Production of Pantothenic Acid and Inositol by *Chlorella vulgaris* and *C. pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

Two unicellular green algae, *Chlorella vulgaris* and *C. pyrenoidosa*, were compared with respect to pantothenic acid and inositol content at different times during a 3-week culture cycle. Pantothenic acid was found in the cells and in the external medium at all times: the concentration in the cells (mmcg./mg. dry weight) decreased sharply toward the end of the first week and then less abruptly through most of the remaining 2 weeks. However, due to increase in the cell mass, the absolute yield (mmcg./ml. harvested culture) increased. The concentration in the external medium, after a relatively small decline during the first week, rose substantially and at the end of the culture period far exceeded that in the cells. Inositol content of the cells increased slowly throughout the culture period. No extracellular inositol was detected at any time. *C. pyrenoidosa* excels *C. vulgaris* as a source of both compounds.

PREVIOUS investigations have revealed that the unicellular green algae, *Chlorella vulgaris* and *C. pyrenoidosa*, compare favorably with conventional vegetable dietary sources of several important vitamins, *e.g.*, biotin, folic acid, niacin, pyridoxine, riboflavin, and thiamine (1, 2). Morimura has reported consistent variations in vitamin content at different stages during the division and reproduction cycle of *C. ellipsoidea* grown in synchronous culture for short periods (3), and others have found that the vitamin content of *C. vulgaris* and of *C. pyrenoidosa* changes during 3 weeks of normal nonsynchronous growth (1, 2). Under the latter conditions, the content of biotin, folic acid, riboflavin, and thiamine (relative to dry weight of the cells) rises to a maximum and then declines during the 3-week culture period while the content of niacin and pyridoxine decreases continuously. However, because of the 16- to 17-fold gain in total cell mass, the absolute yield (mmcg./ml. of harvested culture) of all these vitamins, except folic acid, increases throughout the 3 weeks. The absolute yield of folic acid tends to decrease during the later portion of the culture period.

Of the six vitamins above, only riboflavin occurs in substantial quantities in the supernatant culture medium, the amount sometimes equaling or exceeding that in the cell mass after 3 weeks. The folic acid and the niacin content of the supernatant may be approximately 5% of that in the cells; the corresponding figure for pyridoxine may approximate 20%. Neither thiamine nor biotin appears in the supernatant in concentrations detectable by the microbiologic assay methods that were employed.

The present report deals with the pantothenic acid and inositol content of *Chlorella*. The rationale for pursuing this line of investigation has been reviewed by the authors (1, 2, 4) and others (5, 6). The view has been expressed that the vitamin content of *Chlorella* is sufficient to give the alga "premium value . . . as human or animal food" but is insufficient to render the plants suitable for recovery of vitamin concentrates (7). However, the latter point, being merely a matter of technology and of the economics of supply and demand, could well be subject to revision in the future.

Received April 5, 1966, from the School of Pharmacy, San Francisco Medical Center, University of California, San Francisco. 94122.

Accepted for publication May 23, 1966.

This work was supported entirely by intramural research funds from the University of California.

EXPERIMENTAL

Chlorella Cultures and Procedures

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* employed and the procedures adopted as standard for working with them in this laboratory have been described in detail elsewhere (4). In brief, experimental cultures were inoculated with a sufficient number of organisms from a 4-day liquid culture (inoculated from the most recent of a series of at least six 4-day cultures) to give an initial population of 100 cells/mm.^{3} (100,000/ml.); light was supplied continuously from a Mazda source (intensity 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 specific source of illumination and the geometry of the culture vessels have been described (4). 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

Pantothenic Acid.—Harvested *Chlorella* cells were steamed for 30 min. in 0.1 *M* sodium acetate buffer at pH 6.8 and enzymatically digested by exposure for 4 hr. at 37° to a chicken liver extract[1]

[1] Prepared as described by Nielands and Strong (8).

TABLE I.—PANTOTHENIC ACID AND INOSITOL CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

Days	5	7	11	14	18	21
	C. vulgaris					
Vitamin	A. Content Expressed as mmcg./mg. Dry Wt.					
Panto. ac., cells	27.79	13.18	3.76	2.71	1.51	3.09
Panto. ac., supnt.	12.64	8.89	10.06	12.05	12.35	14.72
Panto. ac., total	40.43	22.07	13.82	14.76	13.86	17.81
Inositol	1,550	1,860	1,640	1,680	2,090	2,290
	C. pyrenoidosa					
Days	5	7	11	14	18	21
Vitamin	A. Content Expressed as mmcg./mg. Dry Wt.					
Panto. ac., cells	16.09	9.20	2.85	2.35	3.13	3.16
Panto. ac., supnt.	15.21	10.03	14.19	15.62	22.21	26.32
Panto. ac., total	31.30	19.23	17.04	17.97	25.34	29.48
Inositol	2,030	1,950	1,880	2,300	2,140	2,370
	C. vulgaris					
Days	5	7	11	14	18	21
Vitamin	B. Content Expressed as mmcg./ml. of Culture					
Panto. ac., cells	9.89	10.47	8.11	7.46	6.82	13.30
Panto. ac., supnt.	4.66	7.40	21.80	33.04	44.58	73.22
Panto. ac., total	14.55	17.87	29.91	40.50	51.40	86.52
Inositol	650	1,510	3,620	5,170	7,610	9,510
	C. pyrenoidosa					
Days	5	7	11	14	18	21
Vitamin	B. Content Expressed as mmcg./ml. of Culture					
Panto ac., cells	6.89	8.30	7.12	7.65	14.90	16.88
Panto. ac., supnt.	6.49	10.55	33.15	49.75	101.40	135.50
Panto. ac., total	13.38	18.85	40.27	57.40	116.30	152.38
Inositol	890	1,870	4,810	7,550	9,680	12,250

TABLE II.—PANTOTHENIC ACID AND INOSITOL CONTENT OF *C. vulgaris* RELATIVE TO THAT OF *C. pyrenoidosa* AT DIFFERENT HARVEST TIMES

	Days											
	A. mmcg./mg. Dry Wt.						B. mmcg./ml. Culture					
Vitamin	5	7	11	14	18	21	5	7	11	14	18	21
Pantothenic ac., cells	1.73	1.43	1.32	1.15	0.48	0.98	1.44	1.26	1.14	0.98	0.46	0.79
Pantothenic ac., supnt.	0.83	0.89	0.71	0.77	0.56	0.55	0.72	0.70	0.66	0.66	0.44	0.54
Pantothenic ac., total	1.29	1.15	0.81	0.82	0.55	0.60	1.09	0.96	0.74	0.71	0.44	0.57
Inositol	0.76	0.95	0.87	0.73	0.98	0.97	0.73	0.81	0.75	0.68	0.79	0.78

and alkaline phosphatase.[2] Cellular debris was removed by filtration and the residual extract was assayed for pantothenic acid, using *Lactobacillus plantarum* ATCC 8014 as the test organism in bacto-pantothenate medium U.S.P. (9).

Inositol.—Inositol was extracted according to the method of Wooley as modified by Snell (10). This involved refluxing the cells in 18% HCl for 6 hr., evaporating *in vacuo* to near dryness, and neutralizing. After filtration, the extract was assayed for inositol, using *Saccharomyces carlsbergensis* ATCC 9080 as the test organism in bacto-inositol assay medium (11).

Reliability of the Assays.—Two extracts were prepared from each sample at each harvest time, and each extract was assayed (in duplicate) at two levels. The S.D. for each such set of eight values was determined. All values for pantothenic acid were within the range ±10% of the mean. All inositol assay values for any given single extract were within the same range but to include all S.D. values from multiple extracts the range was ±15%.

RESULTS AND DISCUSSION

The over-all data, averaged from results of three successive experiments, are presented in Table I. Inositol was found only in the cell mass. At none of the six harvest times spaced over the 3-week culture period could it be detected, by the microbiologic assay technique employed, in the culture fluid. In this respect, its pattern of distribution is similar to that found previously for biotin and thiamine (2). In contrast substantial amounts of pantothenic acid occurred in the culture milieu, and during the latter half of the growth period the concentration in the medium was several times that in the cells, whether results were expressed relative to the dry weight of the cell mass (Table I, A) or in terms of absolute yield (Table I, B).

Examination of Table I, A, reveals a distinct difference in the patterns of pantothenic acid and of inositol content of the cells, relative to dry weight, in both species of *Chlorella*. Pantothenic acid is present in relatively high concentrations during the early phases of the culture period, and then the concentration decreases sharply between the fifth and the seventh days after which it continues to decrease, although less abruptly, for about another week to week and a half when it tends to reach a minimum and then to rise slightly during the remaining few days.

Inositol displays much less variation in level, increasing less than 50% between days 5 and 21 in *C. vulgaris* and approximately 15% in *C. pyrenoidosa*. These figures are in sharp contrast to the decrease of about 95% in pantothenic acid concentration between days 5 and 18 in *C. vulgaris* and of about 85% between days 5 and 14 in *C. pyrenoidosa*.

[2] Obtained from the Mann Research Laboratories, New York, N. Y.

The concentration of pantothenic acid in the culture milieu, expressed in relation to dry weight of the cells, swings through a smaller range but it too decreases (about 30%) between days 5 and 7, after which it rises slowly during the following 2 weeks. At the end of the culture period, it is substantially higher than at day 5 and is from 5 to 8 times higher than in the cells of *C. vulgaris* and of *C. pyrenoidosa*, respectively, at that time.

Despite the decrease in pantothenic acid content and the relatively stable content of inositol in the cells, relative to dry weight of the cells, the absolute yield of both compounds (Table I, B) increases during the 3-week growth period because of the large (16- to 17-fold) increase in total cell mass. This is true also for the total yield of pantothenic acid, *i.e.*, content in cells plus that in supernatant (Table I, B).

The data summarized above differ in two significant respects from those found earlier for other vitamins. First, the abrupt decrease in concentration of pantothenic acid in the cells (Table I, A) and in total production of pantothenic acid (cells + supernatant), expressed relative to dry weight of cells, has not been observed in analyses for other vitamins. Changes in the concentration of other vitamins, from high to low (or vice versa), during the culture period occur gradually and more or less uniformly over a period of several days (1, 2).

Second, the high levels of pantothenic acid in *C. vulgaris* relative to those in *C. pyrenoidosa* during the early days of growth seem unique. In the authors' studies of eight vitamins, only one other (niacin) was found which occurs in higher concentration in *C. vulgaris* than in *C. pyrenoidosa*. In that case the difference in favor of the former species ranged from about 8% at 1 week to about 47% after 3 weeks, when results were calculated in terms of dry weight, *i.e.*, mmcg. of vitamin/mg. dry weight of cells, although when results were expressed in absolute terms, *i.e.*, mmcg./ml. harvested culture, the yield from *C. vulgaris* exceeded that from *C. pyrenoidosa* by 14 to 18%, irrespective of the time of harvest. In contrast, at day 5 the concentration of pantothenic acid in cells of *C. vulgaris* exceeds that in cells of *C. pyrenoidosa* by as much as 73% when expressed in terms of dry weight (Table II, A) and by 44% when calculated as absolute yield from the cells (Table II, B). This advantage of *C. vulgaris* decreases during the next several days but the species still holds a 15% lead at the end of the second week (Table II, A). However, by the end of the third week the two species are essentially equal in pantothenic acid content of the cells. Examination of Tables I and II reveals that the advantage *C. vulgaris* seems to hold over *C. pyrenoidosa* in pantothenic acid content of the cells stems in part from the fact that in cultures of the latter species relatively more of the total pantothenic acid content is found in the external medium. This is shown more specifically by Table III. Thus, except for the first week of the growth period the total pantothenic acid (cells + supernatant) is substantially higher in cultures of *C. pyrenoidosa* than in those of *C. vulgaris*, and the difference in favor of the former increases with time (Tables I and II). At each harvest time (except one) the percentage of the total pantothenic acid found in the supernatant was greater in cultures of *C. pyrenoidosa* than in those of *C. vulgaris* (Table III).

TABLE III.—PANTOTHENIC ACID IN RESIDUAL CULTURE SOLUTION EXPRESSED AS PER CENT OF THE TOTAL (CELLS + SUPERNATANT) CONTENT

Harvest Day	*C. vulgaris*	*C. pyrenoidosa*
5	31.3	48.6
7	40.1	52.2
11	72.8	83.3
14	81.6	86.9
18	89.1	87.6
21	82.6	89.3

It is suggested that the high concentration of cellular pantothenic acid in cultures of *C. vulgaris* relative to *C. pyrenoidosa* at day 5 followed by continuous diminution of the apparent advantage of the former thereafter is essentially a syndrome that stems coincidentally from the culture conditions and the harvest times chosen. Others have shown that in cultures of *C. ellipsoidea* undergoing synchronous growth the cells are largest during the "ripening and maturing" stage which precedes release of daughter cells and that this is a stage of development when pantothenic acid content of cells is greatest (3, 12). At that stage the cells have high respiratory activity, suggesting enhanced formative metabolism and an important role for pantothenic acid as a component of coenzyme A (12). Concomitant with the increase in cell size and in pantothenic acid content, there is a striking decrease in the ratio of dry weight/volume during the early ripening phase, suggesting more active absorption of water by the algal cells during that process (13). The accompanying decrease in glucosamine content of the cell wall (14) may indicate significant alteration in the permeability and other characteristics of the cell.

In the present work it was observed that early harvests, in contrast to later ones, appeared to contain a greater percentage of larger cells, perhaps comparable to the "ripening and maturing" stage of development reported by others to precede release of daughter cells. Accordingly, a study was made of the size distribution and settling rates of cells harvested at different times. The results will be the subject of a later communication, and it will suffice for the present to mention that the percentage of "large" cells, *i.e.*, 6 μ or more in diameter does indeed vary during the growth period, increasing approximately threefold between the time of inoculation and the third day and nearly fivefold in the first 5 days. By the seventh day postinoculation, the percentage (but not the absolute number) of "large" cells has decreased to a value below that found in the inoculum.

To determine rate of settling, cells were spun down, washed with 0.002 M K_2SO_4, spun down again, and resuspended in 2% NaCl. Transmission was determined with a Klett-Summerson colorimeter. (Initial Klett reading of suspensions = 200.) The decrease in Klett values after 1 hr. for suspensions prepared from 3-, 4-, 7-, and 11-day harvests was 140, 70, 41, and 20, respectively, and suggests that cells from the younger cultures were less dense, and thus remained in suspension longer, than those from older cultures. This is consistent with the observation of a decrease in the ratio dry weight/volume during the "ripening" stage (13).

In view of the foregoing observations of size distribution and settling rates in our cultures con-

TABLE IV.—PANTOTHENIC ACID AND INOSITOL CONTENT OF *Chlorella* AND OF SOME CONVENTIONAL FOODS (mcg./100 Gm. OF EDIBLE PORTION)[a]

Item	Pantothenic Acid	Inositol
C. vulgaris, dried 5-day harvest	2,779	155,000
C. vulgaris, dried 7-day harvest	1,318	186,000
C. vulgaris, dried 21-day harvest	309	229,000
C. pyrenoidosa, dried 5-day harvest	1,609	203,000
C. pyrenoidosa, dried 7-day harvest	920	195,000
C. pyrenoidosa, dried 21-day harvest	316	237,000
Beans, dried lima	830	?
Beef, liver	5660–8,180	340,000
Beef, muscle	1,100	11,500
Bread, whole wheat	570	64,400–103,000
Broccoli	1,400	?
Cabbage	?	95,000
Cauliflower	920	?
Cheese	350–960	25,000
Eggs	2,700	22,000
Milk, whole	290	50,000
Oats	1,300	100,000
Oranges	340	210,000
Peas, fresh	600–1,040	162,000
Peas, dried	2,800	?
Peanuts, roasted	2,500	180,000
Potatoes, white	400–650	29,000
Potatoes, sweet	940	66,000
Salmon	660–1,100	17,000
Soy beans	1,800	?

[a] Values for food from Sebrell, W. H., and Harris, R. S., "The Vitamins," vol. III, Academic Press Inc., New York, N. Y., 1954; and "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962.

sidered in conjunction with the very detailed observations on physical characteristics of cells at different stages of development cited in the literature (3, 12), it seems reasonable to suggest that, under the conditions of the experiments in this laboratory (relatively small inoculum, adaptation to new medium, different light intensity, etc.), there may be a tendency toward partial or simulated synchronized growth at the time of the first harvest—day 5. Expressed another way, it may be plausible to suggest that a larger percentage of cells of the total population divides about the third to fifth day postinoculation than at earlier or later times. Since *C. pyrenoidosa* develops more rapidly than *C. vulgaris* during the early phases of culture and enters the log phase and reaches the stationary phase sooner (1, 4), it is not unreasonable to assume that at the time of first harvest (day 5) cultures of *C. vulgaris* were just approaching or entering the critical time of the major burst of division (percentagewise, although not in terms of total number of cells dividing or produced) and that cultures of *C. pyrenoidosa* had already passed that peak.

Bearing in mind that pantothenic acid level is greatest in cells in the "ripening and maturing" stage of development (3, 12), the above hypothesis is consistent with the higher cell content of that constituent found in *C. vulgaris* in the first half of the culture period. It is interesting to note also that Morimura (3) reported formation of pantothenic acid to be related primarily to the process of "ripening" rather than directly to the process of photosynthesis or "growing." If greatest production occurs during the phase preceding release of daughter cells, it may not be unreasonable to assume that upon release of the daughter cells some of the vitamin would also be released to the external medium. If such does in fact occur, since *C. pyrenoidosa* cultures develop more rapidly than those of *C. vulgaris*, it could account for the higher percentage of the total pantothenic acid found in the external medium of the *C. pyrenoidosa* cultures (Table III).

The pantothenic acid and inositol content of the two *Chlorella* species and of several conventional dietary items is shown in Table IV. In comparing *Chlorella* with the other items listed, it should be remembered that data for *Chlorella* are for dried cells but that values of the other items (except lima beans, oats, dried peas, peanuts, and soy beans) are for fresh material. Therefore, since the water content of *Chlorella* is about 90%, values expressed in terms of fresh weight would be about 10% of those recorded.

SUMMARY

In cultures of *C. vulgaris* and of *C. pyrenoidosa* the cellular content of inositol, expressed relative to dry weight of the cells, increased throughout the 21-day culture period—the increase being less than 50% between days 5 (first harvest) and 21 in *C. vulgaris* and about 15% in *C. pyrenoidosa*. The increase in total yield (mmcg./ml. of harvested culture) was approximately 14-fold during the same period in both species. At all harvest times, *C. pyrenoidosa* exceeded *C. vulgaris* in inositol content. At no harvest time was inositol detected in the external culture medium.

In contrast, pantothenic acid was recovered from the cells and from the external culture solution at all harvest times. The concentration in the cells, relative to dry weight decreased sharply during the first week, then less abruptly for another week or week and a half. Except during the first week, when there was a decline, the concentration in the external solution increased throughout the culture period, and at terminal harvest the concentration in the external medium was severalfold that in the cells.

During the first week postinoculation *C. vulgaris* excelled *C. pyrenoidosa* in yield of pantothenic acid, but after that initial period the relative positions of the two species as sources of the vitamin were reversed.

REFERENCES

(1) Pratt, R., and Johnson, E., *J. Pharm. Sci.*, **53**, 151 1964).
(2) *Ibid.*, **54**, 871(1965).
(3) Morimura, Y., *Plant Cell Physiol.* (*Tokyo*), **1**, 63 (1959).
(4) Pratt, R., and Johnson, E., *J. Pharm. Sci.*, **52**, 979 (1963).
(5) Bush, V., in foreword to *Carnegie Inst. Wash. Publ.*, (*No. 600*), III (1953).
(6) Morimura, Y., and Tamiya, N., *Food Technol.*, **8**, 179 (1954).
(7) Fisher, A. W., Jr., *Carnegie Inst. Wash. Publ.* (*No. 600*), 311(1953).
(8) Neilands, J. B., and Strong, F. M., *Arch. Biochem. Biophys.*, **19**, 287(1948).
(9) "Microbiological Assay of Vitamins and Amino Acids," Difco Laboratories, Detroit, Mich., 1963, p. 46.
(10) Snell, E. E., in "Vitamin Methods," vol. I, György, P., ed., Academic Press Inc., New York, N. Y., 1950, p. 439.
(11) "Microbiological Assay of Vitamins and Amino Acids," Difco Laboratories, Detroit, Mich., 1963, p. 36.
(12) Otsuka, H., *J. Gen. Appl. Microbiol.*, **7**, Suppl. 1 (1963).
(13) Yuhara, T., and Hase, E., *ibid.*, **7**, 70(1961).
(14) Mihara, S., *Plant Cell Physiol.* (*Tokyo*), **2**, 25(1961).