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SYNCHRONOUS CULTURE OF *CHLORELLA*

II. CHANGES IN CONTENT OF VARIOUS VITAMINS DURING THE COURSE OF THE ALGAL LIFE CYCLE

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1. *Chlorella ellipsoidea* was grown synchronously and the changes in content of various vitamins during the algal life cycle were followed either by chemical or microbiological assay methods.

2. In terms of μg per gram of cell dry weight, the content of some vitamins (niacin, biotin, inositol and choline) remained almost constant throughout the algal life cycle, while that of others (vitamin B_6-complex, pantothenic acid, folic acid, thiamine and riboflavin) was found to decrease more or less markedly during the "growing phase" and increase at later phases of "ripening". The content of *p*-aminobenzoic acid increased only at an early stage of "ripening", and that of ascorbic acid increased only at the stages in which photosynthesis occurred most actively.

3. These results were discussed in an attempt to interprete their relationship with the previously reported observations pertaining to the physiological and biochemical events occurring in the life cycle of the alga.

In connection with the project of possible utilization of unicellular green algae such as *Chlorella* and *Scenedesmus* as food or feed, there have been a number of investigations dealing with the content of various vitamins in these algae (*1-10*). It has been reported for some vitamins (*3, 4, 5, 9*) that their content in the algae changes considerably according to the culture conditions. In view of the development and elaboration of the technique of synchronous culture of *Chlorella* in our laboratory (*11, 12, 13, 25*), it seemed worth-while to investigate whether and in what manner the content of various vitamins changes during the course of the life cycle of this alga. The experiments and data bearing on this subject are presented herewith.

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METHODS

The experimental organism was *Chlorella ellipsoidea*, and the culture methods used were virtually the same as those reported previously (*10, 11, 12, 13*). Synchronous culturing was started from D_a-cells[1] (smaller and strongly photosynthesizing cells) (*11*), with a culture medium of following composition: per liter, 5.0 g KNO_3; 2.5 g $MgSO_4 \cdot 7H_2O$; 1.25 g KH_2PO_4; 0.0028 g $FeSO_4 \cdot 7H_2O$; 1 ml ARNON's A_5 solution (*14*). Temperature: 16° C; light intensity: 10 kilolux (using daylight fluorescent lamps). When the cells attained the L_3-stage ("ripened light cells"), they were transferred to the dark and kept for 72 hours (at 16° C) under aerobic conditions, so that the cells further matured to the L_4-stage ("fully matured light cells") and eventually divided into D_n-cells ("nascent dark cells"). At each stage of cell development (D_n, D_a, D-L, L_1, L_2, L_3 and L_4), 0.025–0.1 g (dry weight) of algal cells were collected by centrifugation and subjected to the vitamin analysis. The vitamins analyzed and the assay methods adopted were as follows:

Thiamine: Bioassayed turbidimetrically according to the method of SARETT *et al.* (*15*), using *Lactobacillus fermenti 36* as the test organism.

Riboflavin: Bioassayed acidimetrically according to BARTON-WRIGHT (*16*), using *Lactobacillus helveticus* (*casei e*) as the test organism.

Niacin: Bioassayed acidimetrically with *Lactobacillus arabinosus 17-5*, using the assay medium of Difco Laboratories (*17, 18*).

Ascorbic acid: According to ROE *et al.* (*19*), an extract obtained from algal cells with 5% metaphosphoric acid containing 1% thiourea was passed through norit, and after incubation with 2,4-dinitrophenylhydrazine, treated with sulfuric acid, and the resulting red color was measured spectrophotometrically.

Inositol: Bioassayed with a *Neurospora* mutant, *N. crassa* (M. 37401), using the assay medium of BEADLE (*20*). The growth response was measured by the dry weight of the mycelia formed.

Choline: Bioassayed with a *Neurospora* mutant, *N. crassa* (M. 34486), using the assay medium of HOROWITZ *et al.* (*21*).

Biotin: Bioassayed acidimetrically with *Lactobacillus arabinosus 17-5*, using the assay medium of Difco Laboratories (*17, 18*).

Pantothenic acid: Bioassayed acidimetrically with *Lactobacillus arabinosus 17-5*, using the assay medium of Difco Laboratories (*17, 18*).

Vitamin B_6-complex: Bioassayed with a *Neurospora* mutant, *N. sitophila* (M. 299), using the assay medium of STOKES *et al.* (*22*).

p-Aminobenzoic acid: Bioassayed turbidimetrically according to LANDY *et al.* (*23*), using *Acetobacter suboxydans* as the test organism.

Folic acid: Determined according to the method of ALLFREY *et al.*

[1] Pertaining to the notation of various stages of the algal life cycle, reference will be made to the foregoing paper of this series (present number of this Journal, p. 49).

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(*23, 24*), in which a water extract of the algal cells (boiled for 15 minutes) was treated with permanganate and the resulting 2-amino-4-hydroxypteridine-6-carboxylic acid measured fluorometrically.

RESULTS

The results obtained are summarized in Figs. 1, 2, 3 and 4, in which the vitamin content at successive stages of cell development is expressed in terms of μg per g of cell dry weight. As may be seen from Fig. 1, the μg/g-content of niacin, biotin, inositol and choline did not vary appreciably during the course of the life cycle. These results indicate that the formation of these vitamins occurred almost in pace

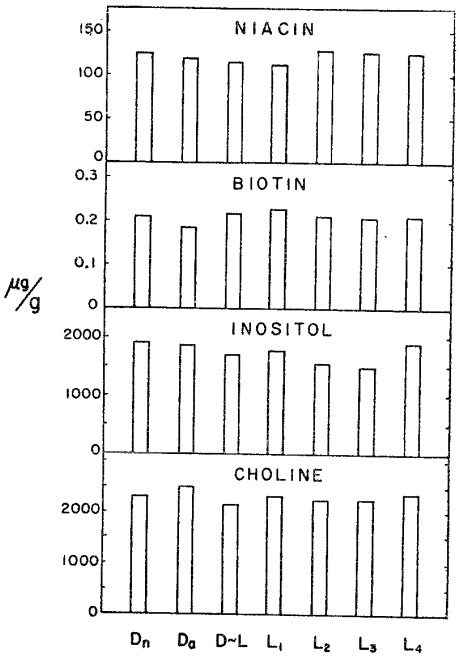

Fig. 1. Niacin, biotin, inositol and choline content at successive stages of cell development (μg/g of cell dry weight).

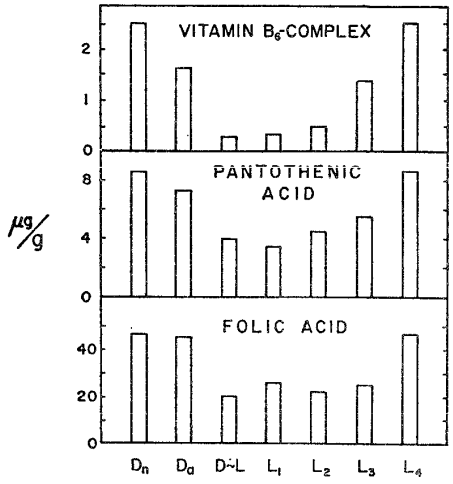

Fig. 2. Vitamin B_6-complex, pantothenic acid and folic acid content at successive stages of cell development (μg/g of cell dry weight).

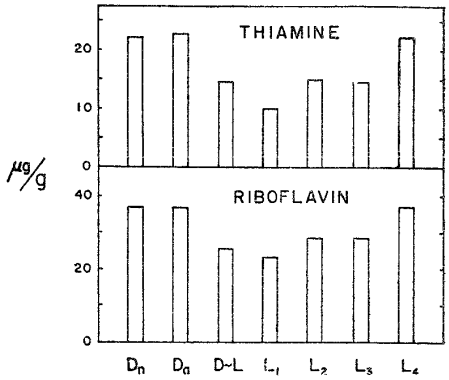

Fig. 3. Thiamine and riboflavin content at successive stages of cell development (μg/g of cell dry weight).

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with the increase in cell mass which took place during the period from the D_n-cell stage to the L_3-cell stage. On the other hand, there were some vitamins, of which the μg/g-content decreased more or less markedly during the "growing phase" (from the D-stage to the beginning of the L-stage) and increased at later phases of L-cells. These were the vitamin B_6-complex, pantothenic acid, folic acid, thiamine and riboflavin (see Figs. 2, 3). Apparently, the formation of these vitamins is related to the process of "ripening", rather than directly to the process of photosvnthesis or of "growing".

Somewhat different features were observed in the cases of *p*-aminobenzoic and ascorbic acids (Fig. 4). In the former case, the content increased only during the stages from L_1 to L_3 and then decreased to a level which remained approximately the same through stages L_4, D_n, D_a to L_1. Ascorbic acid, on the other hand, increased considerably during the stages from D_a to L_1, in which the photosynthetic process occurred most actively, and decreased abruptly at the later stage of L-cells (L_4).

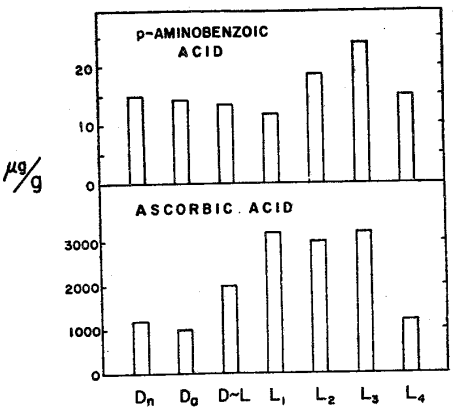

Fig. 4. *p*-Aminobenzoic acid and ascorbic acid content at successive stages of cell development (μg/g of cell dry weight).

The content of various vitamins determined in our experiments are summarized in Table I, in which the data for *Chlorella pyrenoidosa* and *Scenedesmus obliquus* reported by other workers are also presented for comparison.

DISCUSSION

On the basis of the data presented above, the mode of formation of various vitamins during the life cycle of *Chlorella* may be pictured as illustrated schematically in Figures 1 to 4. The point of interest in our observations is the fact that the formation of some vitamins (*p*-aminobenzoic acid, vitamin B_6-complex, pantothenic acid, folic acid, thiamine and riboflavin) occurs more actively in the "ripening" stage than in the "growth" stage, while some other vitamins (ascorbic acid, niacin, biotin, inositol and choline) are formed in pace with the process of "growth" or photosynthesis.

It is interesting to note that *p*-aminobenzoic acid and folic acid, which are known to be involved in the synthesis of purines and pyrimidines, are formed mainly during the process of "ripening". A similar mode of formation was observed with the vitamin B_6-complex which is an

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TABLE I
Vitamin Content of Algal Cells (Chlorella and Scenedesmus)

	Content in μg/g in			
	Chlorella ellipsoidea	*Chlorella pyrenoidosa*		*Scenedesmus obliquus*
Thiamine	10–23	10–24	1.8–18*	2.7
Riboflavin	23–37	36–58		38–43
Niacin	112–125	120–240		73–107
Ascorbic acid	1,000–3,200		1,200–2,400**	
Biotin	0.19–0.23	0.15		0.2
Inositol	1,600–2,100			
Choline	2,200–2,500	3,000		
p-Amino-benzoic acid	12–24			
Folic acid conjugate	22–47			6.0
Folinic acid conjugate				19
Vitamin B_6-complex	0.3–2.5			
Pyridoxine		23		1.8
Pantothenic acid	3.5–8.6	8–20		12–17
Authors	present author	listed by FISHER (*8*)	*V. WITSCH (*3*) **MILNER (*7*)	MEFFERT (*9*)

important factor of the amino acid metabolism (decarboxylation, transamination, etc.). The work previously reported from our laboratory (*25*) showed that with the progress of "ripening" the respiratory activity of algal cells increased considerably, while their photosynthetic activity decreased markedly, a fact indicating that an immense variety of formative metabolism which requires the energy of respiration is occurring in the "ripening" cells. The fact that the content of pantothenic acid (a component of the coenzyme A molecule) increased with the progress of "ripening" may indicate the important role played by coenzyme A in various oxidative and synthetic processes involved in the "ripening" process. The increase in thiamine and riboflavin in *light*-cell stages may also be interpreted as being related to the enhanced formative metabolism occurring in these cells. A convincing explanation for the constancy of the contents of inositol, choline, niacin and biotin throughout the whole course of the algal life cycle, cannot be given until more information becomes available about the roles and fates of these vitamins in the physiology of algal cells.

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used in the microbiological assays. The writer also wishes to acknowledge the helpful assistance of Miss TAMIKO OH-HAMA and Miss MIZUE YOKOTA for some of the analytical results. This work was supported by grants from the Rockefeller Foundation, the Ministry of Education and Ministry of Health and Welfare. To these bodies we extend our grateful thanks.

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