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SHORT-TIME $C^{14}O_2$-INCORPORATION EXPERIMENTS WITH SYNCHRONOUSLY GROWING CHLORELLA CELLS†

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INTRODUCTION

The different morphological and physiological changes occurring in the development of individual cells must be accompanied by shifts in their metabolic patterns. Synchronously growing cultures of unicellular organisms offer an advantageous material for studies of the metabolic processes characteristic of different phases of cell life. Some work along these lines, using Chlorella as test organisms, has been published by TAMIYA and co-workers (1-7). They have developed a technique for the synchronous growth of Chlorella and have used it as a basis for biochemical studies of developmental processes. A different technique to induce synchronization in Chlorella cultures has been used by SOROKIN and MYERS (8,9) and LORENZEN and PIRSON (10-12).

In this laboratory numerous studies have been made of the distribution of $C^{14}O_2$ after photosynthesis in populations of Chlorella heterogeneous with respect to the stages of cell development (13). The experiments reported here were designed to study the distribution of $C^{14}O_2$ after short periods of photosynthesis (2 to 5 minutes) in relatively uniform cell populations of Chlorella at different stages of cell development. Differences in metabolic processes characteristic of

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different states in the development of the Chlorella cell have been found. Differences in the rates of incorporation of $C^{14}O_2$ and its resulting distribution were observed. Observation on the technique of growing Chlorella cells synchronously are made.

METHODS

Synchronous Growth

The conditions described by TAMIYA et al. (1-4), adapted to the culture equipment used in our laboratory for the usual Chlorella cultures, were followed to induce synchronous growth of the algae. The algae were grown in the continuous-culture apparatus described by HOLM-HANSEN et al. (14). It consists of a temperature-controlled column containing about 1000 ml of algal suspension, which is aerated with 5% CO_2 in air. The column itself is a double-walled tube with the algae in the outer layer, which has a thickness of about 0.75 cm, while the inner tube contains circulating water to maintain any desired temperature. The tube is surrounded by a bank of eight 20-watt fluorescent lights yielding a constant light intensity. The light intensity can be adjusted by varying the distance of the lights from the tube. The automatic dilution of the culture with fresh nutrient solution to maintain a constant density of algae was eliminated. Instead, new nutrient solution was supplied manually at the appropriate times from a reserve flask.

The culture of Chlorella pyrenoidosa was started by inoculating the tube with a couple of cells from a stock culture to give an initial algae population of 0.5 to 1.0 ml wet packed cells per liter. The culture medium had the following composition: KIO_3, 2.0 g; $MgSO_4 \cdot 7\ H_2O$, 2.5 g; KH_2PO_4, 1.1 g; $Ca(NO_3)_2$, 0.02 g; EDTA (15), 1 ml; Hoagland's 'A5' solution, 1 ml; distilled water to make up 1 liter (2.16).

The culture was grown at a temperature of 25°C and a light intensity of 10,000 lux for 4 to 5 days. At that time the algae population had a density of about 15 ml packed cells per liter and contained a high percentage of cells with a small cell diameter. Several initial experiments had shown that the percentage of small cells in the population increases with increasing density of the culture.

The lights were then turned off and the culture left in the dark for 2 days. The synchronous growth of the culture was initiated by diluting 100 ml of this Chlorella suspension with 900 ml of nutrient solution and growing at 20°C with a light intensity of 12,000 lux. This time of growth initiation is defined as '0 hr' and the subsequent stages of synchronous growth are designated by the time interval from this dilution. In initial experiments a temperature of 15°C was used, following TAMIYA's conditions. However, this low temperature was not satisfactory with our Chlorella strain and under our experimental conditions for continuous synchronous growth of the culture; even though the first cycle after induction seemed to be unaffected, upon further growth of the culture at 15°C the cell contents appeared granulated at the second or third cell-division cycle. This was caused by fat droplets accumulating in the cells. In addition, the growth and division of the cells were inhibited. The effect of the low temperature could be reversed by increasing the temperature to 25°C. At 20°C these effects were not observed. However, by reduction of the temperature to 20°C a sufficient slowing down of the cell growth was obtained to facilitate the observation and sampling of Chlorella cells in relatively uniform stages of the growth-and-development cycle.

Under the above conditions one cell cycle extended over a period of about 36 hr, during which time the number of cells, the sizes of cells, and the volume of packed cells have been determined. This period is characterized by a gradual increase of the average cell diameter and of the volume of packed cells per unit volume of the culture, and by a constant number of cells per unit volume of the

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culture. The best description of the stages of the synchronously growing culture is given in the variation curves for the cell diameter. Figures 1a, b, and c represent the variation curves obtained for the cell cultures used for the short-time photosynthesis experiments described below. For each of these curves the diameters of 200 cells were measured in a microscope with an ocular micrometer. The smallest cells, derived from the cell division, usually show ellipsoidal shapes. No choice for the direction of diameter was made; the cells were measured in the direction in which they happened to lie in the scale of the ocular micrometer.

Thirty-six hours after synchronous growth was initiated (by dilution and turning on the light) the culture contained a high percentage of dividing cells. If it is desired to obtain an additional cycle of synchronous growth, the light should be turned off at this time. An appropriate initial stage for further synchronous growth is thus obtained since under these conditions the cells present can not grow further. Synchronous growth may again be started after a dark period of 36 hr at 25°C by dilution and illumination as was done at '0 hr'.

$C^{14}O_2$ Incorporation Experiments

Samples were removed from the synchronously growing Chlorella culture at several time intervals (thus at different stages of cell development) to determine possible differences in the photosynthetic fixation of $C^{14}O_2$. The cells were centrifuged and rediluted to a 1% wet packed cell suspension by adding a diluted nutrient solution (1 part nutrient solution and 4 parts distilled water.) (Two experiments with undiluted nutrient solution gave essentially the same results, but only a small portion of the final extract could be used for the analysis by paper chromatography.)

For these experiments the apparatus described by MOSES and CALVIN (17) was used. It consists of cylindrical glass vessels with a flat bottom of about 3.5 cm internal diameter, in which 1 ml of algae suspension forms a layer about 1 mm deep.

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The lid of the vessel in provided with an inlet and an outlet tube, the former reaching nearly to the bottom of the vessel. These vessels are arranged in a water bath at a temperature of 20°C over a bank of eight 6-watt fluorescent tubes yielding a light intensity of 10,000 to 20,000 lux at the bottom of the vessel. After a period of adaption of 30 min the 1 ml algae suspension is shaken in the light, while [redacted] air plus 1% CO_2 is blown over its surface. Immediately before addition of the radioactive carbon dioxide, the tube supplying the air plus 1% CO_2 was disconnected, 100 ul of $NaHC^{14}O_3$ (0.026 M, 40uC) was injected into the suspension, and after 2 or 5 minutes respectively the cells were killed by adding 4 ml boiling 100% ethanol (18). Generally duplicate experiments were done.

Analysis of Extracts

After extraction with the 80% ethanol resulting from the killing procedure which removed amino acids, organic acids, free sugars, pigments, and lipids, the cells were extracted with 20% ethanol for 5 minutes at 50°C, to extract mainly sugar phosphates. The volumes of the initial total suspension, the 80% ethanol extract, and the 20% ethanol extract were determined and samples of each dried with a drop of acetic acid were onto aluminum planchettes and counted with a Nuclear Chicago D 47 thin-window counter with automatic sample changer.

Aliquots of the combined 80% and 20% ethanol extracts were analyzed by paper chromatography. All 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 (19). Radioactive substances were located by exposure of the chromatograms to Dupont X-ray film No. 507K. Substances were identified by their chromatographic position, by cochromatography with known markers, and by reactions with appropriate sprays. They were counted directly from paper chromatograms with a Mylar-window Scott-type Geiger-Muller tube connected to a scaler.

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RESULTS

The results of one of these experiments (Chlorella D.,) in which samples were taken at the 11-hr, 18-hr, and 32-hr stages, are presented in Tables I and II. The corresponding variation curves for the cell diameter in the synchronously growing culture are given in Fig. 1a. At the 11-hr stage the first increase in the diameter of the cells can be seen. This increase in cell size is pronounced at the 18-hr stage. The 32-hr stage precedes the onset of cell division in the culture. Several other experiments gave comparable results.

Table I shows that the total amount of C^{14} fixation per unit volume of cells more than doubled with the progressing cell development. The fixation of $C^{14}O_2$ reached the highest rate immediately before cell division occurred. At the same time the proportion of radioactivity appearing in the 80% ethanol- and 20% ethanol-soluble material decreased. This is particularly evident in the 5-min photosynthesis experiments, where the percentage of radioactivity in the soluble material is reduced by one-fifth.

The pattern of C^{14} incorporation shows great differences in the different stages of the cell development (Table II). The most striking differences are found for sucrose, for which the percentage of incorporation of $C^{14}O_2$ increases considerably in the 32 hr stage (immediately before cell division), and for alanine, which shows a much higher proportion of incorporation at 11 hr and 18 hr than at 32 hr. These differences in the amount of incorporation of C^{14} in sucrose and alanine are found in both the 2-min and 5-min photosynthesis experiments. In the 2-min experiment, parallel to the decrease in the incorporation of C^{14} in alanine at the 32 hr stage in comparison with the 18-hr stage, there can be observed a decrease for other amino acids, such as threonine, serine, glycine, aspartic acid, citrulline, and tyrosine. These characteristic differences are observed only with cultures showing normal cell-size variations with synchronous growth and not showing abnormalities such as granulation and fat deposition.

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The same changes in the pattern of C^{14} incorporation in different stages of the cell development have been observed in repetitions of this experiment. The amounts of radioactivity found in the sucrose and alanine spots of two further experiments are represented in Table III. The change in the incorporation pattern is expressed in a striking increase of the ratio sucrose:alanine.

DISCUSSION

Cultures of unicellular organisms growing under constant conditions usually contain cells at all stages of the cell life cycle. To obtain cells growing synchronously special treatments must be used, which will cause all cells to grow and divide at the same time. These treatments usually involve the application to the culture of conditions that enhance, slow down, or arrest the development of the cells or of portions of the cells in special stages of their life cycle. Exposure to optimal conditions initiates the synchronous growth of the culture. The first phase of this synchronized growth is a phase of recovery from the imposed stress and unbalance (20). The contribution of such recovery process to differences observed in different stages of synchronous growth is difficult to determine. This difficulty constitutes a certain restriction on the use of synchronized cultures in studies on metabolic changes during the life cycle of the cell. The different methods for inducing synchronous growth differ in this respect only slightly. In the method of synchronization of Chlorella used by TAMIYA and co-workers (1-7) the prior treatment causes an exhaustion of energy or some substrate necessary for cell growth. SOROKIN and MYERS (8,9) and LORENZEN and PIRSON (10-12) used a periodic treatment of intermittent illumination. In their procedure, synchronization is lost without repeated treatment, which shows that at least a portion of the cells is influenced by the period imposed.

TAMIYA and coworkers induced the synchronization of Chlorella cells by an initial culture phase of about 1 week at a reduced light intensity of 400 or 800

lux. They observed that the mass increase of the small Chlorella cells derived from cell division is dependent on a sufficient supply of light. Therefore, the method for induction of synchronization may be dependent upon the inhibition of cell growth by light reduction. In our experiments it was observed that a culture with a high percentage of small cells could be obtained by growing the initial culture in a light intensity of 10,000 lux to high cell density, in which case the light intensity per cell is small. Under these conditions the factors restricting the growth of the cells may be either a reduced light [illegible] or deficiency for some nutrient element in the culture.

With exception of some preliminary experiments, the first sample for the photosynthesis experiments with $C^{14}O_2$ was taken from the synchronously growing culture 11 to 12 hours after applying optimal conditions. It is assumed that at this time the recovery processes have been finished. This is even more probable for the stages at 18 to 24 hr and 30 to 36 hr, between which the comparison of the results is mostly made.

In their investigations using synchronously growing Chlorella cultures TAMIYA and co-workers (1-7) found profound changes in different stages of cell life. The rate of light-saturated photosynthesis constantly decreased during the life cycle irrespective of whether the rate was referred to a unit of dry weight of cells or to a unit number of cells. During the period of their observations (28 hr in the light) the average cell volume increased about 3-1/2 times, while the rate of photosynthesis per cell decreased more than twice for O_2 production and more than six times for CO_2 consumption. This was reflected in a change of photosynthetic quotient (O_2/CO_2), which was around unity at the beginning of the development and reached a value of 3.3 in the large cells prior to cell division. The relative chlorophyll content was highest at the beginning of the life cycle and decreased during the increase in cell mass. The respiratory activity per dry weight of cells

was found to increase during the course of the development to about 2.5 times the initial value.

In her experiments with synchronously growing cultures of a high-temperature strain of Chlorella pyrenoidosa under diurnally intermittent illumination (9 hr light, 15 hr darkness) SOROKIN (9) observed during the first 3 to 4 hr of cell growth in the light an increase of the rate of apparent photosynthesis referred to volume of packed cells, followed by a decrease during the further course of cell development. Referred to a single cell, the rate of the increase in the photosynthetic activity during the first 3 hr of cell development is twice as high as the increase in the photosynthetic activity of the cell at the later stages of development, between 5 and 8 hours. The disagreement between these results and the results of TAMIYA and co-workers (1,2) are explained by the observation that a change in temperature from the conditions of the culture to those of the manometric experiment (15°C to 25°C), as in the experiments of TAMIYA and co-workers, may greatly affect the results of measurements of the rate of photosynthesis.

From their studies on the rate of both endogenous and glucose respiration SOROKIN and MYERS (8) concluded that the respiration rate rises immediately after the start of the development of a cell, soon (under their conditions after about 1 hr) reaches a maximum, and undergoes a slow decline until the mother cell ruptures into daughter cells. Direct activation of respiratory enzymes by light could not be excluded as contributing to the initial rise of respiratory activity. Striking differences were observed in the shape of the curve for the rate of endogenous respiration, dependent on the time interval between harvesting cells and taking manometric readings.

Further important changes in the metabolism of cells in different development stages have been reported by IWAMURA (4), NIHEI (5,6) and HASE et al.

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(7). The content of ribonucleic acid in per cent of dry weight was found to be realtively constant throughout the whole course of cell development. In contrast, the percentage of desoxyribonucleic acid decreased more or less in the growing phase, and increased markedly in the 'sporulation phase', prior to division of mother cells into a number of autospores. The stage immediately prior to nuclear division was further characterized by a light-induced O_2 evolution in the presence of phosphate and the absence of CO_2, and a corresponding phosphate fixation probably in the form of metaphosphate. The content of crude protein (in percentage of dry weight) remained almost constant throughout the life cycle. A slight increase for total carbohydrate (in percentage of dry weight) has been observed in later stages of the development; the portion of sucrose seems to have been significantly increased in the stage prior to cell division.

In the short-time C^{14}-incorporation experiments described in this paper, an increasing incorporation referred to volume of packed cells with progressing development of the cell was found. Moreover, in the later stages of cell developments, shifts in the incorporation pattern have been observed. The percentage of radioactivity in the 80% and 20% ethanol-insoluble material and the incorporation of C^{14} in sucrose, mainly at the expense of incorporation in alanine, have been increased. These last results show some parallelism to the results of HASE et al. (7), who found differences in carbohydrate content during the life cycle. Their results also emphasize, in agreement with the results of TAMIYA and coworkers, that important metabolic changes take place in the stages prior to cell division. The synthesis of proteins during the increase in cell mass, revealed by a high percentage of incorporation of C^{14} in alanine, is replaced in these stages by other synthetic processes, including carbohydrate synthesis, and--according to the results of IWAMURA (4)--synthesis of desoxyribonucleic acid.

Similar changes, as far as the short-time incorporation of C^{14} in sucrose is concerned, have been observed in regenerating cells of the liverwort Riella

(STANGE [illegible], unpublished results), a cell material preparing for cell division and comparable in this respect with the Chlorella cells immediately prior to cell division. Like the Chlorella cells in respect to desoxyribonucleic acid, the regenerating cells of Riella have been shown to synthesize nucleic acids at the same time as carbohydrates (21).

The production of carbohydrates at this stage of development may be understood as an accumulation of reserve substances, as in higher plants at the transition from the vegetative to the reproductive phase, and (or) as a preparation of material for the new cell walls. The mechanism for the shifts in the metabolic pattern is unknown.

Further analysis of these processes would require experiments with longer incorporation times and with further examination of the insoluble material of the cell.

SUMMARY

After a tenfold dilution of an initial culture, grown at 25°C and 10,000 lux to a density of 18 ml packed cells per liter and containing a high percentage of small cells, cells of Chlorella pyrenoidosa have been grown synchronously at 20°C and 12,000 lux. In 2- and 5-min photosynthesis experiments in the presence of $C^{14}O_2$, the incorporation of C^{14} per unit volume of packed cells was increased in progressing stages of cell development. In later stages of the cell development prior to cell division, the portion of radioactivity in the 80% and 20% ethanol-insoluble material was higher than during the initial growth of the cells. At the same time a large increase of C^{14} incorporated in sucrose and a decrease of C^{14} incorporated in alanine have been observed.

Table I. Incorporation of C^{14} after 2-min and 5-min photosynthesis with 40μc of $NaHC^{14}O_3$ in different stages of cell development of synchronously growing *Chlorella* cells. (*Chlorella* IX) (Total activity fixed by 1 ml of 1% cell suspension)

Stage of Synchronous growth	2-min photosynthesis				5-min photosynthesis			
	Total activity (cpm x 10^{-3})	% of total activity			Total activity (cpm x 10^{-3})	% of total activity		
		80% ethanol	20% ethanol	80 +20% ethanol		80% ethanol	20% ethanol	80 + 20% ethanol
11 hr	1 115	50.2	30.9	81.0	1 891	56.3	20.2	76.5
	1 167	47.0	36.0	83.0				
18 hr	1 739	56.1	26.6	82.7	2 503	56.6	16.0	72.6
	1 394	57.5	26.5	84.0				
32 hr	3 013	45.7	24.1	69.9	3 901	49.4	11.3	60.6
	2 707	50.3	28.3	78.7				

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Table II. Incorporation pattern of C^{14} after 2-min and 5-min photosynthesis with $NaHC^{14}O_3$ in different stages of cell development of synchronously growing Chlorella cells (Chlorella IX). (% distribution of radioactivity on paper chromatograms*).

Spot No.	Substance	2-min photosynthesis 11 hr	18 hr	32 hr	5-min photosynthesis 11 hr	18 hr	32 hr
1	origin	0.45	0.43	1.65	0.31	0.39	1.14
2	diphosphates	8.50	5.88	6.11	4.46	3.34	2.81
3	uridine diphosphoglucose	2.94	3.59	4.19	2.22	3.61	2.52
4	monophosphates	30.62	23.97	33.52	14.18	17.76	16.32
5	phosphoglyceric acid	9.43	9.74	10.30	4.96	6.73	5.89
6	P-glycolic acid	0.71	0.64	0.51	0.26	0.44	0.29
7	oligosaccharide	2.47	3.08	4.64	3.66	3.77	2.79
8	maltose	0.39	0.58	0.59	0.51	0.61	0.49
9	sucrose	0.22	0.77	5.73	1.84	2.31	12.59
10	glucose	0.67	0.73	0.38	0.86	0.86	0.82
11	fructose	-	-	0.53	1.19	0.64	0.25
12	threonine	1.09	1.01	0.60	1.06	1.15	0.88
13	serine	3.45	6.57	3.73	4.84	4.81	4.91
14	glycine	0.43	1.23	0.64	0.56	0.65	0.40
15	glutamic acid	3.12	4.75	4.94	8.56	12.04	15.43
16	aspartic acid	9.54	10.23	3.98	15.71	10.09	11.64
17	citric acid	0.50	0.98	0.69	0.61	0.94	0.99
18	malic acid	6.15	8.01	7.72	6.55	5.70	8.60
19	citrulline	0.77	0.44	0.28	0.37	0.31	0.31
20	alanine	14.09	12.25	4.10	21.02	16.36	6.45
21	tyrosine	0.44	0.90	0.50	0.91	0.89	0.59
22	glutamine	0.35	0.34	0.73	1.36	1.19	1.08
23	glycolic acid	1.76	1.68	1.75	1.10	1.29	1.11
	4-6 unknown spots	1.89	2.01	2.20	2.76	4.15	1.68

* This list contains only spots on the paper which had been covered by the film.

Table II (Continued)

Some substances, e.g. valine and leucine, under the conditions used for the paper chromatographic separation run near to the border of the paper and have not been counted.

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Table III. Incorporation of C^{14} into sucrose and alanine after 2-min photosynthesis with $NaHC^{14}O_3$ in different stages of cell development of synchronously growing Chlorella cells. (cpm of sucrose and alanine spots on paper chromatograms+).

Experiment	Stage of synchronous growth	Sucrose	Alanine	Ratio Sucrose / Alanine
Chlorella II 15°C	2 hr	1069	1650	6.65
	24 hr	1210	5300	0.23
	48 hr	4491	764	5.88
Chlorella V 20°C	12 hr	1119	3248	0.34
	24 hr	1404	4144	0.34
	36 hr	2717	871	3.12

+ In both experiments samples with the same number of cells but increasing volume of packed cells have been used. Under these conditions we did not find the increase of C^{14} fixation per unit volume of cells reported in Table I. Under the experimental conditions used, increasing volumes of packed cells had an influence on total fixation per unit volume of cells at the same stage of synchronous growth.

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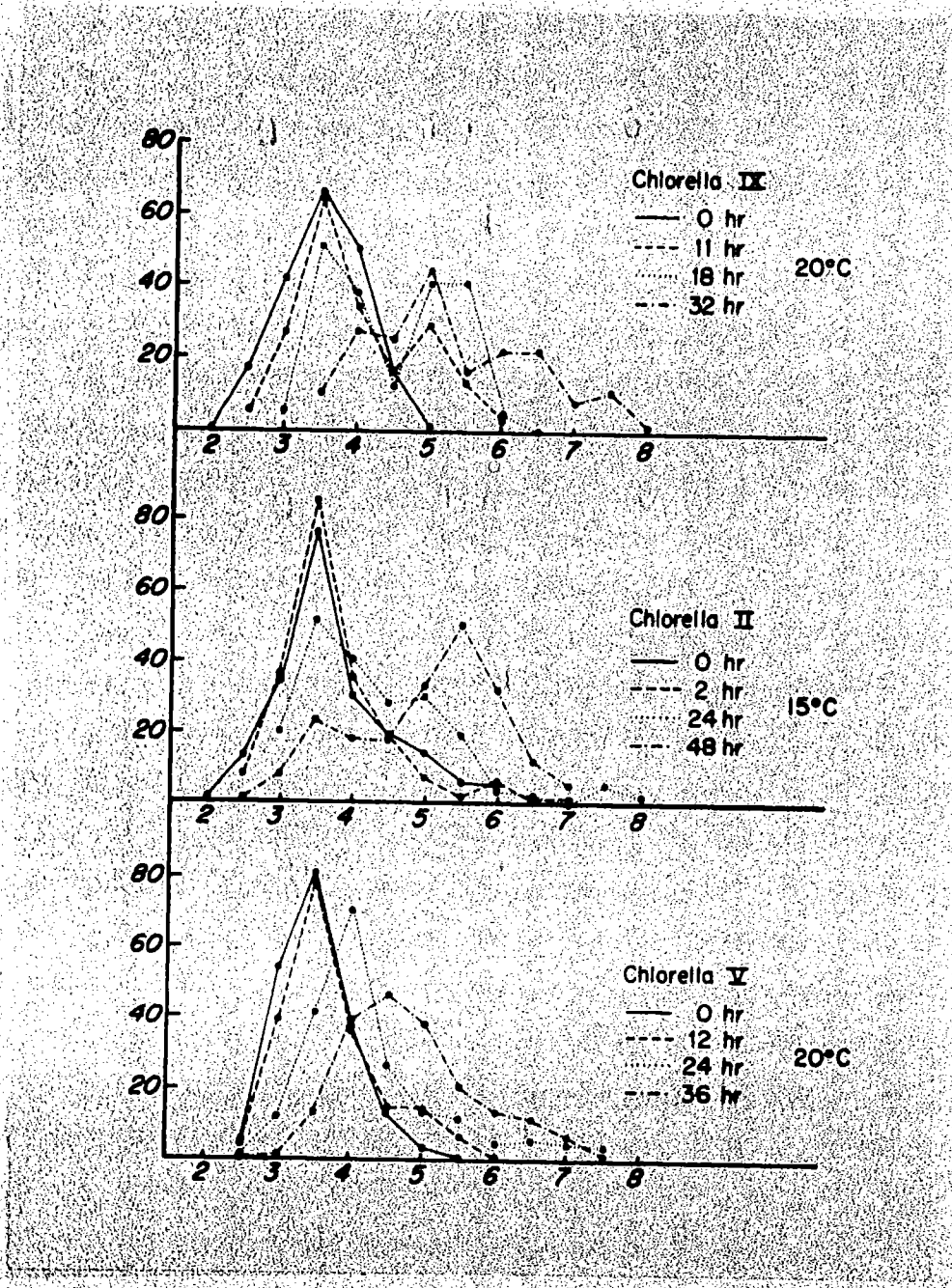

Fig. 1 a-c. Variation Curves of Cell Diameters (Microns)

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REFERENCES

1 H.TAMIYA, T.IWAMURA, E. HASE, AND T.NIHEI, Biochim. et Biophys. Acta, 12 (1953) 23.

2 T.NIHEI, T.SASA, O.MIYACHI, K.SUZUKI, and H.TAMIYA, Arch. Mikrobiol., 21 (1954) 158.

3 T.IWAMURA, E.HASE, Y.MORIMURA, and H.TAMIYA, Ann. Acad. Scient. Fennicae Ser. A, II, 60 (1955) 89.

4 T.IWAMURA, J. Biochem. (Tokyo), 42 (1955) 575.

5 T.NIHEI, J. Biochem. (Tokyo), 42 (1955) 245.

6 T.NIHEI, J. Biochem. (Tokyo), 44 (1957) 389.

7 E.HASE, Y.MORIMURA, and H.TAMIYA, Arch. Biochem. Biophys., 69 (1957) 149.

8 C.SOROKIN and J.MYERS, J. Gen. Physiol., 40 (1957) 579.

9 C.SOROKIN, Physiol. Plantarum, 10 (1957) 659.

10 H.LORENZEN, Flora, 144 (1957) 473.

11 A.PIRSON, H.LORENZEN, Z. Botan., 46 (1957) 53.

12 H.LORENZEN Ber. deut. botan. Ges., 71 (1958) 89.

13 J.A.BASSHAM and M.CALVIN, The Path of Carbon in Photosynthesis, Prentice-Hall, Inc. (1957).

14 O.HOLM-HANSEN, F.HAYES, and F.SMITH, In University of California Radiation Laboratory Chemistry Division Quarterly Report, UCRL-3595, Oct., 1956, p.58.

15 L.JACOBSEN, Plant. Physiol., 26 (1951) 411.

16 J.S.BURLEW, Algae Culture from Laboratory to Pilot Plant, Carnegie Institute of Washington Publication 600 (1953).

17 V.HOOPS and M.CALVIN, Proc. Nat. Acad. Sci., 44 (1957) 260.

18 M.CALVIN and A.BENSON, Science, 107 (1948) 476.

19 A.BENSON, J. A. BASSHAM, M.CALVIN, T.GOODALE, V.HAAS, and W.STEPKA, J. Am. Chem. Soc., 72 (1950) 1710.

20 A.CAMPBELL, Bacteriol. Rev., 21 (1957) 263.

21 L.STAMME, Z. Botan., 45 (1957) 197.