
	21 (00)–21 (30)	4.10	—	29.2	72.5	—	0.402	—	0.94
	02 (00)–02 (30)	0.79	4.82	4.22	17.7	0.272	0.238	0.59	0.55
II	18 (10)–18 (40)	3.50	23.5		51.0	0.461	—	1.00	—
	21 (00)–21 (30)	2.93	25.0	—	96.9	0.258	—	0.56	—
	23 (30)–24 (00)	3.98	27.4	—	95.2	0.289	—	0.63	—
III	16 (20)–16 (50)	0.71	6.10	6.86	17.0	0.353	0.404	0.73	0.59
	18 (00)–18 (30)	3.46	30.5	42.7	62.8	0.486	0.681	1.00	1.00
	21 (00)–21 (30)	2.79	29.1	45.3	77.8	0.374	0.582	0.77	0.85
	23 (30)–24 (00)	3.34	32.2	52.8	97.2	0.331	0.544	0.68	0.80

[a] Cultures were grown for two synchrony cycles prior to the pulse experiment with $^{35}SO_4$. During the experiment, cell densities doubled from 80,000–100,000 to 160,000–200,000 cells/ml, as shown in Figure 3. The specific activity of sulfate in the external medium during the 30-min pulse was 1.13×10^6, 0.82×10^6, and 1.35×10^6 cpm/μmole for each pulse (*cf.* Figure 3) in expt I–III, respectively.

synchronized cultures of *Astasia* were pulsed with radioactive sulfate at several times during the synchrony cycle. It can be seen from Figure 3 that a full doubling of cell number was achieved in all three experiments, following the usual lag after the onset of the warm period. For each pulse, the specific activity of cysteic acid in the TCA-soluble pool of the cell was determined, as well as the amounts and distribution of radioactivity in whole cell and in mitochondrial protein. The data obtained from these experiments are presented in Table IV.

In data not shown here, it was found that over 85% of the radioactivity in the soluble pool of amino acids was in cysteine and 15% in methionine. In *E. gracilis* there is also about six times as much cysteine as methionine in the TCA-soluble pool (Kempner and Miller, 1965). Goodman and Schiff (1964) reported the presence of cysteine, cystine, and glutathione in the TCA-soluble pool of *E. gracilis*, but could not detect homocysteine, methionine, or *S*-adenosylmethionine. Since methionine was present in *Astasia* in relatively small amounts and was eluted (as methionine sulfone) close to a large peak of aspartic acid, the specific activity of methionine in the TCA-soluble pool could not be determined. As mentioned above, however, the data of Table III suggest that methionine had the same specific activity as cysteine. The cysteic acid could, however, be purified and its specific activity determined directly (Table IV). The specific activity of the cysteic acid pool at the end of a 30-min pulse increased throughout the warm period, reaching a level over three times that attained during a 30-min pulse in the cold period.

Knowing that the sulfur amino acid composition of mitochondrial protein (0.69 mμmole of cysteine and 1.22 mμmoles of methionine/μg of protein nitrogen), does not change significantly during the cell cycle, and having measured the specific activity of cysteic acid in the TCA-soluble pool of the cell, one can then compute the amount of cysteine newly incorporated into mitochondrial protein during each pulse. The amount of cysteine newly incorporated into whole cell protein can be similarly computed. As an example of such computation, consider the data for the pulse from 16 hr 0 min to 16.5 hr (*i.e.*, near the end of the cold period) of expt I in Table IV. There were 2.78 cpm/μg of protein nitrogen in the TCA pellet of purified mitochondria. The specific activity of cysteic acid in the TCA-soluble pool was 13.0 cpm/mμmole. Dividing these two numbers leads to the result that 0.214 mμmole of sulfur amino acids was newly incorporated/μg of mitochondrial protein nitrogen during this 30-min interval at the end of the cold period. For each experiment shown in Table IV, the data are also presented as relative rates of biosynthesis, with the rate early in the warm period (when the cells are entering prophase) taken as unity. It is evident that at the beginning of the warm period there is a burst of mito-

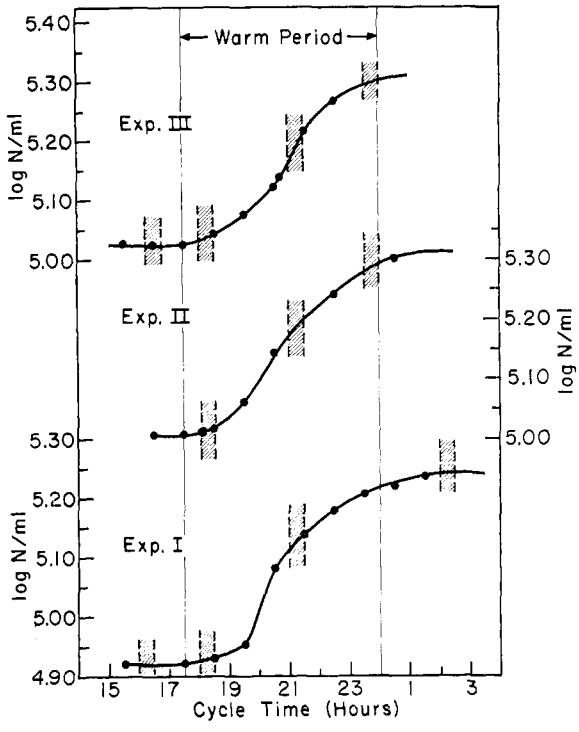

FIGURE 3: Patterns of doubling of cell number for the experiments of Table IV. Each culture had two synchrony cycles prior to conducting the pulse experiment. The time of each pulse is indicated by the dotted lines. It should be noted that the cold period extends from 0- to 17.5-hr cycle time, and that only the end of one cold period, the ensuing warm period, and the beginning of the next cold period are shown.

chondrial protein biosynthesis. Following this burst, the rate decreases throughout the warm period until it is comparable to the rate observed during the cold period. The relative rates of synthesis of total cell protein, computed from the incorporation of cysteic acid for each 30-min interval, are similar to the relative rates of synthesis of mitochondrial protein throughout the cell cycle.

Discussion

It is necessary to examine the validity of two assumptions implicit in the computations shown in Table IV before discussing the implications of these experiments. These assumptions are: (1) there is no significant turnover of mitochondrial protein during any 30-min interval; (2) there is a single pool of sulfur amino acids *in situ* from which all proteins are synthesized.

There is general agreement that the rate of protein turnover in growing organisms is very low (Mandelstam and Halvorson, 1960). Furthermore, since Luck (1963) could not detect any turnover of mitochondria in exponentially growing *Neurospora* over a period of three generations, it seems safe to assume that there was no significant turnover in the synchronized *Astasia* cells in any 30-min interval.

Several investigations have suggested the existence of more than one pool of precursors in a variety of cell types (Cowie and McClure, 1959). Mitochondria may be capable of synthesizing amino acids (Kinsey and Wagner, 1966) as well as RNA (Neubert and Helge, 1965) and DNA (Luck and Reich, 1964). In recent studies on DNA replication in the mitochondria of *Neurospora*, Reich and Luck (1966) obtained evidence suggesting that mitochondria drew on a pool of DNA precursors which was different than that used for nuclear DNA synthesis. Direct measurements of the amino acid pool of isolated mitochondria have been attempted (Das *et al.*, 1964), but such measurements are difficult to assess because of leakage from the mitochondria during isolation and purification. Even if the specific activity of the pool of amino acids in the mitochondria could be assayed satisfactorily, one could not use such data for computation of mitochondrial protein biosynthesis because of the possible extramitochondrial origin of some mitochondrial proteins (Truman, 1964; Beattie *et al.*, 1966). Since we were primarily interested in the relative rates of mitochondrial protein synthesis at various times of the cell cycle, it was only necessary to assume that if there were two pools of sulfur amino acids, the ratio of specific activities between the two pools was constant for each pulse period. The observation that the specific activities of KCl-soluble proteins of the mitochondria were approximately equal to the specific activities of the KCl-insoluble mitochondrial proteins (Table II) suggests that if there are two sulfur amino acid pools in *Astasia*, they have about the same specific activity at the end of a 30-min pulse. It is therefore safe to assume that the computations presented in Table IV accurately reflect the relative rates of biosynthesis of protein at various times of the cell cycle. Furthermore, one can show that the absolute magnitudes of the rates of mitochondrial protein biosynthesis are not unreasonable. First, we compute from the data of Table IV (using a value of 135 as the average molecular weight of cysteine and methionine) that mitochondrial protein contains about 4% by weight of sulfur amino acids. This is close to the value of 3.3% found for whole *Euglena* protein by Kempner and Miller (1965). Second, we can compute the amount of newly synthesized mitochondrial protein in a 30-min pulse. Since the cells contain about 50 μg of protein nitrogen/10^6 cells, the cells will have synthesized $50(2^{0.5/6.5} - 1)$ μg of protein nitrogen at the end of 30 min of exponential growth with a generation time of, say, 6.5 hr (*i.e.*, the duration of the warm period). Thus, in a 30-min interval, about 1.5 μg of newly synthesized protein nitrogen will have been synthesized by 10^6 cells. From Table IV, the measured amount of newly synthesized sulfur amino acid/30 min per μg of mitochondrial protein nitrogen is about 0.46 mμmole. Since 4% of the mitochondrial protein is sulfur amino acids, this amounts to 0.24 μg of newly synthesized protein

nitrogen/30 min per μg of mitochondrial protein nitrogen, or about 15% of the rate of protein synthesis of the whole cell. According to Ringo (1963), there are about 250 mitochondria/*Astasia*, each about 0.25 μ^3 in volume. The volume of an *Astasia* cell is about 1670 μ^3 (James, 1963). Thus, the mitochondria occupy about 4% of the cell volume. Under the conditions of these experiments, however, probably more than one-half of the cell volume is occupied by paramylon granules. There is, therefore, reasonable quantitative agreement between the rate of mitochondrial protein biosynthesis computed from the isotopic data and the rate which would be computed on the basis of cell mass considerations.

In a large variety of cell types, the cell cycle can be divided into four phases, commonly referred to as G_1 (pre-DNA synthesis), S (DNA synthesis), G_2 (post DNA synthesis), and M (mitosis) (see, for example, Stanners and Till (1960), and references therein). There is, however, only preliminary evidence in the literature concerning the timing of biosynthesis of cell organelles with respect to the cell cycle. At present it is impossible to state whether organelles such as mitochondria or chloroplasts grow and divide at a uniform rate throughout the cell cycle or whether organelle growth is more rapid in certain phases of the cycle. Cook (1966), studying chloroplast replication in cultures of *E. gracilis*, synchronized by a repetitive light–dark cycle, reported an increase in the number of chloroplasts per cell during the early dark period (when division occurs), and suggested that the chloroplasts divided just before cytokinesis. In their studies on changes in dry weight, cell volume, and oxygen consumption of synchronous cultures of yeast, Scopes and Williamson (1964) noted that oxygen consumption increased very rapidly following each division phase, whereas dry weight increased relatively uniformly with time. Scopes and Williamson (1964) suggested that one interpretation of their data was that the discontinuous increases in oxygen consumption were reflections of periodic mitochondrial duplication or *de novo* synthesis. In the present study, the rate of sulfur amino acid incorporation into the mitochondria of synchronized cultures of *Astasia* has provided us with a direct and sensitive assay for determining the relative rates of biosynthesis of mitochondrial protein at various times in the cell cycle. The results indicate that mitochondrial protein biosynthesis continues throughout the life cycle in this protozoan, but not at a constant rate. Early in the warm period, at a time corresponding to prophase for most of the cells, there is an increased rate of mitochondrial protein synthesis. This results in cells which have more mitochondrial protein per cell, but without counts of the number of mitochondria per cell one cannot say whether there is an increase in the number or size of the mitochondria, or both. James (1965), in his most recent studies of oxygen consumption by synchronized cultures of *Astasia*, reports a continuous increase in the rate of respiration per cell throughout the cold period, followed by a rapid increase at the beginning of the warm period. If oxygen consumption by *Astasia* is directly proportional to the mitochondrial mass, the present data on mitochondrial protein synthesis would explain the rise in oxygen consumption rate observed by James (1965). It should be pointed out that the rate of mitochondrial protein synthesis appears to be correlated with the rate of whole cell protein synthesis. It was earlier shown (Blum and Padilla, 1962), and confirmed in the present study, that whole cell protein synthesis continues in *Astasia* throughout the cold period and increases rapidly early in the warm period. In synchronized *Astasia*, therefore, it appears that mitochondrial protein is synthesized at a relatively uniform rate throughout the cell cycle except for a period corresponding to early prophase, when there is a significantly faster rate of protein synthesis.

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