Production of Thiamine, Riboflavin, Folic Acid, and Biotin by *Chlorella vulgaris* and *Chlorella pyrenoidosa*

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Comparison of *Chlorella vulgaris* and *Chlorella pyrenoidosa* with respect to content of thiamine, riboflavin, folic acid, and biotin at different times in the growth period revealed that (*a*) the content of all four vitamins, relative to dry weight of cells, rose to a maximum and then declined; (*b*) the absolute amount of vitamin present (mmcg./ml. of harvested culture) increased continuously, except for folic acid, which decreased in the latter portion of the culture period; (*c*) of the four vitamins, only riboflavin appeared in the external culture medium in significant quantities; (*d*) in general, *C. pyrenoidosa* excels *C. vulgaris* in content of the four vitamins; (*e*) the thiamine, riboflavin, folic acid, and biotin content of both *Chlorella* species compares favorably with that of conventional vegetable dietary sources.

Changes in the concentration of niacin and of the pyridoxine–pyridoxal–pyridoxamine complex (vitamin B_6) in the unicellular green algae *Chlorella vulgaris* and *Chlorella pyrenoidosa* at different periods in the growth cycle have been studied previously, and it has been shown that the niacin and B_6 content of both species compares favorably with that of conventional foods from the vegetable kingdom (1). The present report deals with the occurrence of four other vitamins—thiamine, riboflavin, folic acid, and biotin—in the same species of algae. The rationale for pursuing this line of investigation has been presented in previous papers by the authors (1, 2) and others (3, 4).

EXPERIMENTAL

Organisms and Culture Conditions

The strains of *C. vulgaris* and of *C. pyrenoidosa* employed and the procedures for maintaining stock cultures and experimental cultures have been described elsewhere (1, 2). In brief, experimental cultures were inoculated with sufficient organisms from a 4-day liquid culture to give an initial population of 100 cells/mm.^{3} (100,000/ml.); light intensity (Mazda source) was 600 f.c. at the position of the culture vessels; aeration was provided by a mixture consisting of 5% CO_2 and 95% air continuously passed through the cultures in finely dispersed bubbles, and the temperature was 20.5 ± 0.5°. The culture medium consisted of KNO_3, 0.025 *M;* $MgSO_4 \cdot 7H_2O$, 0.02 *M;* KH_2PO_4, 0.018 *M;* $FeSO_4 \cdot 7H_2O$, 5×10^{-5} *M;* potassium citrate, 5×10^{-5} *M;* Zn (as $ZnSO_4 \cdot 7H_2O$), 0.4 p.p.m.; Cu (as $CuSO_4 \cdot 5H_2O$), 0.004 p.p.m.; Mn (as $MnSO_4 \cdot 4H_2O$), 0.4 p.p.m.; and B (as H_3BO_3), 0.02 p.p.m.

Microbiologic Assays

Thiamine.—Thiamine was extracted from the harvested cells by the U.S.P. method, as adapted by Snell (5). Briefly, this entailed hydrolysis of the harvest with 0.1 *N* H_2SO_4 and then digestion with aspergillus oryzal enzyme.[1] The extracts were assayed microbiologically, using *Lactobacillus fermenti* ATCC 9338 as the test organism in Bacto-thiamine assay medium, according to the procedures of Sarett, Cheldelin, and co-workers (6, 7), as reported by Snell (5).

Riboflavin.—Riboflavin was extracted by digesting the cells in 0.1 *N* HCl (8) and was assayed microbiologically, employing *Lactobacillus casei* ATCC 7469 in Bacto-riboflavin assay medium (9).

Folic Acid.—Extraction of folic acid was accomplished with the aid of hog kidney conjugase, as recommended by Bird *et al.* (10). The extract was assayed against *Streptococcus faecalis* ATCC 8043 in Bacto-folic acid TE medium, prepared according to the formula of Teply and Elvehjem (11). To obtain consistent and reproducible results, it was necessary to maintain stock cultures of the test organism on liver-tryptone agar (12).

Biotin.—To extract biotin from the cells, the harvest was autoclaved at 120° and 15 p.s.i. in 3 *N* H_2SO_4 for 2 hr. (13), and the assay was performed with *Lactobacillus plantarum* ATCC 8014 in Bacto-niacin assay medium (14).

RESULTS

The values in Table I represent averaged data from three separate experiments run at intervals of 6 to 8 weeks. The standard deviations of the results of the assays for each vitamin were comparable in all instances for a given vitamin, irrespective of the species of *Chlorella* or the time of harvest. Therefore, it seems justifiable to present averaged values for standard deviation as closely representing dependability of the data. It should be pointed out that the vitamin assay was only the final step in a long series of manipulations, including inoculation of cultures, adjustment of gas-flow and dispersion of organisms in the culture medium during a 3-week culture period, harvesting the cells, determining dry weight of same, and extraction of vitamins. Thus, the standard deviations listed reflect more than simply the reproducibility of the assays *per se*. In

Received February 15, 1965, from the School of Pharmacy, San Francisco Medical Center, University of California, San Francisco.
Accepted for publication March 12, 1965.
This work was supported entirely by intramural funds from the University of California.

[1] Marketed as Taka-Diastase.

TABLE I.—THIAMINE, RIBOFLAVIN, FOLIC ACID, AND BIOTIN CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

Vitamin	*C. vulgaris* 4–5 Days	7 Days	11 Days	14 Days	18 Days	21 Days	*C. pyrenoidosa* 4–5 Days	7 Days	11 Days	14 Days	18 Days	21 Days
A. Content Expressed as mmcg./mg. Dry Wt.[a]												
Thiamine	12.3	19.4	15.5	10.0	9.0	9.4	19.0	24.5	21.8	14.8	12.0	10.6
Riboflavin	24.7	43.4	40.0	46.4	35.4	28.7	...	51.1	40.6	40.5	28.9	27.4
Folic acid	9.3	22.0	15.1	11.5	8.6	6.4	14.0	28.3	22.3	14.6	9.1	6.4
Biotin	0.8	1.9	2.4	1.8	2.0	2.0	1.2	2.3	2.5	2.1	1.9	1.8
B. Expressed as mmcg./ml. of Culture[b]												
Thiamine	3.3	15.3	30.0	26.9	35.3	43.3	5.4	20.9	51.2	47.8	52.0	53.5
Riboflavin	7.2	35.2	77.9	128.9	136.0	128.8	12.6	43.6	96.0	132.9	115.6	136.5
Folic acid	1.3	17.2	29.9	35.5	29.4	25.4	4.0	30.4	50.3	46.7	37.8	31.9
Biotin	0.1	2.1	4.7	4.9	6.8	7.9	0.3	2.5	5.8	6.6	7.9	9.0

[a] Average standard deviation: thiamine = 3.125, riboflavin = 4.775, folic acid = 3.185, biotin = 0.286. [b] Refers to amounts of the several vitamins found in the cells harvested per milliliter of culture. Of the four vitamins, only riboflavin was detected in the supernatant solution. (See *Results*.)

effect, they are an indication of the reproducibility of the entire experiment.

In both species of *Chlorella*, the concentration of the vitamins studied, expressed as mmcg./mg. dry weight of organisms, increased in young cultures and reached a maximum about the end of the first week or, in the case of biotin, week and a half (Table I.A). During the remainder of the 3-week culture cycle, the concentration of thiamine, riboflavin, and folic acid (vitamins B_1, B_2, and B_c, respectively) decreased substantially. In contrast, within a few days after attaining the maximum, the biotin content of the organisms decreased slightly and then was subject to only minor variations during the remainder of the culture period (Table I.A). However, with the exception of folic acid, the absolute amount of each of the vitamins (mmcg./ml. of culture) increased throughout the culture period, especially during the second week of growth (Table I.B), because of the large increment in total mass of the organisms.[2] Contrary to the pattern characteristic of the other vitamins included in this study and also found previously for niacin and B_6 (1), there was a decrease in the absolute amount of folic acid, *i.e.*, mmcg./ml. of culture, during the latter part of the culture period. In *C. vulgaris*, after a large increase in yield during the first 2 weeks, nearly 30% of the accumulated vitamin disappeared during the third week. A similar pattern was evident in cultures of *C. pyrenoidosa*, but the peak accumulation was reached in a shorter time, consistent with the more rapid attainment of full development noted previously in this species (2). Loss of folic acid in *C. pyrenoidosa* was almost 37% during the last 10 days of the 3-week culture period (Table I.B).

Since the above data refer solely to amounts recoverable from the chlorella cells, they need not necessarily reflect the total vitamin synthesis in the cultures. Therefore, the supernatant solutions remaining after separation of the organisms were also assayed in two of the runs. No folic acid was detectable in the residual medium recovered from cultures of either species of *Chlorella* at the 7-day or the 14-day harvests, but small amounts were present in the supernatant residuum from the 21-day harvests. The quantities found were 4.8 and 4.6% of the amounts present in cells of *C. vulgaris* and of *C. pyrenoidosa*, respectively, at that time. These percentages are too low to account to any significant degree for the approximately one-third decrease in cellular yield of folic acid during the later stages of the growth period. Determination of the fate of the vitamin unaccounted for will require further investigation. Information presently available is not adequate to indicate whether the decrease is due to (*a*) release of the vitamin into the external solution, followed by destruction or inactivation; (*b*) metabolization within the cells at a rate that exceeds biosynthesis during the later stages of the culture period; or (*c*) other possibilities.

TABLE II.—RIBOFLAVIN IN RESIDUAL CULTURE SOLUTION AFTER REMOVAL OF CHLORELLA CELLS[a]

Harvest	*C. vulgaris*	*C. pyrenoidosa*
7-day	Trace	36.2
14-day	60.3	71.6
21-day	59.8	108.9
27-day	Not sampled	115.7

[a] Content expressed as per cent of absolute quantity (mmcg./ml. harvested culture) in cells.

Neither thiamine nor biotin was present, in amounts detectable by the microbiologic methods employed, in the supernatant solutions from cultures of either *Chlorella* species at any of the harvest times. Of the four vitamins under study, only riboflavin was found in appreciable quantities in the residual culture media. The data in Table II indicate that the vitamin was lost from the cells to the external medium throughout the culture period and that, at least in *C. pyrenoidosa*, the quantity in the residual solution at the final harvest exceeded that in the cells. It should be pointed out that during the culture period there was no decrease in the absolute yield (mmcg. riboflavin/ml. of harvested culture) in the cells and that, in fact, there was a continuous increase during most of the period.

[2] The total mass of cells harvested (mg. dry wt./ml.) from cultures of *C. vulgaris* increased nearly 3.5 times (0.806 mg. to 2.747 mg./ml. = 3.4) during the second week of growth and more than 5.5 times (0.806 mg. to 4.57 mg./ml. = 5.67) during the last 2 weeks. Corresponding values for *C. pyrenoidosa* were 0.855 mg. to 3.281 mg./ml. = 3.8-fold increase in the second week, and 0.855 mg. to 5.053 mg./ml. = 5.9-fold increase in the last 2 weeks. The figures are based on averaged data from three separate runs performed successively over a period of about 6 months and are consistent with data from experiments performed more than 1 year earlier (2).

TABLE III.—VALUE OF *C. vulgaris* RELATIVE TO *C. pyrenoidosa* AS A SOURCE OF THIAMINE, RIBOFLAVIN, FOLIC ACID, AND BIOTIN

Vitamin	mmcg./mg. Dry Wt. 4–5 Days	7 Days	11 Days	14 Days	18 Days	21 Days	mmcg./ml. Culture 4–5 Days	7 Days	11 Days	14 Days	18 Days	21 Days
Thiamine	0.65	0.79	0.71	0.66	0.75	0.90	0.61	0.73	0.59	0.56	0.68	0.81
Riboflavin	. . .	0.85	0.99	1.14	1.22	1.05	. . .	0.81	0.81	0.97	1.18	0.94
Folic acid	0.66	0.78	0.68	0.79	0.95	1.00	0.33	0.57	0.59	0.76	0.79	0.80
Biotin	0.67	0.83	0.96	0.86	1.05	1.11	0.33	0.84	0.81	0.74	0.86	0.88

TABLE IV.—THIAMINE, RIBOFLAVIN, FOLIC ACID, AND BIOTIN CONTENT OF CHLORELLA (mg./100 Gm. DRY WT.) AS REPORTED FROM DIFFERENT LABORATORIES

Vitamin	*C. vulgaris* Present Work Peak	3-Wk.	*C. pyrenoidosa* Present Work Peak	3-Wk.	Fisher[a]	*C. ellipsoidia* Morimura[b]	*Chlorella* Species Morimura and Tamiya[c]
Thiamine	1.94	0.94	2.45	1.06	1.0–2.4	1.0–2.3	0.4
Riboflavin	4.34	2.87	5.11	2.75	3.6–5.8	2.3–3.7	2.1–2.8
Folic acid	2.20	0.64	2.83	0.64	. . .	2.2–4.7[d]	48.5
Biotin	0.24	0.20	0.25	0.18	0.015	0.019–0.023	. . .

[a] Work of various authors reported by Fisher, A. W., Jr., and Burlew, J. S., *Carnegie Inst. Wash. Publ.*, (No. 600) 303(1953). [b] Morimura, Y., *Plant Cell Physiol. Tokyo*, **1**, 63(1959). [c] Morimura, Y., and Tamiya, N., *Food Technol.*, **8**, 179(1954). [d] Folic acid conjugate.

TABLE V.—THIAMINE, RIBOFLAVIN, FOLIC ACID, AND BIOTIN CONTENT OF CHLORELLA AND SELECTED CONVENTIONAL FOODS (mcg. VITAMIN/100 Gm. OF EDIBLE PORTION)[a]

Food Item	Thiamin (Vit. B_1)	Riboflavin (Vit. B_2)	Folic Acid (Vit. B_c)	Biotin
C. vulgaris (max. concn.)	1940	4340	2200	240
C. vulgaris (21-day harvest)	940	2870	640	200
C. pyrenoidosa (max. concn.)	2450	5110	2830	250
C. pyrenoidosa (21-day harvest)	1060	2750	640	180
Apple (raw)	40	30	4	0.9–6.2[c]
Banana	40	50	35	4.4–18.0[c]
Barley	120	80	45	?
Beef	60	180	6–14	2.6–7.7[c]
Beet greens (raw)	80	180	31–39	2.7–26.0[c]
Beets (raw)	20	50	26–50	0.3–2.2[c]
Cabbage (raw)	60	50	11–75	2.4–29.0[c]
Eggs (fresh)	100	290	3–8	9.0–35[c]
Lamb	160	220	3	2.1–6.9[c]
Liver, beef (raw)	260	3330	290	96.0–260.0[c]
Milk (cow), whole	40	170	nil.	5.0–41.0[c]
Molasses, cane, blackstrap	280	250	?	9.1–12[c]
Peas (dried)	770	280	12–35	9.4–38.0[c]
Potato, white (raw and peeled)	110	40	4–6	0.6–2.7[c]
Rice, white	70	30	15	?
Rice, whole[b]	320	50	12–35	?
Salmon (fresh)	100	230	?	5.3–19.0[c]
Soybeans (dried)	1070	310	208	?
Spinach (raw)	110	200	48–115	6.9–67[c]
Tomatoes (fresh)	60	40	2–15	4.0–67.0[c]
Wheat bran[d]	460	230	100	?
Wheat germ	2050	800	?	?
Yeast, bakers (compressed)	450	2070	74–265[e]	?
Yeast, brewers (dried)	9690	5450	355	?

[a] Values for foods from "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962; Sebrell, W. H., and Harris, R. S., "The Vitamins," vols. II and III, Academic Press Inc., New York, N. Y., 1954; and Jones, A., and Morris, S., *Analyst*, **74**, 29 (1949). [b] Brown rice. [c] Higher value is for dried product. [d] Bran flakes breakfast cereal. [e] Dried sample.

Thus, it appears that, at least under the environmental conditions provided, the two species of *Chlorella* studied may synthesize riboflavin in quantities that substantially exceed their requirements.

Table III, derived from the analytical data in Table I, shows that at virtually all times during the 3-week culture period *C. pyrenoidosa* excelled, or was at least substantially equivalent to, *C. vulgaris* as a source of thiamine, folic acid, and biotin, whether yield is expressed relative to dry weight of cells or in terms of total amounts of vitamin present (mmcg./ml. harvested culture). In general, there was a tendency for the vitamin content of *C. vulgaris*, relative to that of *C. pyrenoidosa*, to improve with time because, as noted previously, cultures of the latter species develop more rapidly than those of the former and approach or reach the stationary phase while cultures of *C. vulgaris* are still in the logarithmic phase. Only in riboflavin content (relative to dry weight) did *C. vulgaris* surpass *C. pyrenoidosa*, and then only during the third week of culture when growth of the latter organism had slowed significantly. Because of the greater cell mass produced in cultures of *C. pyrenoidosa*, the total yield of riboflavin from this organism, in general, exceeded that from *C. vulgaris*.

Only sporadic and scattered reports of the vitamin content of *Chlorella* species have appeared in the literature. But it seemed of interest to compare the values found in the present experiments with those recorded by others. Table IV provides such a comparison. In examining the table, cognizance should be taken of the fact that different species (sometimes unspecified) have been studied by different workers, that generally the ages of the cultures have not been stated, and that often the culture solutions employed have differed from one laboratory to the next. Despite these sources of variance, there appears to be reasonable agreement in the ranges reported, with two notable exceptions—namely, the exceedingly high value for folic acid reported by Morimura and Tamiya and the relatively high values for biotin reported in the present experiments.

Table V indicates the concentrations of thiamine, riboflavin, folic acid, and biotin in the two species of *Chlorella* reported here and in some representative conventional foods. Values for chlorella are for dried cells, whereas those for most of the other noncereal grain items, unless specified to the contrary, are for fresh material. Since the water content of chlorella is approximately 90%, values expressed in terms of fresh weight of the algae would be about 10% of those listed in the table. The two species of *Chlorella* appear to compare favorably with conventional vegetable (and some animal) dietary sources of the four vitamins.

SUMMARY

In *C. vulgaris* and *C. pyrenoidosa* the concentrations of thiamine, riboflavin, folic acid, and biotin relative to dry weight of cells rise to a maximum during approximately the first third to half of the 3-week culture period and then decline. Despite the decrease in *concentration* during the latter portion of the growth period, there is a substantial increase in the *absolute quantity* of thiamine, riboflavin, and biotin in the cultures because of the large increment in total cell mass, but folic acid does not conform to the general pattern and decreases in *absolute amount* as well as in *concentration*.

None of the vitamins studied, except riboflavin, appears to accumulate in the external medium in appreciable quantities. But the amount of riboflavin in the external medium increases continuously in cultures of both species of *Chlorella* and, in cultures of *C. pyrenoidosa*, equals or exceeds the amount present in the cells at the end of a 3-week growth period.

In general, *C. pyrenoidosa* excels *C. vulgaris* as a source of the vitamins studied whether yield is expressed relative to dry weight of cell mass or in terms of total amount present in the culture. The superiority is greatest in young cultures and diminishes as age of the cultures increases.

As potential sources of the four vitamins studied, both species of *Chlorella* compare favorably with conventional dietary vegetable sources.

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