(15) Price, A. H., Erf, L., and Bierly, J., *J. Am. Med. Assoc.*, **167**, 1612(1958).
(16) Gray, W. D., *Am. Pract.*, **10**, 660(1959).
(17) Herndon, J. F., Rice, E. G., Tucker, R. G., Van Loom, E. J., and Greenberg, S. M., *J. Nutr.*, **64**, 615(1958).
(18) Migden, J., *J. Am. Geriat. Soc.*, **7**, 928(1959).
(19) Eickholt, T. H., and White, W. F., *J. Pharm. Sci.*, **54**, 1211(1965).
(20) Finney, D. J., "Statistical Method in Biological Assay," Charles Griffin and Co. Ltd., London, Hafner Publishing Co., New York, N. Y., 1952.
(21) Burns, J. H., "Biological Standardization," Oxford University Press, London, England, 1937.
(22) Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Hafner Publishing Co., Inc., New York, N. Y., 1957.
(23) Eickholt, T. H., and White, W. F., *J. Pharm. Sci.* **53**, 1418(1964).

Vitamin C and Choline Content of *Chlorella vulgaris* and *C. pyrenoidosa*

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The choline content of *Chlorella vulgaris* and of *C. pyrenoidosa* (expressed as micrograms per milligram dry cell weight) remained relatively constant during a 3-week growth period. However, the concentration in the latter species exceeded slightly that of the former at all harvest times. In both species, after reaching a peak early in the culture period (5–7 days), the concentration of ascorbic acid decreased continuously. The total yield of both vitamins (micrograms per milliliter of culture) increased continuously because of the increment in cell mass.

THIS NOTE records data concerning yields of vitamin C (total ascorbic acid) and of choline from two species of unicellular green algae and is part of a continuing study of production of vitamins and accessory growth factors by *Chlorella vulgaris* and *C. pyrenoidosa* (1–5).

METHODS

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* employed, the method of maintaining stock cultures, the composition and inoculation of stock and experimental culture solutions, and the details of environmental conditions provided and of harvesting experimental cultures have been described previously (1). In summary, an inorganic medium consisting of minerals and micro-elements was inoculated with the algae and was continuously aerated with a CO_2–air mixture. Continuous illumination (600 f.c.) was provided from a Mazda source and the temperature was maintained at 20.5 ± 0.5°. Cells were harvested at intervals during the 3-week culture period and vitamin C and choline content were determined.

Assays—*Vitamin C Activity*—Since ascorbic and dehydroascorbic acids exhibit equal antiscorbutic activity, the method of Roe and Osterling (6) was applicable and was employed to determine "total ascorbic acid." This entailed extraction of cells with a metaphosphoric acid mixture and treatment with a decolorizing agent[1] which oxidized ascorbic to dehydroascorbic acid and clarified the solution. The oxidized form was then coupled with 2,4-dinitrophenylhydrazine and the resulting derivative, when treated with H_2SO_4, produced a red color which could be quantitated photometrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was assayed in duplicate for total ascorbic acid. The standard deviations of the assays were within ±5% of the mean; most were within ±2%.

Choline—Cells were autoclaved in 3% H_2SO_4 at 15 lb. pressure for 2 hr. and then were neutralized with BaOH, following the method of Horowitz and Beadle (7). After filtration, the extract was passed through a Permutit column to separate choline from methionine and to eliminate other substances which might interfere with the assay.

Choline content of the extract was determined using *Neurospora crassa* ATCC 9277 as the test organism in bacto-choline assay medium (8). Growth response of the organism was determined gravimetrically.

Aliquots of each cell suspension were extracted in duplicate and each extract was then assayed in duplicate. For all assays the standard deviation was within the range ±10% of the mean, generally falling in the range ±5% or less.

RESULTS

The maximum ascorbic acid content detected in the cells of both organisms was about 1.1 to 1.2 mcg./mg. of cell dry weight. This peak occurred in the early period of growth (5 to 7 days) and then the concentration declined continuously throughout the remainder of the 21-day culture period (Table I, A). Choline appeared more constant in concentration. The apparent trend in the average values for choline in *C. vulgaris* is not supported by the data from individual runs. Variations were random. Neither compound was detectable in the harvested culture medium.

When results are expressed in terms of micrograms per milliliter of culture, both compounds are seen to have increased continuously throughout the culture period (Table I, B). This is a reflection of the continuous increase in cell mass as growth progressed.

The levels of choline and the peak levels of ascorbic acid (Table I, A) compare favorably with data reported by Morimura (9) for short-term (2–3 days) synchronized cultures of *C. ellipsoidea*. He also found that the choline content remained fairly constant, but reported that ascorbic acid "increased considerably during the stages . . . in which the photosynthetic process occurred most actively." Confirmatory evidence for this view is provided by the facts that (*a*) in the present

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[1] Marketed as Norit A by American Norit Co., Inc., Jacksonville, Fla.

TABLE I—VITAMIN C AND CHOLINE CONTENT OF *C. vulgaris* AND *C. pyrenoidosa*[a]

Vitamin	*C. vulgaris* 5 Days	7 Days	11 Days	14 Days	18–21 Days	*C. pyrenoidosa* 5 Days	7 Days	11 Days	14 Days	18–21 Days
A. Content Expressed as mcg./mg. Dry Wt.										
C	0.984	1.181	0.679	0.507	0.469	1.104	0.885	0.585	0.515	0.386
Choline	1.783	2.365	2.400	2.617	2.611	2.952	2.912	2.767	2.676	2.879
B. Content Expressed as mcg./ml. of Culture										
C	0.345	1.186	1.675	1.626	1.936	0.613	1.111	1.504	1.818	2.065
Choline	0.538	2.383	5.941	8.386	10.736	1.505	3.797	8.142	9.532	15.065

[a] Values recorded are averages from four separate experiments, except for 5-day results which are averaged from three experiments.

TABLE II—CHOLINE AND VITAMIN C CONTENT OF *Chlorella* AND OF SOME CONVENTIONAL FOODS (mg. VITAMIN/100 Gm. DRY WEIGHT)[a]

Item	Choline	Vitamin C
C. vulgaris (7-day)	236	118
C. vulgaris (21-day)	261	47
C. pyrenoidosa (7-day)	291	110
C. pyrenoidosa (21-day)	288	39
Beef (round steak)	325	0
Lamb (chop)	300	0
Liver (beef)	1883	31[b]
Pork (chop)	166	0
Eggs (yolk)	3018	0
Milk	105	1[b]
Wheat (whole)	93	0
Barley	143	0
Rice (polished)	93	0
Soybean	305	Tr.
Cabbage	267	50[b]
Carrots	103	4[b]
Oranges	...	49[b]
Parsley	...	193[b]
Pepper, bell	...	120[b]
Potato, white	121	17[b]
Spinach	253	59[b]

[a] Values for foods from "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962, and from Sebrell, W. H., and Harris, R. S., "The Vitamins," Academic Press Inc., New York, N. Y., vols. I and II, 1954. [b] Milligrams of vitamin/100 Gm. raw edible portion.

experiments of longer duration, ascorbic acid content of the cells declined continuously after achieving a peak level early in the culture period, and (*b*) the rate of photosynthesis continuously decreases with advancing age of the culture.

In the current study, the final concentration of ascorbic acid in the cells of *C. vulgaris* was approximately 40% of the early maximum. Previous work has shown that under similar conditions (differing only in light intensity) the rate of photosynthesis at day 21 is only about 43% of the rate determined on the fourth day (10). Calculations (10), based on data reported by Sargent (11), indicate that the decrease in rate of photosynthesis in cultures of *C. pyrenoidosa* may be somewhat greater than for *C. vulgaris*, and it may be significant that in the present experiments the ascorbic acid content in cells from 21-day-old cultures of *C. pyrenoidosa* was only about 35% of the early peak concentration.

The above observations and Morimura's report (9) seem to confirm in the thallophytes the conclusion, based on work with higher plants in 1928, that light has a beneficial effect on the production of ascorbic acid (12). Many of the numerous subsequent direct and indirect verifications of that report and of an association between ascorbic acid and photosynthesis in higher plants have been cited by Mapson (13).

Table II shows that *Chlorella* compares favorably with several conventional dietary sources of choline but less favorably with the better known sources of vitamin C.

In comparing vitamin C content of the *Chlorella* species with that of the dietary sources listed, allowance must be made for the fact that values for *Chlorella* are for dried material, whereas those for the other sources are for fresh material. Recent determination of fresh and dry weights of *Chlorella* revealed approximately 75% water content (70% in *C. vulgaris* and 78% in *C. pyrenoidosa*). Therefore, vitamin C content of the two algae, expressed in terms of fresh weight would be about 25% of the figures given in the table.

REFERENCES

(1) Pratt, R., and Johnson, E., *J. Pharm. Sci.*, **52**, 979 (1963).
(2) *Ibid.*, **53**, 151(1964).
(3) *Ibid.*, **53**, 1135(1964).
(4) *Ibid.*, **54**, 871(1965).
(5) *Ibid.*, **55**, 799(1966).
(6) Roe, J. H., and Osterling, M. J., *J. Biol. Chem.*, **152**, 511(1944).
(7) Horowitz, N. H., and Beadle, G. W., *ibid.*, **150**, 325 (1943).
(8) "Microbiological Assay of Vitamins and Amino Acids," Difco Laboratories, Detroit, Mich., 1963, No. 247, p. 29.
(9) Morimura, Y., *Plant Cell Physiol.*, **1**, 63(1959).
(10) Pratt, R., *Am. J. Botany*, **30**, 404(1943).
(11) Sargent, M. C., *Plant Physiol.*, **15**, 275(1940).
(12) Heller, V. G., *J. Biol. Chem.*, **76**, 499(1928).
(13) Mapson, L. W., in "The Vitamins," Sebrell, W. H., and Harris, R. S., eds., Academic Press Inc., New York, N. Y., 1954, vol. 1, p. 220.