# Production of Pyridoxine and Niacin by *Chlorella vulgaris* and *C. pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

**Two species of Chlorella were compared with respect to their content of niacin and of the pyridoxine-pyridoxal-pyridoxamine complex ($B_6$) after different periods of incubation. Despite decreasing concentration of the vitamins (mmcg./mg. dry weight of cell material) in both species during the 3-week culture period, the total yield of each vitamin (mmcg./ml. of harvested culture) increased due to the substantial gain in total cell mass. The two species are roughly comparable in their ability to produce niacin but differ with respect to $B_6$. The niacin and $B_6$ content of both species compares favorably with that of conventional foods from the vegetable kingdom.**

Serious doubts have been expressed that present agricultural methods and conventional sources can provide adequate food to support the world population in three or four generations if the present rate of human reproduction continues unabated. In several countries, including the United States, responsible scientists have considered mass culture of unicellular green algae as a means of coping with this problem, and experiments—both laboratory and pilot plant scale—have been conducted, generally with species of *Chlorella* (1).

The basal composition of *Chlorella* cells with respect to the ratio of protein/lipid/carbohydrate changes with the age of the culture, but not all species change equally. Comparison of *C. vulgaris* and of *C. pyrenoidosa* in a standard medium and in one reputed to favor production of lipid revealed that (*a*) both species grow poorly in the modified medium; (*b*) therefore, higher absolute yields of both protein and lipid are produced in the standard medium, despite the lower percentage yield; (*c*) the protein content (as per cent of dry weight) of both species decreases with age, although there is an increase in the absolute amount of protein, *i.e.*, mg./ml. of culture; and (*d*) at all ages and in both media *C. pyrenoidosa* excels *C. vulgaris* as a source of lipid, whether results are expressed in terms of percentage of dry weight or of absolute yield (2).

The present report deals with the content of two vitamins and their associated vitamers in cells of two species of *Chlorella* cultured in the standard medium. The vitamins studied were niacin and $B_6$ (pyridoxine-pyridoxal-pyridoxamine).

A few studies of the vitamin content of algae have been reported (3–6), and the view has been expressed that *Chlorella* and some other algae contain vitamins sufficient to give those plants "premium value. . .as human or animal food" although insufficient to render the plants suitable for recovery of vitamin concentrates (7). Morimura (6) studied variations in vitamin content at different times during the division and reproduction cycle of *C. ellipsoidea* grown in synchronous culture for short periods. However, no systematic evaluation of *Chlorella* growing nonsynchronously and normally over a period of several days or weeks has been published, especially with respect to changes in vitamin content that may occur with age of the culture. It should be emphasized that the data that follow deal exclusively with the vitamin content of the cells at the times of harvest and do not indicate total production of the vitamins by *Chlorella*. Designations of mmcg. of $B_6$ or of niacin per milliliter of culture refer to the amounts of the vitamins found in the cells harvested per milliliter of culture and do not include vitamins present extracellularly in the supernatant. In most harvests the niacin content of the supernatant was 4.5 to 6.5% of the amount found in the cells. The corresponding figures for $B_6$ were 18.5 to 25%.

## EXPERIMENTAL

### Organisms and Culture Conditions

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* used in this study; the method of maintaining stock cultures; the composition, preparation, and inoculation of stock and experimental culture solutions, and the details of cultivation, illumination, and harvesting of experimental cultures have been described previously (2).

### Microbiologic Assays

**Vitamin $B_6$ Activity.**—The method of Atkin, *et al.* (8), using *Saccharomyces carlsbergensis* ATCC 4228 as the test organism, was found to yield satisfactorily reproducible results. The coefficients of variability (standard deviation expressed as a percentage of the mean) calculated for multiple cultures harvested at five different times during the culture period and assayed separately ranged from

Received May 20, 1963, from the School of Pharmacy, University of California, San Francisco.
Accepted for publication June 27, 1963.
This work was supported entirely by intramural funds of the University of California.

TABLE I.—VITAMIN $B_6$ AND NIACIN CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

| Vitamin | *C. vulgaris* 6–8 days *1* | 13–15 days *2* | 20–22 days *3* | *C. pyrenoidosa* 6–8 days *4* | 13–15 days *5* | 20–22 days *6* |
|---|---|---|---|---|---|---|
| | *A* Content Expressed as mmcg./mg. Dry Wt. | | | | | |
| $B_6$ | 16.05 | 13.51 | 11.51 | 25.28 | 20.55 | 12.80 |
| Niacin | 258.00 | 225.00 | 189.00 | 240.00 | 183.50 | 128.00 |
| | *B* Content Expressed as mmcg./ml. of Culture | | | | | |
| $B_6$ | 16.23 | 40.47 | 54.30 | 22.50 | 73.06 | 72.40 |
| Niacin | 253.00 | 645.98 | 850.69 | 213.60 | 652.41 | 723.97 |

0.5 to 7.7%. The actual values were: 8 days, 0.5%; 11 days, 4.6%; 15 days, 2.6%; 18 days, 7.6%; and 22 days, 7.7%.

**Niacin.**—Niacin was extracted from the cells according to the procedure recommended by Snell (9), and assays were performed with *Lactobacillus plantarum* ATCC 8014 in Difco niacin assay medium. Greater reproducibility was achieved in niacin assays than in those for $B_6$ activity, possibly because of the greater stability and greater ease of extraction of niacin. The maximum coefficient of variability calculated from the data for niacin content was 3%. Another factor contributing to greater uniformity of assays for niacin content of *Chlorella* cells undoubtedly was the greater retention of niacin than of pyridoxine by the cells during the culture period.

## RESULTS

Table I presents averages of the results from experiments conducted under conditions that have been adopted as standard in this laboratory[1] in the columnar culture vessels described previously (2). Tables II–IV are derived from Table I.

It has been pointed out, with reference to the reporting of protein and lipid contents of *C. vulgaris* and *C. pyrenoidosa* (2), that values expressed in terms of percentage of dry weight sometimes provide only a partial picture and are, therefore, misleading with respect to the total or absolute yields that can be obtained from the two species at different times. Cultures with the highest percentage yields are not necessarily those with the largest cell population or cell mass. Frequently, therefore, the highest yields, in terms of absolute amount of protein or lipid recoverable, are obtained under conditions that produce abundant growth of organisms but a lower percentage content. The same principle applies with respect to vitamin content and yield.

In both species of *Chlorella*, $B_6$ and niacin content (expressed as mmcg./mg. dry weight, which may be considered analogous to percentage content referred to above) progressively decrease during the second and third weeks of growth (Table I *A*). However, despite the decline in concentration relative to dry weight, there is a continuous and substantial increase in the absolute amount of each of them because of the large increment in total mass of the cultures, especially during the second week of growth (Table I *B*). The magnitude of these changes can be more readily visualized when expressed as percentage increment or decrement, as in Table II.

[1] Inoculum: 100 cells/mm.$^3$ (100,000/ml.) from 4-day cultures in liquid medium. Light: 600 f.-c. (Mazda source). Aeration: 5% $CO_2$ + 95% air continuously passed through culture in finely dispersed bubbles. Temperature: 20.5 ± 0.5° C. Culture medium: $KNO_3$, 0.025 *M*; $MgSO_4 \cdot 7H_2O$, 0.02 *M*; $KH_2PO_4$, 0.018 *M*; $FeSO_4 \cdot 7H_2O$, 5 × $10^{-6}$ *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.; B (as $H_3BO_3$), 0.02 p.p.m. See *Reference 2* for details.

TABLE II.—PER CENT CHANGE IN PROTEIN AND VITAMIN CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

| | *C. vulgaris* 2nd wk. *1* | 3rd wk. *2* | *C. pyrenoidosa* 2nd wk. *3* | 3rd wk. *4* |
|---|---|---|---|---|
| | *A* % Change in Concn. | | | |
| Protein (% dry wt.) | − 12.7 | − 7.7 | − 7.8 | −36.3 |
| $B_6$ (mmcg./mg.) | − 15.8 | −14.8 | − 18.7 | −37.7 |
| Niacin (mmcg./mg.) | − 12.8 | −16.0 | − 23.5 | −30.0 |
| | *B* % Change in Absolute Amount | | | |
| Protein (mg./ml.) | +247.4 | +41.3 | +226.5 | + 1.3 |
| $B_6$ (mmcg./ml.) | +149.3 | +34.2 | +224.0 | − 0.9 |
| Niacin (mmcg./ml.) | +155.3 | +31.6 | +205.0 | +11.0 |

TABLE III.—VALUE OF *C. vulgaris* RELATIVE TO *C. pyrenoidosa* AS A SOURCE OF VITAMIN $B_6$ AND NIACIN AT DIFFERENT HARVEST TIMES

| Vitamin | Dry Wt., mmcg./ml. 2 wks. *1* | 3 wks. *2* | mmcg./ml. Culture 2 wks. *3* | 3 wks. *4* |
|---|---|---|---|---|
| $B_6$ | 0.66 | 0.90 | 0.57 | 0.75 |
| Niacin | 1.06 | 1.18 | 0.93 | 1.07 |

The numbers in Table II indicate the percentage loss or gain in vitamin content during the second week with respect to the levels existing at the end of the first week (columns *1* and *3*) and during the third week relative to the values at the end of the second week (columns *2* and *4*). Figures for protein are included for comparison. The upper and lower parts of the table deal, respectively, with changes in the concentration of different components relative to dry weight and with changes in the absolute amounts of the components in the cells recovered from the cultures. (It is important to bear in mind that the numbers in Table II *A* indicate per cent change in values that, for protein, already are expressed in percentages and, for the vitamins, are essentially the equivalent of percentages).

The relative values of the two species of *Chlorella* as sources of the two vitamins at 2 and 3-week harvest times are shown in Table III. At both harvest times, *C. pyrenoidosa* excels *C. vulgaris* with respect to $B_6$ content, whether results are expressed relative to dry weight (columns *1* and *2*) or in terms of total yield per milliliter of culture (columns *3* and

TABLE IV.—INTERDEPENDENCE OF CONCENTRATIONS OF NIACIN, PYRIDOXINE, AND PROTEIN IN *Chlorella*[a]

| | | *C. vulgaris* | | | *C. pyrenoidosa* | | |
|---|---|---|---|---|---|---|---|
| | | 1 wk. *1* | 2 wks. *2* | 3 wks. *3* | 1 wk. *4* | 2 wks. *5* | 3 wks. *6* |
| *A* | Niacin/$B_6$ | 16.07 | 16.67 | 16.42 | 9.49 | 8.93 | 10.00 |
| *B* | Protein/niacin | 2,341 | 2,342 | 2,571 | 2,650 | 3,192 | 2,914 |
| *C* | Protein/$B_6$ | 37,632 | 39,008 | 42,224 | 25,158 | 28,515 | 29,140 |

[a] Calculated in terms of dry weight, *i.e.*, mmcg. niacin/mg. dry weight of cells divided by mmcg. pyridoxine/mg., etc.

TABLE V.—VITAMIN $B_6$ AND NIACIN CONTENT OF *Chlorella* AND OF SELECTED CONVENTIONAL FOODS (MCG. VITAMIN/100 GM. OF EDIBLE PORTION)[a]

| Food Item | $B_6$ | Niacin |
|---|---|---|
| *C. vulgaris* (dried 6–8 day harvest) | 1,605 | 25,800 |
| *C. vulgaris* (dried 13–15 day harvest) | 1,351 | 22,500 |
| *C. vulgaris* (dried 20–22 day harvest) | 1,151 | 18,900 |
| *C. pyrenoidosa* (dried 6–8 day harvest) | 2,528 | 24,000 |
| *C. pyrenoidosa* (dried 13–15 day harvest) | 2,055 | 18,300 |
| *C. pyrenoidosa* (dried 20–22 day harvest) | 1,280 | 12,800 |
| Apple | 26 | 200 |
| Banana | 320 | 700 |
| Barley | 320–560 | 3,100 |
| Beef | 230–320 | 2,900–5,500 |
| Beet greens | 37 | 400 |
| Cabbage | 120–290 | 300 |
| Eggs, fresh | 22–48 | 100 |
| Lamb | 250–370 | 4,300–5,600 |
| Liver, beef | 600–710 | 13,700 |
| Liver, calves | 300 | 16,100 |
| Milk, whole | 54–110 | 100 |
| Peas, dried | 160–330 | 3,100 |
| Potato | 160–250 | 1,200–1,400 |
| Rice, whole | 1,030 | 4,600[b] |
| Rice, white | 340–450 | 1,600 |
| Salmon, fresh | 590 | 7,200 |
| Soybeans, dried | 710–1,200 | 2,300 |
| Tomatoes, canned | 710 | 700 |
| Tuna, canned | 440 | 12,800 |
| Spinach | 60 | 300–600 |
| Wheat bran | 1,380–1,570 | 8,700[c] |
| Wheat, germ | 850–1,600 | 4,600 |
| Yeast, bakers' | 620–700 | 28,200 |
| Yeast, brewers' (dried) | 4,000–5,700 | 36,200 |

[a] Values for foods abstracted from Sebrell, W. H., and Harris, R. S., "The Vitamins," Vol. III, Academic Press, New York, N. Y., 1954, and from "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962. [b] Brown rice. [c] Bran flakes breakfast cereal.

*4*). However, the disparity between the two species diminishes during the third week. This is not surprising, since growth is more rapid in cultures of *C. pyrenoidosa* than in those of *C. vulgaris* during the first 2 weeks after inoculation, while the reverse is true during the third week. Despite the different growth patterns of the two species and contrary to the findings regarding $B_6$, there appears to be little difference between the two species with respect to niacin content.

It has been pointed out that in both species of algae the content of $B_6$ and of niacin, relative to dry weight, decreases continuously throughout the 3-week culture period (Table I *A*) The data in Table IV indicate that the decreases in concentration of the two entities are not independent and that each of the two organisms tends to maintain a different but characteristic ratio of niacin to $B_6$ (line *A*). The niacin/$B_6$ ratio characteristic for *C. vulgaris* under the standard culture conditions mentioned above is approximately 16; for *C. pyrenoidosa* it is about 9 or 10.

Despite the potential sources of variability in the raw data, the values calculated from them relative to *C. vulgaris* and recorded in line *B* (columns *1–3*) of Table IV are remarkably uniform. A similar approach to uniformity is seen in the results for *C. pyrenoidosa* (columns *4–6* of the same line). The numbers (representing mmcg. protein/mg. dry weight ÷ mmcg. niacin/mg. dry weight) can be interpreted as indicating that the protein/niacin ratio tends to remain uniform during the 3-week growth period—approximately 2,400:1 in *C. vulgaris* and perhaps slightly higher (about 2,700:1) in *C. pyrenoidosa*. However, the difference recorded is too small to be considered significant at this time.

Protein/pyridoxine ratios calculated in the same way (Table IV, line *C*) appear to be less uniform than the protein/niacin ratios (line *B*) and do not seem to show random variation but instead to show increase with the age of the cultures. However, if there is a constant and uniform relation between the concentration of niacin and of $B_6$ and between protein and niacin in *Chlorella*, there should also be a constant relationship between protein and $B_6$. Stated another way, when the niacin/$B_6$ and protein/niacin relationships just mentioned obtain, one can write the formulation

$$\frac{\text{niacin}}{B_6} \times \frac{\text{protein}}{\text{niacin}} = \frac{\text{protein}}{B_6}$$

Therefore, we consider the seeming progressive increase in protein/$B_6$ ratios an artifact of coincidence that arises despite the random variations in lines *A* and *B*. We propose that under the conditions of these experiments *C. vulgaris* tends to maintain a protein/$B_6$ ratio of about 39,000 or 40,000:1 during the active growth period and that *C. pyrenoidosa* is characterized by a somewhat lower ratio—the order of approximately 27,000 or 28,000:1.

Table V shows the $B_6$ and the niacin content of *C. vulgaris* and of *C. pyrenoidosa* harvested at different times and of representative conventional items of diet. In examining the data in the table, allowance must be made for the fact that figures for *Chlorella* are for dried cells, whereas values for most of the other items (except rice, wheat, brewers' yeast, peas, and soybeans) are for fresh material. The water content of *Chlorella* is about 90%. Thus values expressed in terms of the fresh weight of *Chlorella* would be about 10% of those recorded in the table. It is apparent that *Chlorella* compares favorably with conventional dietary vegetable sources of the two vitamins.

Experiments were also performed with *Chlorella* cultured in a nitrogen-deficient medium—conditions under which the organisms grow very poorly but produce a higher percentage (but not absolute amount) of lipid (2). Yields were so low in this medium that neither niacin nor $B_6$ activity could be satisfactorily determined with the procedures employed.

## SUMMARY

In *C. vulgaris* and *C. pyrenoidosa* there is a continuous decrease in the concentration of niacin and of vitamin $B_6$ (mmcg./mg. dry weight of cells) during the second and third weeks of a 3-week culture period. However, despite the decrease in *concentration* of the two vitamins in the cells, there is a substantial increase in the *absolute amount* present in the cultures because of the large increase in total mass of cells during the same period.

*C. pyrenoidosa* excels *C. vulgaris* as a source of $B_6$ activity whether results are expressed in terms of concentration in the cells or of total yield from the harvested cells, although the superiority of the former species diminishes with increasing age of the cultures. The two species are approximately equivalent with respect to niacin content.

The niacin/$B_6$ ratio remains constant in both species during the culture period, but the ratio maintained by *C. vulgaris* is higher than that maintained by *C. pyrenoidosa*. Similarly, *C. vulgaris* contains more protein relative to $B_6$ than does *C. pyrenoidosa*.

As a source of $B_6$ and niacin, both species of *Chlorella* compare favorably with dietary vegetable sources.

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# Scope of Acetonitrile as a Solvent in the Nonaqueous Titration of Organic Medicinals

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**Acetonitrile has been shown to be a useful solvent for the assay of a variety of salts of organic bases. Its scope as a solvent must be investigated for each individual compound, since solubility cannot be predicted on the basis of chemical similarity. This solvent has also been demonstrated to be particularly valuable in the assay of tablets and capsules because so few excipients cause interference in it.**

ACETONITRILE has been shown to be a satisfactory solvent medium for the titration of a limited number of organic compounds (1–3). However, its use as a primary solvent for salts of medicinals had been previously applied on a small scale by Mizukami and Hirai (4). In other investigations the role of acetonitrile in the titration of organic medicinal agents has been restricted to that of a stabilizing solvent (5–7).

From the results of a recent publication (8), which dealt with the titratability of tablet excipients in a variety of organic solvents, it was decided to select those solvents in which minimal interference by excipients occurred and to determine the scope of each as a titration medium for the analysis of medicinals and their pharmaceutical forms. Acetonitrile was one of those solvents chosen for this investigation.

Received June 3, 1963, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada, and the Food and Drug Directorate, Ottawa, Ontario, Canada.
Accepted for publication July 26, 1963.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.
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The authors are indebted to the following drug manufacturers for the generous supplies of crystalline materials: Abbott Laboratories, Ltd., Montreal, for methamphetamine HCl and pramoxine HCl; Ayerst McKenna and Harrison, Montreal, for isothipendyl HCl; Burroughs-Wellcome, Montreal, for methoxamine base and chlorcyclizine HCl; Ciba and Co., Montreal, for tripelennamine HCl; Eli Lilly and Co., Toronto, for piperocaine HCl; W. S. Merrell Co., Weston, Ontario, for diperodone HCl and doxylamine succinate; Parke Davis and Co., Brockville, Ontario, for diphenhydramine HCl; Poulenc, Ltd., Montreal, for the hydrochlorides of chlorpromazine, promethazine, and prochlorperazine; Sandoz Pharmaceuticals, Montreal, for thioridazine HCl; E. R. Squibb & Sons, Ltd., Montreal, for triflupromazine HCl; The Upjohn Co., Don Mills, Ontario, for methoxyphenamine HCl and pyrathiazine HCl; John Wyeth and Brother, Windsor, for promazine HCl.

## EXPERIMENTAL

### Apparatus

An A. C. titrometer, Precision Scientific Co., equipped with a glass-calomel electrode combination, 5-ml. microburets graduated to 0.01 ml., and electromagnetic stirrers were employed.

### Reagents

Acetone A.C.S., acetonitrile A.C.S., chloroform A.C.S., glacial acetic acid A.C.S., anhydrous