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SUMMARY

A study of the incorporation of $^{14}CO_2$ into cell components of synchronously growing *Chlorella pyrenoidosa* has shown that DNA is synthesized primarily during the latter stages of the cell cycle prior to cell division.

RNA was synthesized at an approximately equal rate during each of the three phases of the cell growth studied. No major differences were noted in the incorporation of $^{14}CO_2$ into the soluble cell components in these long-term incorporation studies.

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INTRODUCTION

Shifts in the metabolism of individual cells presumably are associated with the various morphological and physiological changes that occur during the development of these cells. In investigations on the sequence of metabolic changes underlying the cyclic processes of cell growth and division, the nucleic acids are of special interest because of their functions in cell growth and reproduction. Incorporation of $^{14}CO_2$ in different stages of the synchronous growth of Chlorella cells has been used as a method to investigate possible metabolic differences related to different stages of the life cycle of the cell[1]. We have used the steady-state apparatus described by BASSHAM[2] to carry out experiments on the long-term incorporation of $^{14}CO_2$ to label the so-called insoluble material of the cells. This insoluble material, particularly the nucleic acids, has been analyzed by a fractionation procedure previously described[3]. Rates of synthesis or of turnover of RNA were estimated by determining the amount of radioactivity incorporated and the specific activity as a function of time. The ^{14}C activity incorporated into the bases of RNA has been determined after hydrolysis and their separation on paper chromatograms. The amount of ^{14}C appearing in the thymine of the DNA fraction was taken as a measurement of DNA synthesis.

METHODS

Synchronous growth

The culture of *Chlorella pyrenoidosa* was grown in the continuous-culture apparatus described by HOLM-HANSEN *et al.*[4] without the automatic dilution device. The culture medium had the following composition: KNO_3, 5.0 g; $MgSO_4 \cdot 7\ H_2O$, 2.5 g; KH_2PO_4, 1.1 g; $Ca(NO_3)_2$, 0.02 g; Fe-Versen-ol[5], 1 ml; modified Arnon's A-4 solution, 1 ml; distilled water to make 1 liter[6].

The synchronization of the cells was brought about by regular light and dark changes[7-10]. Full synchronization of the culture was induced by using a temperature of 25°, and alternating 18 h light and 18 h dark periods. The initial culture was started by inoculating the tube with a sample of cells from a stock culture in a shaker flask growing at a light intensity of 13,000 lux under continuous illumination. The initial algae population in the tube of the continuous-culture apparatus had a density of about $20 \cdot 10^6$ cells/ml (0.1%) and was exposed to eight 20 watt fluorescent lights yielding a constant light intensity of 12,000 lux. At the beginning of each light phase the culture was diluted manually to the initial density.

The continuous sterility of the culture was checked by inoculating an agar plate each time a sample was taken from the tube. No bacterial contamination was observed in the culture used for the experiments reported here.

The synchronous growth of the culture was followed by determining the cell number per unit volume in a haematocytometer and by measuring the diameter of 200 cells with an ocular micrometer each time a sample was taken, and at the ends of the light and dark phases.

$^{14}CO_2$ - incorporation experiments

In the long-time incorporation experiments, varying between 1 h and 27 h incorporation, the algae were supplied with $^{14}CO_2$ in the steady-state apparatus described by BASSHAM[2]. At the desired time, samples of the synchronously growing culture were removed from the continuous-culture tube and centrifuged and the algae were resuspended in fresh nutrient solution of the same composition at a 0.5% concentration (volume wet packed cells/volume). Subsequently, 70-80 ml of this suspension was transferred into the algae chamber of the steady-state apparatus.

The algae chamber was illuminated from both sides by photospot lamps (G.E. RSP 2) with a light intensity of 25,000 lux. The temperature in the algae suspension was maintained at 25° by circulating water from a water bath. The density of the culture was measured in a side loop of the algae chamber by a photocell and the culture was diluted with nutrient solution to the original value whenever the density increased by approximately 8% over the original density.

A stream of gas (2% CO_2 in air initially) was circulated through the whole closed system of the apparatus, and bubbled at a constant rate through the algae suspension. $^{14}CO_2$ was added to this gas by turning the stopcock of a loop containing the desired amount of $^{14}CO_2$ (an average 5 mC for 4 h incorporation). After the gas bubbled through the algae, it passed through instruments which measure CO_2, ^{14}C, and O_2. Each measurement was automatically recorded. The growth chamber and measuring instruments can be isolated from the larger part of the system, termed the reservoir, by turning a stopcock. In this way rate measurements of CO_2 and $^{14}CO_2$ uptake and O_2 evolution can be made by observing the rate of change of their concentrations on the recorder. From the known sensitivities

of these instruments and the volume of the system, one can calculate rates of gas exchange, specific radioactivity, and--by assuming an average carbon composition of 52.3%--the increase in substance[2]. The increase in cell material was also calculated from the total $^{14}CO_2$ utilized and its specific activity. Such measurements were made every hour. All calculations involving ^{14}C uptake and specific activity have been normalized for $^{14}CO_2$ of a specific activity of 1 μC/μmole. The actual specific activity used, however, was 2.0 to 3.3 μC/μmole.

At the end of the desired time interval, a 30 ml sample of the algae suspension was taken from the algae chamber, centrifuged, and, after decanting the supernatant nutrient solution, resuspended in 1 ml distilled water and killed by adding 4 ml boiling ethanol. The time between taking the sample and killing the algae varied between 10 and 12 min.

Fractionation of the samples

Since in Experiments I and II not all the samples could be analyzed simultaneously, the three samples with the same incorporation time but of different stages of the cell cycle have been worked up together.

The cells were homogenized in a Kontes Duall-type all-glass homogenizer and the fractionation procedure for the insoluble material of *Chlorella* cells described earlier[3] was followed. After the removal of the ethanol-, water-, and ether-soluble materials, the RNA and DNA were degraded to soluble components with ribonuclease and deoxyribonuclease, respectively.

The extraction procedure consisted of six extractions each with 5 ml boiling 80% ethanol, two extractions with 5 ml 20% ethanol heated 5 min at 40°, three extractions with 5 ml water heated 5 min at 70°. The residue was treated for 3 h with 3 mg ribonuclease (Armour, crystallized) dissolved in 3 ml of distilled water at 37° to yield the RNA-1 fraction.

Then the suspension was centrifuged and the residue was washed with 5 ml of distilled water. This treatment with ribonuclease was repeated in the same way twice. For the last wash 0.005 M $MgSO_4$ was used. Following treatment with ribonuclease, the residue was digested with 0.4 mg deoxyribonuclease (Worthington Biochemical Corp., U.S.A.) in 3 ml of 0.005 M $MgSO_4$, pH 5 (0.1 M acetate buffer) for 3 h at 37° and the suspension was centrifuged afterwards. In some cases, the digestion with deoxyribonuclease was repeated twice in the same way. To exclude any effects through contamination of the samples by bacteria, one drop of toluene was added to the enzymic digestions.

To determine the radioactivity of the total suspension and of the single fractions, small aliquots were dried onto aluminum planchettes and counted with a Mylar-window Scott-type Geiger-Muller tube connected to a scaler.

Analysis of the nucleic acid fractions

The nucleic acid content of the nucleic acid fractions was determined by measuring the uv absorption in a Beckman spectrophotometer. The amount of RNA present (in mg) in each RNA fraction was calculated from the 260 mμ extinction, using an absorbancy of 34.4 for the nucleotides obtained from a 0.1% RNA solution. This extinction was calculated from the known extinction coefficients of the nucleotides[11], and it was assumed that the RNA had the following base ratio: guanylic acid, 0.30; adenylic acid, 0.23; cytidylic acid, 0.255; and uridylic acid, 0.215[12]. RNA of this composition contains 35.7% carbon. If an equimolar distribution of the four bases were assumed, the absorbancy would be 29.4 and the carbon percentage would be 33.6%. The uv absorption of the 20% ethanol

and the water fractions was also checked to determine any extraction of nucleic acid in these fractions.

The nucleic acid fractions were analyzed further by paper chromatography. 100 µl of the RNA fractions was hydrolyzed by N HCl at 100° for 4 h (in some cases, 2 h). 500 µl of the DNA fractions (in some cases of the RNA fractions too) were hydrolyzed with 6 N HCl for 2 h at 120° in order to detect free thymine. These hydrolyzates had to be evaporated to dryness three times after redissolving in water twice to remove HCl which interfered with the chromatography.

The chromatograms were made on oxalic acid-washed Whatman No. 4 filter paper and were developed in phenol-water in the first dimension and in n-butanol-propionic acid-water in the second dimension. Since under these separation conditions adenine and thymine run to almost the same position, this area of the chromatogram was eluted and rechromatographed with water adjusted to pH 10 with ammonium hydroxide; this solvent system clearly separates adenine and thymine.

Radioactive substances were located by exposure of the chromatogram to Kodak single-coated x-ray film. The radioactivity of the single spots on the chromatograms was counted directly on the paper with a Mylar-window Scott-type Geiger-Muller tube. The total activity in the RNA fraction has been calculated from the sum of these counts and a conversion factor of 11.2 to convert counts to disintegrations.

RESULTS

Three experiments were carried out with $^{14}CO_2$. A schematic summary of the experimental design is given in Fig. 1. Since there is generally good agreement between the results of Experiments I and II, the results for only Experiment II are presented in Fig. 2 and Table III.

In Experiments I and II, samples were taken from the synchronous culture and transferred to the steady-state apparatus at 3, 8, and 13 h after the beginning of the light phase. The sample taken 3 h after onset of light was called "A," the sample after 8 h "B," and the sample after 13 h "C." In each case, after an initial adaptation for 15 min, $^{14}CO_2$ was added to the system. Aliquots were removed after 1, 2, and 4 h of incorporation of $^{14}CO_2$ in the steady-state apparatus. In naming these samples, the incorporation time is given as a subscript to the letter.

In Experiment III, a sample of the synchronous culture was taken at the end of the dark phase and transferred to the steady-state apparatus. The light phase was started and after 15 min of adaptation $^{14}CO_2$ was added. This sample was called "L." Aliquots were removed after 7.5 h and 15 h. The lights were then turned off, and the last aliquot was taken after 12 h in the dark or 27 h after administration of $^{14}CO_2$.

Synchronous growth

For all three experiments algae of the same culture were used. This culture was started August 3 from a continuously illuminated stock culture in a shaker flask. Experiment I was carried out August 5, in the second light phase, 36 h after the start of the culture. In this experiment the cells proved to be not yet completely synchronized--about 10% of the cells started cell division 12 h after onset of light, whereas the rest of the cells entered cell division 5 h later at the end of the light phase.

Experiment II was carried out September 27 and Experiment III September 30, after the culture had grown under regular light and dark changes for 55 and 58 days, respectively. In both these experiments the cells were growing completely synchronously; no division occurred during the light phase.

Figure 2 presents variation curves for the cell diameter obtained for the cell culture of Experiment II. Whereas the values at 0 h, 3 h, 8 h, 13 h, and 18 h were measured in samples from the continuous culture tube, the values at 7 h, 12 h, and 17 h were determined from the samples taken from the steady-state apparatus.

Most of the increase in cell size had occurred in the time between 3 and 7 h after onset of the light phase. (In Experiment I, a significant shift of the curve to the right was observed after 3 h.) There is a phase in the further course of cell development in which little or no increase of volume takes place. However, CO_2 uptake continues and the cells increase in mass. At the end of the light phase the first cell divisions in the culture can be seen. The shift of the curves for 17 h and 18 h to the right indicate a further increase in volume preceding cell division. This increase in volume preceding cell division has been observed also by SOEDER[13]. Since it occurs also in the dark, it cannot be related to the production of new substance[13], but is probably a mechanical preparation for sporulation.

We believe it is not generally possible to conclude from the overlapping of these variation curves at the beginning and the end of the light phase that the synchronization has been incomplete, as assumed by METZNER and LORENZEN[14]. The inherent variation of cell diameter in a

synchronous culture determines the shape of the distribution curve. The degree of shifting of the variation curves to the right is determined by the extent of growth of the single cells.

A better criterion for the degree of synchronization, applicable in all cases, is a comparison of the cell multiplication factor with the proportion of cells dividing into different numbers of autospores. For example, in one experiment the cell number at the beginning of the light phase was $21.5 \cdot 10^6$ cells/ml and at the end of the following dark phase $176 \cdot 10^6$ cells/ml. Thus, the factor of cell multiplication was 8.2. At the beginning of the dark phase the number of cells dividing in 4, 8, or 16 autospores was determined from 100 dividing cells: 15% of the cells divided into 4 autospores, 71% into 8 autospores, and 14% into 16 autospores. This means that a multiplication factor of 8.5 should be expected. The agreement between the multiplication factors expected and observed shows that all the cells in the culture had divided.

Photosynthetic rate

The rates of exchange of gases during the incorporation of $^{14}CO_2$ for Experiments I and II are shown in Table I. The values derived from the measurement of CO_2 pressure and ^{14}C uptake seldom differed by more than 3 to 5%. A decrease in the photosynthetic rate occurred during each incorporation period in the steady-state apparatus. In addition, a decrease of the carbon uptake was observed with progressing cell development when carbon uptake of the samples with the same incorporation time but from different stages of synchronous growth were compared. The rate for the first hour was about 20% less for cell stage C than for A.

The dry weight of algae removed with each sample from the steady-state

apparatus is listed in Table I. This has been calculated from the $^{14}CO_2$ uptake rate by assuming an average percentage carbon composition of 52% and a wet weight/dry weight ratio of 6. The carbon percentage undoubtedly changes during the development of the cells. The calculated dry weight removed shows a clear increase during each incorporation period. This result was unexpected, since it was assumed that the concentration of the culture was kept constant by keeping the density as measured by the photocell at a constant value. Apparently, changes in the composition of the cells occur during the incorporation period, which lead to a decrease in absorption of light by the same bulk of cell material.

Data in the final columns of Table I present the total μmoles of carbon (or μC of ^{14}C) incorporated into the sample removed and the percentage labeling of the total carbon of the cells. The total $^{14}CO_2$ incorporated and the growth of the cells have been derived from the change of $^{14}CO_2$ activity in the entire steady-state system during the growth period.

The cumulative growth of the culture in the steady-state apparatus during Experiment III is summarized in Table II. During 15 h photosynthesis, the cell mass increased fourfold. The photosynthetic rate during this experiment was about 1/2 of that observed in Experiments I and II.

In Experiment III, in which the culture was growing 15 h continuously in the steady-state apparatus, a yellowing of the culture and a strong accumulation of starch in the cells was observed after about 8 h of growth in the steady-state apparatus. This was in contrast to the conditions in the stock culture at the same time. These modifications of the cell composition are probably due to the high light intensity to which the algae in the steady-state apparatus have been exposed and which had

been elected to compensate for the high density of the algae suspension used for the incorporation experiments. This explanation for the increase in dry weight of algae per volume during the incorporation periods is supported by the fact that the content in RNA of the aliquots removed does not show the same increase, and shows a decrease when expressed in terms of the total algae weight (see Table IV).

Incorporation of ^{14}C into the different fractions

The incorporation and distribution of ^{14}C in several components of the cells in different stages of the cell cycle and after three incorporation times are summarized in Table III. The results are presented as μC found for the total algae suspension removed, and as percentage of the total radioactivity found in each fraction.

In these long-term incorporation experiments, shifts in the distribution of ^{14}C in the various fractions were relatively small. Significant shifts in the distribution of ^{14}C were found with different stages of cell maturation in our previous short-term studies. As expected, the biggest shift in the long-term experiments as compared with the short-term experiments is a large decrease in the percentage of ^{14}C found in the ethanol extracts from 80% in a 2-minute photosynthesis experiment to about 20% in a 4-h or 7.5-h experiment.

In Experiment III, the 27-h sample in comparison with the other two samples shows that after 12 h darkness a considerable decrease in radioactivity occurs in the water and the ethanol-ether fractions, while there is a constant percentage of radioactivity present in the 80% ethanol fraction. In addition, the total ^{14}C present had decreased about 28%.

The amount of radioactivity in the RNA-1 fraction cannot be taken as a measurement for ^{14}C incorporated into RNA, since this fraction was contaminated by labeled starch and needed further separation by paper chromatography after acid hydrolysis. In elaborating the method for fractionation of the insoluble material of Chlorella cells[3], the ^{14}C had been incorporated after one injection of only 400 μC $NaH^{14}CO_3$. Under these conditions only negligible amounts of glucose were detected on the chromatogram after acid hydrolysis of the RNA-1 fraction.

No consistent differences were noted in the residue.

Incorporation of ^{14}C into RNA

The amount of radioactivity incorporated into RNA was determined by counting the radioactivity of the separated spots of the components of RNA on the paper chromatograms of the RNA-1 fraction. These spots were identified as adenine, guanine, ribose, cytidylic acid, and uridylic acid, and as minor radioactive spots which contained cytidine, uridine, and uracil. From the sum of the radioactivity of these compounds and the amount of RNA in the fraction, the relative specific activity (or degree of labeling) in the RNA of the algae has been determined. These values for the RNA fractions, which are summarized in Fig. 3 and in Table IV, have been obtained by dividing the dpm of RNA-1 fraction by the mg of RNA of the RNA-1 fraction as determined by spectrophotometry.

It was found that appreciable but inconsistent uv absorption occurred in the 20% ethanol fraction and particularly in the water fraction. It could be shown by dialysis that at least 75% of this uv absorption was due to higher molecular weight compounds. The data in Table IV for the mg RNA and percentage RNA were obtained by totaling

the 260 mμ absorption of the three fractions. In most cases 70 to 80% of the 260 mμ absorption was in the RNA fraction and most of the remainder was in the water wash. Low values of 260 mμ absorption in the RNA-1 fraction were accompanied by high values in the corresponding 20% ethanol and water fractions. The uv absorption in the RNA-2 fraction after the second digestion by ribonuclease amounted to 5% of that in the RNA-1 fraction and was neglected. The 240/260-mμ ratio of the RNA-1 fraction was 0.48. These ratios calculated for a statistical tetranucleotide are 0.55 and 0.51 respectively, and for a base composition of 30% guanine, 23% adenine, 25.5% cytidine, and 21.5% uridine, these ratios are 0.57 and 0.53.

There is a similar linear increase in the relative specific activity of RNA from 1 h to 4 h incorporation in all three stages (A, B and C) of the cell cycle. This indicates that the rate of synthesis is uniform over the section of the cell cycle tested in Experiments I and II. At the beginning of the $^{14}CO_2$ incorporation period the precursors for the RNA are not saturated with ^{14}C and the curves, when drawn linearly, do not intercept the axes at zero time. From these curves it can be estimated that approximately 15 to 20 minutes is required to saturate these precursors. The linear increase of relative specific activity also suggests that the RNA precursors are directly derived from CO_2.

The degree of labeling of RNA has been divided by the degree of labeling of the entire culture (see Table I), and the results have been tabulated in Table IV. It is readily apparent that the degree of labeling of the RNA fractions is markedly less than the degree of labeling calculated for the entire algal culture. The comparatively low labeling of the RNA fraction can probably be explained by two factors: (1) an

appreciable time may be required for the intermediates involved in nucleic acid synthesis to become saturated with ^{14}C, and (2) under the conditions of culture in the steady-state apparatus, the RNA content as a percentage of cell weight decreased; thus the new RNA synthesized was small and the resulting degree of labeling unexpectedly low.

The RNA content per cell increased as the cell matured from the cell type characteristic of sample A to cell type characteristic of sample C under the normal synchronous culture conditions, and as a result the RNA content as a percentage of cell weight remained nearly constant (compare sample A-1, B-1, and C-1, Table IV). As has been noted above, although it was intended that the conditions of cell culture in the steady state should permit normal growth, a decrease in photosynthetic rate and a loss of pigment per unit mass was observed. In addition, a marked decrease in the percentage of RNA in the cells while in the steady-state apparatus was also observed.

In Experiment III, a total incorporation time of 15 h was used. Twice as much ^{14}C was incorporated into RNA at 7.5 h as had been incorporated at 4 h (based on dpm/mg algae, as unfortunately, no uv-absorption data were obtained for the 7.5-h sample). The relative specific activity of the 15-h sample was approximately 60% and the activity per mg algae was the same as at 7.5 h. At 15 h, the degree of labeling of the entire cell culture was calculated to be about 75%; thus the RNA was about 80% as labeled as the entire cell culture. After 12 h dark, the specific activity of the RNA fraction was the same, but the total amount of RNA had decreased by about 20% during the dark phase.

The distribution of radioactivity between the single components of RNA in different stages of the cell cycle is shown in Table V. No striking

changes were found which depended on stage of cell development or on incorporation time. The average distribution of ^{14}C is compared with the expected distribution based on: (1) RNA which is a statistical tetranucleotide, all bases equally labeled, and (2) nucleic acid of the composition reported for Scenedesmus[12].

Incorporation of ^{14}C into DNA

The amount of ^{14}C appearing in the thymine of the DNA fraction was taken as a measurement for the synthesis of DNA. Since thymine is unique to DNA, any contamination by the bases of RNA does not interfere. The results of the three experiments are summarized in Table VI.

In Experiment II labeled thymine appeared only in stage C. No labeled thymine could be detected in the stages A and B. The synthesis of DNA had occurred only in the last third of the light phase. In Experiment I small amounts of labeled thymine appeared in growth stages A and B, but in this experiment the cells were not yet completely synchronized, and about 10% of the cells were in the later stages of the growth cycle and started cell division about 12 h after the start of the light phase. The radioactivity increased with incorporation time, especially in Experiment II, stage C.

In Experiment III very little radioactivity is found in thymine of DNA after 7.5 h. The high value for 15 h shows that almost the whole synthesis of DNA had occurred in the second half of the light phase of 15 h. There has been further synthesis of DNA in the following dark phase.

The amount of DNA recovered was very low and amounted to about 0.15% of the dry weight of the cells. Therefore, no estimate could be made of the degree of labeling of the DNA.

DISCUSSION

The principal aims of this investigation were to study the incorporation of $^{14}CO_2$ into the nucleic acids of synchronously growing Chlorella cells. It was found that RNA was synthesized at an approximately equal rate during each of the three phases of the cell growth studied. RNA does not appear to turn over in the absence of net new synthesis. However, in the dark, RNA content of the cells decreases. DNA synthesis, or turnover in the absence of net synthesis, as measured by $^{14}CO_2$ incorporation into thymine, occurred only in the latter stages of the cell prior to cell division.

The dynamics of the DNA content during the cell cycle in microorganisms have been studied by using synchronously growing Escherichia coli, Corynebacterium diphtheriae, and budding yeasts[12]. It was found that the DNA content remains constant until just prior to division. IWAMURA[15], using synchronously growing Chlorella, determined the RNA and DNA content of growing cells, which were estimated by a modification of the nucleic acid extraction methods of OGAR and ROSEN[16]. IWAMURA found that RNA content per cell increased relatively uniformly while the cells were developing in the light. DNA content did not increase in the "growing phase," but a marked rise in content occurred in the "sporulation phase." Since the method used for synchronization by IWAMURA is very different from the methods we used, no comparison of the time scales for the cell development can be made.

LORENZEN and RUPPEL[17] determined the RNA and DNA content of Chlorella cells synchronized by a method similar to that which we used. RNA and DNA content were determined colorimetrically after extraction with 2 N perchloric acid at 70°. The RNA content when expressed in terms of the undiluted suspension was found to increase during the first 10 h of the light phase

and then remain constant when expressed in terms of the volume of the suspension. DNA synthesis commenced about 11 h after the beginning of the light phase. We first observed DNA synthesis 13 to 14 h after the light phase. During the period when DNA is not being synthesized, the content of DNA as a fraction of cell weight should be decreasing. We were unable to directly confirm this by our procedure.

The composition of RNA during the course of the life cycle of Chlorella cells has been studied by IWAMURA and MYERS[18]. The molar content of guanine has always been higher than that of each of the other three bases. In addition, they reported a decrease in the guanine proportion during the time of DNA synthesis whereas adenine and cytidine increased. Such a decrease would be in contradiction to the rule of CHARGAFF[19] that G + U = A + C. We were unable to detect consistent changes in the base ratio by our methods.

As recently shown by SMILLIE and KROTKOV[20], many of the commonly used estimation procedures of nucleic acids as applied to algae are inadequate unless suitably modified. In our procedure, some RNA was apparently extracted by the 20% ethanol-water extract prior to digestion by ribonuclease. Substitution of cold 10% trichloroacetic acid would possibly avoid this loss. In addition, the amount of DNA isolated was lower than expected.

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TABLE I

The photosynthetic rate in different stages of the cell cycle as determined by O_2 evolution and $^{14}CO_2$ uptake.

Experiment	Sample	Hours after Light	O_2 evolution (μmoles/min/g)	$^{14}CO_2$ uptake (μm/min/g)	$^{14}CO_2/O_2$ (X100)	Weight of* sample harvested (mg)	Total radioactivity of sample (μC)	Degree** of Labeling (%)
I	A_1	4	242	184	76	31.8	291	21.0
	A_2	5	237	209	88	33.7	540	36.9
	A_3	6	--	170	--	--	--	--
	A_4	7	181	151	83	39.9	999	57.7
	B_1	9	230	170	74	31.8	291	21.2
	B_2	10	195	188	96	34.0	540	36.5
	B_3	11	--	154	--	--	--	--
	B_4	12	145	122	84	41.8	1074	59.0
	C_1	14	187	151	81	31.1	266	19.7
	C_2	15	224	158	70	32.1	464	33.3
	C_3	16	--	124	--	--	--	--
	C_4	17	185	118	64	35.7	794	51.1
II	A_1	4	200	174	87	29.9	290	22.3
	A_2	5	200	155	78	33.7	530	36.1
	A_3	6	209	136	65	--	--	--
	A_4	7	153	128	76	39.6	950	55.0
	B_1	9	209	147	70	29.1	232	18.4
	B_2	10	184	132	72	32.7	449	31.6
	B_3	11	174	113	65	--	--	--
	B_4	12	140	107	76	37.4	796	46.8
	C_1	14	198	132	67	28.5	219	17.7
	C_2	15	158	124	78	32.9	416	29.0
	C_3	16	204	119	58	--	--	--
	C_4	17	178	90	50	39.0	832	49.0

*The initial dry weight of the sample in g is assumed to be 1/6 of the packed volume of the cells in ml. Subsequent weights of the cells during the experiment were calculated from the cumulative ^{14}C uptake and an assumed cell composition of 52.3% C.

**The degree of labeling is based on the calculated activity incorporated, the calculated weight of the harvested cells, and an assumed cell composition of 52.3% C.

TABLE II

The growth rate, cumulative growth, photosynthetic rate, and calculated degree of labeling for Experiment III.

Time in Light	Growth* Rate (mg/h)	Cumulative* cell weight (mg)	$^{14}CO_2$ uptake (μC/min/g)	$^{14}CO_2/O_2$ (X100)	Calculated Degree of Labeling (%)
0	--	30.2	--	--	0
0 h 50'	3.9	33.3	84	76	9.3
1 h 50'	4.4	37.5	86	64	19.5
2 h 50'	5.1	42.2	87	64	28.4
3 h 50'	5.7	47.7	86	72	36.7
4 h 50'	6.3	53.7	85	71	43.8
5 h 50'	6.6	60.0	79	73	49.7
6 h 50'	6.9	66.9	78	90	54.8
7 h 30'	--	71.5	--	--	57.8
7 h 50'	6.7	73.7	69	--	59.0
8 h 50'	6.6	80.4	62	--	62.4
9 h 50'	6.6	87.0	58	--	65.3
10 h 50'	6.8	93.7	55	--	67.8
11 h 50'	7.3	100.8	56	--	70.0
12 h 50'	6.7	107.9	46	--	72.0
13 h 50'	8.0	115.2	53	--	73.8
14 h 50'	7.6	123.0	48	--	75.4

*The growth rate and cumulative cell weight expressed in mg dry weight were calculated from measurements of $^{14}CO_2$ uptake at hourly intervals. The cumulative cell weight from the total observed $^{14}CO_2$ uptake was 67.9 mg at 7.5 h (55.5% labeled). At 7 1/2 h, 46% of the culture was removed for sample L_1; at 15 h, 62% of the remaining culture was harvested for sample L_2. The lights were turned off, and after 12 additional h, a final sample was removed. The calculations of cumulative growth were corrected for the sample which was removed.

TABLE III

Distribution of ^{14}C in the fractions of Chlorella cells in various stages of the cell growth

Stage of cell growth	Hours of Light	Time of $^{14}CO_2$ incorporation (h)	Total Suspension μC calc. present*	Total Suspension μC found	Fraction Extracted by: 80% Ethanol	20% Ethanol	Ethanol-Water	Ethanol-Ether	RNA-1	Residue	Recovered
					(Expressed as % of μC found in total suspension)						
					Experiment II						
A	4	1	290	293	23.7	1.4	1.8	0.3	1.2	46.5	74.8
	5	2	530	508	24.5	1.5	3.1	0.6	1.0	59.2	89.9
	7	4	950	943	18.0	1.2	2.3	0.8	1.4	56.1	77.8
B	9	1	232	241	26.0	1.2	2.3	0.6	1.1	51.4	82.6
	10	2	449	398	26.2	1.3	4.0	1.1	1.2	62.9	96.8
	12	4	798	769	16.9	1.5	2.8	1.1	1.8	44.4	68.5
C	14	1	219	233	25.5	1.8	2.8	0.7	2.6	44.4	77.7
	15	2	416	375	26.3	2.4	5.3	1.2	2.0	61.2	98.4
	17	4	832	759	20.6	2.0	4.0	1.1	2.1	44.9	75.0
					Experiment III						
	7.5	7.5	757	651	17.9	1.9	5.2	0.9	0.8	--	--
	15	15	1176	1075	19.9	2.6	5.5	0.6	3.3	42.5	74.4
	15 (followed by 12 h dark)	27	--	785	19.4	1.4	2.0	0.1	2.1	--	--

*The μC calculated present was determined from the μC of ^{14}C taken up by the cells in the steady-state apparatus and the aliquot removed (see Table I). The μC found was determined by direct plating and counting an aliquot of the cell suspension.

TABLE IV

Content and specific activity of RNA in different stages of the cell cycle

Algae in sample (mg)	Stage of cycle	Time of $^{14}CO_2$ incorporation (h)	mg RNA*	% RNA**	Total*** activity in RNA (μC)	Relative**** specific activity of RNA (X100)	Relative specific activity of RNA / Degree of labeling of entire cells (X100)
				Experiment I			
31.8	A	1	1.34	4.21	2.07	5.2	24.8
33.7		2	1.31	3.89	4.59	11.8	32.0
39.9		4	1.43	3.58	10.96	25.8	44.7
31.65	B	1	1.44	4.55	2.86	6.7	31.6
34.0		2	1.36	4.00	6.06	15.0	41.1
41.85		4	1.54	3.68	13.54	29.6	50.2
31.1	C	1	1.55	4.98	2.38	5.1	25.9
32.1		2	1.35	4.21	4.53	11.2	33.6
35.7		4	1.46	4.09	10.58	24.3	47.8
				Experiment II			
29.9	A	1	1.33	4.45	2.25	5.7	25.6
33.7		2	1.44	4.27	6.37	14.9	41.3
39.6		4	1.31	3.31	12.88	32.6	59.3
29.1	B	1	1.45	4.98	2.15	5.0	27.2
32.7		2	1.61	4.92	6.26	13.1	41.4
37.4		4	1.31	3.50	11.13	28.6	58.6
28.5	C	1	1.66	5.82	2.71	5.5	31.1
32.9		2	2.04	6.20	7.15	11.8	40.7
39.0		4	1.74	4.46	12.61	24.4	49.8
				Experiment III			
31.1	L	7 1/2	--	--	15.1	--	--
37.2		15	1.73	4.6	17.0	60.3	82.9
		27	1.45		13.2	62.0	85.3

*The mg of RNA was calculated from the 260 mμ absorption and an $E^{1\,cm}_{0.1\%}$ of 34.4

**mg RNA ÷ calc. dry weight of harvested algae.

***Determined from the total activity found in the identified radioactive spots after paper chromatography of the RNA fraction. A conversion factor of 11.2 was used to convert cpm to dpm.

****The total activity in RNA divided by the total μmoles C in RNA (assumed to be 0.357 · wt of RNA).

TABLE V

The distribution of radioactivity between the single components of RNA in different stages of the cell cycle (expressed as % of radioactivity in total RNA).

Exp.	Sample	Adenine	Guanine	Ribose	Cytidylic acid*	Uridylic acid*	Unknown spots 1 (Uridine or Cytidine)	2	3
I	A_1	11.8	12.5	23.3	16.5	25.2	6.1	3.6	1.0
	B_1	10.0	11.5	24.9	17.0	29.0	4.7	1.9	1.0
	C_1	11.6	12.1	24.6	19.6	22.6	5.2	2.7	1.6
	A_2	11.1	13.4	25.5	21.3	22.6	2.8	2.5	0.8
	B_2	12.9	12.9	25.4	20.3	24.3	3.1	0.9	0.3
	C_2	10.6	12.2	24.0	20.0	28.4	3.2	1.4	0.5
	A_4	13.9	11.8	27.8	21.6	16.8	4.9	2.2	1.0
	B_4	14.4	11.4	23.8	20.3	23.1	4.5	1.6	1.0
	C_4	14.7	11.5	25.5	21.2	18.8	4.8	2.5	1.0
II	A_1	10.4	13.3	28.1	16.9	21.6	5.5	2.7	1.5
	B_1	10.1	13.9	27.0	19.3	21.4	5.4	1.9	1.1
	C_1	9.9	13.3	26.7	18.6	18.6	5.4	5.2	2.3
	A_2	11.2	12.9	25.0	18.6	22.9	4.3	3.8	1.4
	B_2	10.13	12.3	24.8	19.6	24.8	4.8	2.3	1.2
	C_2	9.2	12.2	23.7	18.2	23.4	3.6	7.0	2.7
	A_4	13.9	14.6	21.5	20.0	23.1	5.0	1.1	0.8
	B_4	11.5	11.7	22.3	21.5	26.7	4.3	1.1	0.9
	C_4	12.5	12.3	23.1	19.7	25.7	4.4	1.1	1.1
Average		11.66	12.55	24.83	19.46	23.27	4.54	2.53	1.17
Theory	(1)**	13.16	13.16	26.32	23.69	23.69			
	(2)***	12.05	15.75	27.8	24.10	20.6			

*The values for cytidylic acid are probably low and those for uridylic are probably high, due to deamination of the cytidylic acid during hydrolysis.

**Based on statistical tetranucleotide composition for RNA.

***Based on composition reported for Scenedesmus[12].

TABLE VI

The radioactivity of the DNA-thymine in different stages of the cell cycle

(dpm/mg algae)

Experiment	Cell stage	Hours of $^{14}CO_2$ incorporation		
		1	2	4
I	A	ND*	13	67
	B	ND	69	148
	C	121	157	264
II	A	ND	ND	ND
	B	ND	ND	ND
	C	156	373	860
		7.5	15	15 (followed by 12 h dark)
III		172	4650	est. 10,000 (min. 7,500)

*ND = no counts detectable in thymine area of chromatogram.

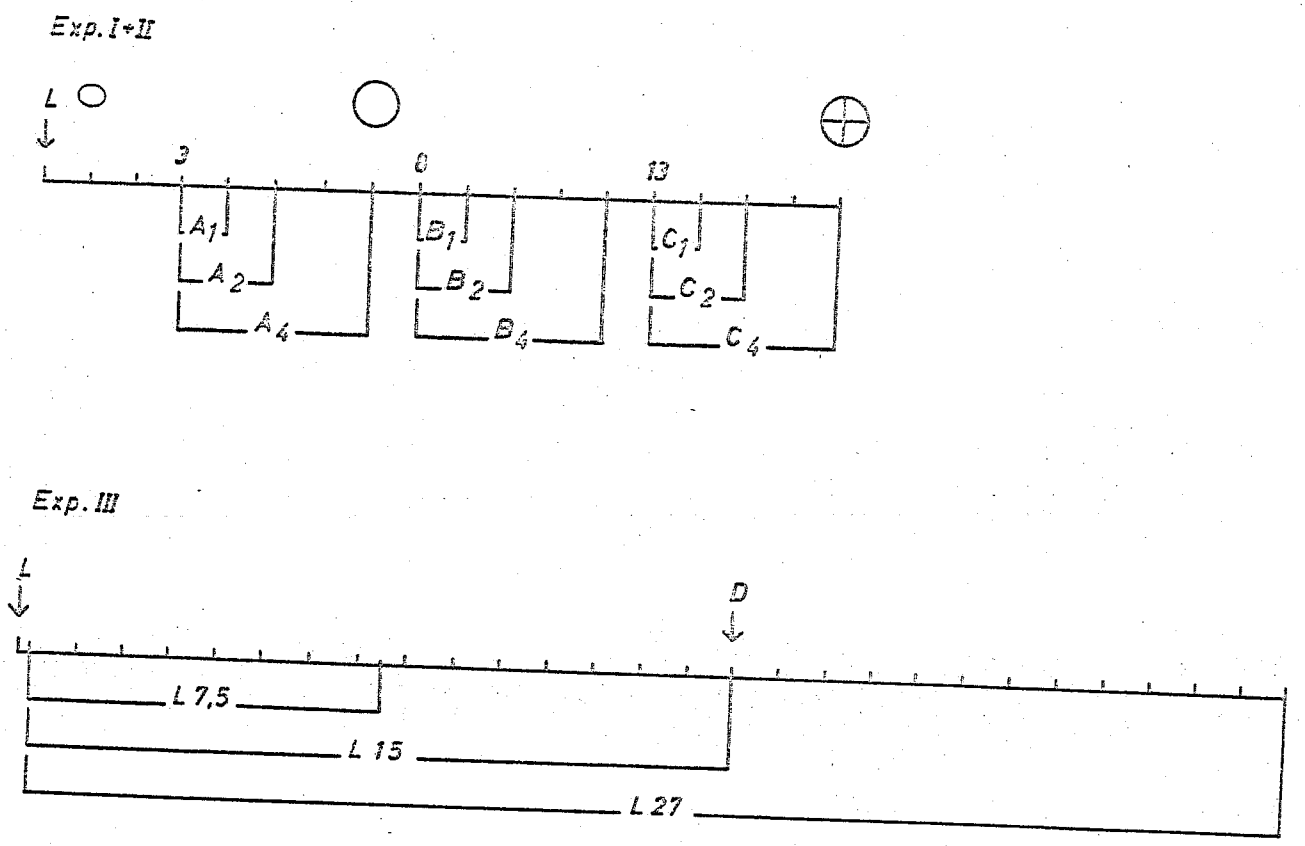

Figure 1. Schematic summary of experimental design for incorporation of $^{14}CO_2$. A_1 designates a sample transferred to the steady-state apparatus 3 h after the beginning of the light-phase and allowed to grow in the presence of $^{14}CO_2$ for one h. A_2 represents a sample allowed to grow in presence of $^{14}CO_2$ for 2 h, etc. The stages of the cell cycle are indicated by , , and indicating the relative size of the cells and the onset of cell division inside the mother cell.

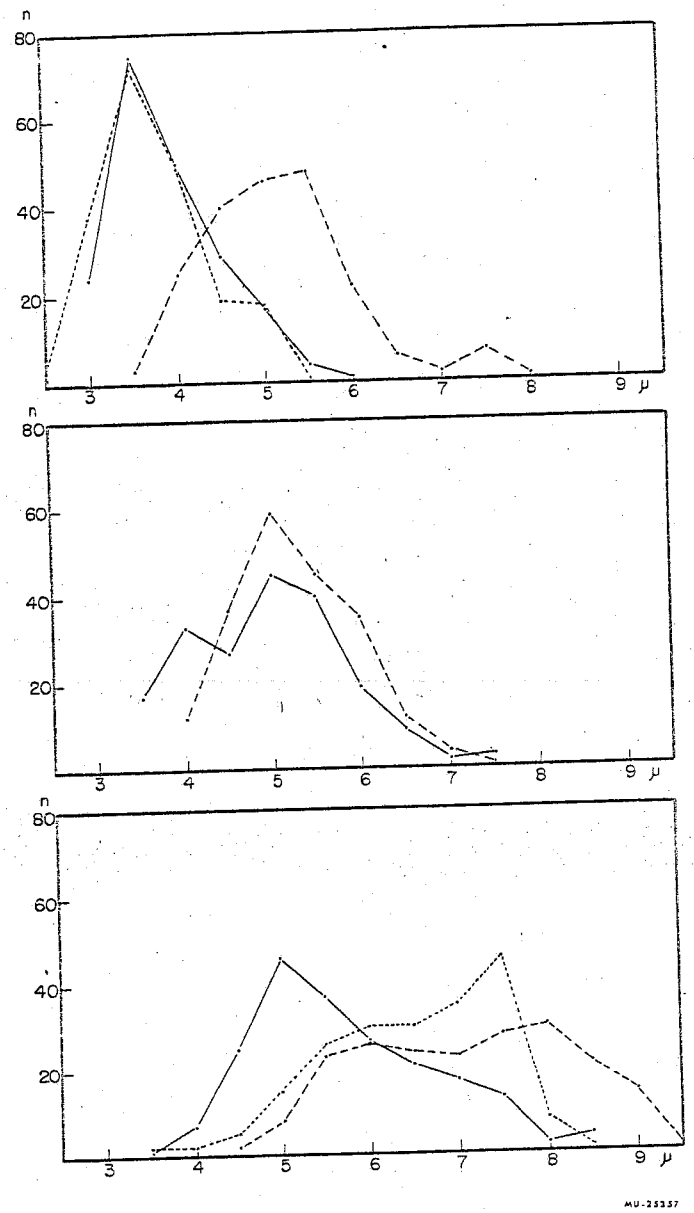

Figure 2. Variation curves for the cell diameter obtained for the cell cultures of Experiment II. Cell sizes were determined at:

Top: 0 h (.....), 3 h (———), 7 h (------) (sample A)
Middle: 8 h (———), 12 h (------) sample B_4, and
Bottom: 13 h (———), 17 h (------) sample C_4, and 18 h (.....)

after initiation of growth by turning on the light.

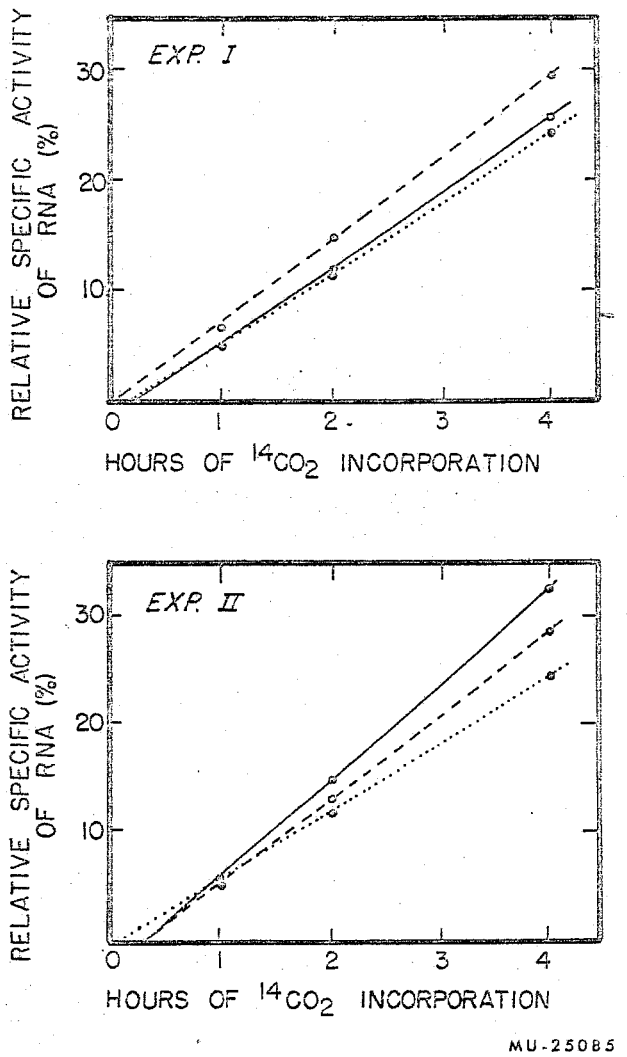

Figure 3. Relative specific activity of RNA vs time of $^{14}CO_2$ incorporation of three stages of Chlorella culture. Stage A _____; Stage B -------; Stage C .....