Production of Protein and Lipid by *Chlorella vulgaris* and *Chlorella pyrenoidosa*

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Cultures of *Chlorella vulgaris* reach the stationary phase of their growth cycle more slowly than cultures of *C. pyrenoidosa* when both are provided with conditions favorable for their growth. But after 3 weeks of incubation, the yields from the two organisms are nearly identical in terms of packed volume, dry weight, and cell population. There is only a relatively small difference in protein content—about 8 per cent in favor of *C. vulgaris*. There is a somewhat larger difference in lipid content, *C. pyrenoidosa* yielding about 11 to 12 per cent more lipid than *C. vulgaris*. Both species produce more lipid and relatively less protein with increasing age. Accumulation of lipid in excess of about 25 per cent of total dry weight is indicative of waning vigor in cultures of *Chlorella*, and a lipid content that equals or exceeds the protein content is indicative of extreme senescence. Both organisms grow poorly in a nitrogen-deficient medium but differ in their response to it. The yield from *C. vulgaris* is about 80 to 86 per cent of the yield from *C. pyrenoidosa* with respect to packed volume, dry weight, and per cent lipid. Only with respect to per cent protein are the two organisms the same. However, in absolute terms (mg./ml. of culture) *C. vulgaris* yields about 14 per cent less protein and nearly 30 per cent less lipid than *C. pyrenoidosa* in such a medium.

THE YIELD of conventional crop plants is markedly affected by the chemical composition and nutritive value of the substrate. But the ratio of protein/lipid/carbohydrate is not altered substantially. In contrast with this, it has been reported that the protein content of *Chlorella pyrenoidosa* can be varied from about 8 to 58% regularly (on one occasion a value of 88% was found) and that the lipid content can be varied from about 4.5% to more than 85% by simply altering the composition of the inorganic culture solution and properly controlling the incident light intensity, duration of culture, and other environmental factors (1). Carbohydrate concentration appears to be less amenable to alteration, the range being from about 5% to approximately 37%. All values were expressed as per cent of dry weight.

The concentration of nitrogen (or availability of nitrogen?) is reported to be a key factor. Spoehr and Milner (2) stated that high lipid values were never attained when the "residual fixed nitrogen concentration was greater than 0.001 molar" in the medium and that an initial nitrogen concentration of 0.025 *M* was sufficient to ensure that the plants would not reduce the level below 0.001 *M* and would not yield a high lipid value. Not all species of *Chlorella* respond uniformly in this respect. Such manipulation of the nitrogen concentration in the medium does not induce equivalent changes in the proportion of fatty acids produced by *C. vulgaris* (3).

The above observations, if regularly reproducible, would have potential practical import as well as theoretical interest. In the United States, Canada, Great Britain, Japan, and Israel there has been considerable interest in mass culture of *Chlorella* and other unicellular algae as potential future sources of food or food supplements. When the human race can no longer afford the inefficient luxury of the vast land areas and relatively long growth periods required to produce grain and meat and other conventional present-day foods, it may become necessary to resort to mass culture of unicellular algae to provide adequate sustenance for a large portion of the world population. It would be of considerable value to have knowledge of practical means for controlling the protein/lipid/carbohydrate ratio of the crop so that a custom-tailored diet, with respect to these major food components, could be produced. It will be important to know also how different species of algae compare with each other and with existing foods as sources of vitamins and of accessory growth factors. Some quantitative studies of vitamin content of algae and a few nutritional studies with animals have been reported (4–7), but no extensive or systematic evaluation has been published, especially with respect to changes that might occur with alteration of the basal composition of the harvest. The present paper deals with protein and lipid yields from *C. pyrenoidosa* and *C. vulgaris* cultured in media reputed to favor high protein and high lipid yields, respectively.

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EXPERIMENTAL

Materials and Methods

Organisms.—The strain of *C. vulgaris* employed

has been maintained in the laboratory since 1931 when it was isolated from a pond in Van Cortlandt Park, New York City. Identification was verified at that time by the late Professor Tracy Hazen of Columbia University. *C. pyrenoidosa* (an Emerson strain designated No. C-1-1-1 in the algal culture collection of the Kaiser Foundation Research Institue, Richmond, Cal.) was obtained through the courtesy of Dr. Mary Belle Allen of that institution.

Culture Conditions.—Inocula for experiments were always taken from 4-day stock cultures (inoculated from a long series of 4-day cultures) in liquid medium and were adjusted to provide an initial population of 100 cells/mm.^{3} (100,000/ml.). Stock cultures were illuminated continuously by a water-jacketed, 500-watt incandescent Mazda lamp. The total light intensity (as measured by a Weston photoelectric cell light meter, model 603) was 1,250 f.-c. at the position of the culture flasks. A mixture of 5% CO_2 and 95% air was bubbled through the cultures continuously. The temperature varied between 20 and 22°.

The standard solution for stock cultures and the reference base for experiments consisted of:

KNO_3	0.025 *M*
$MgSO_4 \cdot 7H_2O$	0.020 *M*
KH_2PO_4	0.018 *M*
$FeSO_4 \cdot 7H_2O$	5×10^{-5} *M*
$K_3C_6H_5O_7 \cdot H_2O$	5×10^{-5} *M*
Zn(as $ZnSO_4 \cdot 7\ H_2O$)	0.400 p.p.m.
Cu(as $CuSO_4 \cdot 5H_2O$)	0.004 p.p.m.
Mn(as $MnSO_4 \cdot 4H_2O$)	0.400 p.p.m.
B(as H_3BO_3)	0.020 p.p.m.

A solution containing the three major salts was sterilized in the autoclave (15 minutes at 18-lbs. pressure). The other salts were incorporated in a single solution which was sterilized by filtration and then added aseptically to complete the formulation. The pH of the uninoculated medium is 4.5 to 4.6. As the culture develops, the pH rises to a value of 7.0 to 7.5 in 2 weeks and tends to remain at that level, even with prolonged incubation.

Columnar Pyrex culture vessels (60.5 cm. tall × 43 cm. diam. and each containing 500 ml. of culture medium) were arranged in a circle around a central, water-cooled incandescent light bank consisting of four clear, 60-watt Lumiline show case lamps, which provided 600 f.-c. continuously at the culture vessels. The temperature was 20.5 ± 0.5°.

A 5% CO_2–95% air mixture was introduced continuously through a sintered-glass sparger at the bottom of each vessel and bubbled up through the cultures in a finely dispersed stream which served the dual purpose of providing CO_2 for photosynthesis and retarding settling of the cells. Even so, daily shaking was necessary to maintain a uniform suspension.

Under exceptional circumstances, the cultures developed a population density of about half a million cells/mm.^{3} (actual maximum count = 506 million/ml.) after 3 weeks of growth; but the usual plateau level attained at that time was about 330 to 340 million cells/ml.

The C-2 formulation of Spoehr and Milner (2), henceforth designated SM, was selected as the high-lipid medium. It contains

$(NH_4)H_2PO_4$	0.000825 *M*
$(NH_4)_2HPO_4$	0.000715 *M*
KCl	0.03 *M*
$MgSO_4 \cdot 7H_2O$	0.01 *M*
KH_2PO_4	0.01 *M*
[Fe]	5×10^{-6} *M*

No trace elements (other than iron) were added by the original formulators, since they used "local tap water" which had a "fairly high mineral content," and they found that "the yields of *Chlorella* indicated that the water was adequate for providing micro-elements." However, media for the present experiments were prepared with distilled water. Therefore, micronutrients were added in the same concentration as for the standard medium. Thus, the concentration of iron added in our experiments was ten times that added by Spoehr and Milner. Since the iron content of the tap water they employed is not known, the precise difference in concentration of iron in the two sets of experiments cannot be stated. But it is reasonable to assume that it was at least several-fold higher in our experiments than in theirs. The initial pH of the SM medium before inoculation is 5.9. This value drops rapidly as the organisms begin to grow and soon stabilizes at about pH 3.5. This is consistent with the earlier observations of Pratt and Fong (8).

Analytical Methods

Upon harvest, the cell suspensions were centrifuged and the cell mass was resuspended and washed in distilled water. In harvesting cultures that had nitrogen supplied as ammonium salts, the cells were washed repeatedly until the supernatant gave a negative reaction with Nessler's reagent.

After the final washing, the cells were resuspended in distilled water (0.1 the original harvested volume). Aliquots of this tenfold concentrated suspension were used for determination of packed volume and dry weight by conventional methods. Cell counts were determined with a haemocytometer from the original cell suspension before centrifuging.

Protein was determined by the standard Kjeldahl micro-method (range 10 to 40 mcg. nitrogen) as outlined by Umbreit, *et al.* (9), with modifications adapted from Johnson (10) and Schmidt (11).

Lipids were determined by a procedure adapted from Milner (12), using a paste of freshly harvested cells [100–500 mg. (dry wt.)]. The cell mass was extracted twice with methanol under reflux; then extracted by refluxing three times each with ethyl ether and methanol, alternately, over a period of 2 days. The several fractions were filtered, pooled, and evaporated to dryness. The dry residue was re-extracted with ethyl ether three times to eliminate methanol-soluble sugars. The final ether extract was evaporated to dryness and brought to constant weight in a vacuum desiccator. A fresh 30-ml. portion of solvent was used for each extraction throughout the procedure.

Precision of Methods and Reproducibility of Data

In work of the type reported below it is important to indicate the degree of reproducibility of the data (adequacy of sampling) and the precision of the analytic procedures employed to obtain them (methodology). Among factors affecting adequacy

of sampling we include all the numerous physical factors and manipulative procedures to which the cultures are subjected.

The coefficient of variability (standard deviation expressed as a percentage of the mean $=v$) was chosen as the most appropriate statistical indicator because it relates not only to the reproducibility of one set of data, but also indicates the reliability and reproducibility of several kinds of data. In general, variabilities of two or more distributions, regardless of their units of measurement, may be considered to be related to each other as the corresponding values of v. The procedures upon which the conclusions presented below are based were determinations of dry weight, nitrogen, and lipid. Run on aliquots of a given sample, such determinations routinely yielded highly reproducible values. The critical factors affecting reproducibility of data are in the cultures. Inherent biologic variation cannot be ruled out of any work with living organisms, but strains of *Chlorella* that have been under long laboratory cultivation appear to be quite stable. We believe that the major sources of variability are in certain physical culture conditions which cannot reasonably be controlled with absolute precision and the extensive but necessary manipulation of the cultures from the time of set-up through the time of harvest and preparation of the cells for analysis. In any given run of replicate cultures, some variation occurs. Values of v calculated for duplicate to quadruplicate samples from each of ten cultures were within the following limits: mg. dry wt./ml., 0 to 1.3%; mcg. nitrogen/ml., 0.5 to 5.1%; mg. lipid/ml., 0 to 5.5%.

When averaged data from several series of cultures in different experiments conducted for different lengths of time are compared, larger coefficients of variability are to be expected. Values of v for the data tabulated under *Results* were within the following limits: mg. dry wt./ml. 14 to 14.2%; protein as per cent of dry weight, 6.7 to 8.6%; and lipid as per cent of dry weight, 7.2 to 18%. Packed volume and cell counts are recorded in some of the following tables primarily to round out the picture; they did not form the basis for the specific conclusions reached. The coefficients of variability for these potential indicators of growth in all of the experiments were within the following limits: packed volume, 9.6 to 23.4%; cell count 17.1 to 17.5%.

RESULTS

Averaged data from 12 replicated experiments are summarized in Table I. The comparative data in Table II parts A and B, (based on the figures in Table I) indicate, respectively, the relative value of the standard and the "high-lipid-inducing" media for each organism and the relative performance of the two organisms in each medium.

The tables show clearly the superiority of the standard over the SM medium for growth of both species of *Chlorella*, as indicated by packed volume, dry weight, and protein content. Only with respect to lipid do the cells produce a lower percentage yield in the standard medium than in SM (Table I and Table II, part A, columns *1–4*). The tables also show differences in the responses of the two organisms to the different media, as discussed below.

Packed Volume and Dry Weight.—After 2 weeks, protein content, dry weight, and packed volume of *C. vulgaris* cultured in standard medium are about three to four times those produced in SM medium (Table II, part A, column *1*). After another week, the differences are approximately 3- to 5.5-fold in favor of the standard (Table II, part A, column *2*). A similar trend, differing only in detail, occurs in cultures of *C. pyrenoidosa* (Table II, part A, columns *3* and *4*). During the third week of incubation, the increase in relative dry weight in the standard *versus* the SM medium is less for *C. pyrenoidosa* than for *C. vulgaris;* during the same period the relative values for packed volume and protein decrease in cultures of *C. pyrenoidosa*, while they increase in cultures of *C. vulgaris*. In other words, the superiority of the standard medium over the "high lipid" medium for supporting *C. vulgaris* is more apparent after 3 weeks than after 2 weeks, but the same is not true for cultures of *C. pyrenoidosa*. This can be ascribed, in part, to more rapid attainment of "maturity" by cultures of the latter organism than of the former when both are in standard medium.

For example, the yield in terms of packed volume and dry weight is essentially the same from both organisms at 3 weeks in standard medium (Table I, columns *2* and *6*; Table II, part B, column *6*). But *C. pyrenoidosa* produces about 90% of its packed volume and about 80% of its dry weight in the first 2 weeks of growth (compare columns *5* and *6*, Table I), while the corresponding figures for *C. vulgaris* are 70 and 64% (compare columns *1* and *2*, Table I). Stated another way, *C. vulgaris* still has 30 to 35% of its growth to produce in the third week under the conditions described, but *C. pyrenoidosa* has only 10 to 20% of its yield remaining to be produced. Because of this difference in the growth pattern of the two organisms in the standard medium, and since neither of them makes a substantial gain in packed volume or dry weight during the third week in SM medium (Table I, columns *3*, *4*, *7*, and *8*),

TABLE I.—YIELDS FROM *C. vulgaris* AND *C. pyrenoidosa* IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA (LIGHT = 600 F.-C.)

	C. vulgaris				*C. pyrenoidosa*			
	Std. Medium		SM Medium		Std. Medium		SM Medium	
	13–14 days (*1*)	20–21 days (*2*)	13–14 days (*3*)	20–21 days (*4*)	13–14 days (*5*)	20–21 days (*6*)	13–14 days (*7*)	20–21 days (*8*)
Packed vol. (ml./65 ml. of culture)	0.848	1.200	0.198	0.215	1.100	1.186	0.24	0.27
Dry weight (mg./ml. of culture)	2.623	4.134	0.842	0.896	3.316	4.182	1.017	1.039
Cell count (per mm.^{3})	213,600	338,857	n.c.[a]	n.c.[a]	183,042	332,750	n.c.[a]	n.c.[a]
Protein (% dry wt.)	52.7	48.58	17.7	15.6	56.46	44.83	16.2	15.6
Lipid (% dry wt.)	21.3	20.45	30.7	37.7	21.8	23.1	37.9	44.8

[a] n.c. = Cells not countable because of adhesion and excessive clumping.

TABLE II.—A, RELATIVE VALUE OF STANDARD (STD.) AND OF "HIGH-LIPID-INDUCING" (SM) MEDIA FOR SUPPORTING *C. vulgaris* AND *C. pyrenoidosa*. B, YIELDS OF *C. vulgaris* WITH RESPECT TO YIELDS OF *C. pyrenoidosa* IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA

	A				B			
	C. vulgaris		*C. pyrenoidosa*		Std. Medium		High-Lipid-Inducing Medium	
	Std./SM[a]		Std./SM[a]		*vulgaris/pyrenoidosa*[b]		*vulgaris/pyrenoidosa*[b]	
	13–14 days (1)	20–21 days (2)	13–14 days (3)	20–21 days (4)	13–14 days (5)	20–21 days (6)	13–14 days (7)	20–21 days (8)
Packed vol.	4.28	5.58	4.58	4.40	0.77	1.01	0.82	0.80
Dry wt.	3.12	4.61	3.26	4.02	0.79	0.99	0.82	0.86
Cell count	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	1.17	1.02	n.d.[c]	n.d.[c]
Protein[d]	2.97	3.11	3.48	2.87	0.93	1.08	1.09	1.00
Lipid[e]	0.69	0.54	0.57	0.52	0.98	0.89	0.81	0.84

[a] Std./SM = Yield in standard medium ÷ yield in SM medium. [b] *vulgaris/pyrenoidosa* = Yield from *vulgaris* ÷ yield from *pyrenoidosa*. [c] n.d. = Not determined because cells in SM medium too clumped to permit accurate counting. [d] Calculated from protein expressed as per cent of dry weight. [e] Calculated from lipid expressed as per cent of dry weight.

the disparity between the two media after 3 weeks will be greater for *C. vulgaris* than for *C. pyrenoidosa*. (See Table II, part A, columns *2* and *4*).

Protein Content.—The protein content (expressed as per cent of dry weight of the cells) of both organisms decreases during the third week, regardless of the medium (Table I). However, when the magnitude of the increase in dry weight during that period is taken into consideration, it is found that in terms of absolute yield of protein *C. vulgaris* shows a substantial gain in the standard medium (1.38 mg./ml. of culture at 2 weeks *versus* 2.01 mg./ml. at 3 weeks), whereas cultures of *C. pyrenoidosa* remain virtually static in this respect (1.872 *versus* 1.874), as do both organisms in SM medium (*C. vulgaris* 0.149 *versus* 0.140 at 2 weeks and 3 weeks, respectively: *C. pyrenoidosa* 0.164 *versus* 0.162).

The protein content of *C. vulgaris* in standard medium at 3 weeks is only 3.75% higher than that of *C. pyrenoidosa* when expressed as per cent of dry weight (Table I) but in relative terms (Table II, part B, column *6*) and in terms of absolute yields (2.01 *versus* 1.87 mg./ml. of culture) is about 8% higher. This is just the reverse of the situation at 2 weeks (Table I, column *5* minus column *1;* and Table II, part B, column *5*) and may be taken as further evidence that cultures of *C. vulgaris* attain full productivity more slowly than those of *C. pyrenoidosa* in the standard medium.

Lipid.—*C. pyrenoidosa* has a higher lipid content than *C. vulgaris* at 2 weeks and at 3 weeks, regardless of the medium employed and of whether values are expressed as per cent of dry weight or in absolute terms of mg. of lipid per ml. of culture; both organisms produce more lipid, relative to their dry weight, in SM medium than in the standard (Table III). The percentage lipid content of both organisms grown in SM medium is approximately two to three times their protein content (Table I).

Chlorella cells in SM medium are distinctly abnormal in comparison with those in standard medium. Normal, healthy cultures are a clear, pure green, and the cells are separate, discrete and, when the cultures are shaken, readily form a uniform suspension. Although there is some tendency for settling out, a large proportion of the cells remain in suspension. The cells do not clump, and haemocytometer counts of cell population can be made satisfactorily. Viewed under the microscope, the cells are smooth and rounded; they present a clear, sharp outline.

In contrast to the above, cultures in SM medium

TABLE III.—LIPID CONTENT OF *Chlorella* CULTURES IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA (LIGHT = 600 F.-C.)

Lipid Content	Std. Medium 13–14 days	Std. Medium 20–21 days	SM Medium 13–14 days	SM Medium 20–21 days
	C. vulgaris			
As % of dry weight	21.3	20.45	30.7	37.7
Mg./ml. of culture	0.559	0.845	0.258	0.338
	C. pyrenoidosa			
As % of dry weight	21.8	23.1	37.9	44.8
Mg./ml. of culture	0.723	0.966	0.385	0.465

acquire a distinctly yellowish cast after 5 to 6 days. About 10 days after inoculation cells no longer form a uniform suspension when the cultures are shaken. They are clumped in irregular aggregates, probably containing from a few thousand to several million cells, and the aggregates cannot be separated sufficiently to permit accurate or reproducible counts using the haemocytometer method. The clumps do not long remain in suspension: either they settle out quickly or rise with the gas stream to the surface of the culture where most of them adhere to the sparger inlet tube or to the side of the culture vessel. Many cultures (especially of *C. vulgaris*) soon become virtually water-clear except for a short space near the bottom of the vessel and for the cells adhering to the sparger tube or culture wall at the surface of the medium. Microscopic examination shows the margins of the cells to be wrinkled and convoluted. The cells are several times larger than cells of comparable age in standard medium and each cell appears to contain many smaller cells, often containing still smaller units, as though division had not been completed or, if completed, had not been followed by release of the daughter cells. The pattern is reminiscent of that seen in aged cultures that have been in standard medium for many weeks. It is obvious that the cultures in SM medium are unhealthy, if not actually moribund.

Others also have commented on the altered appearance of cultures in high-lipid-inducing media, noting their yellowish color, changes in the character of the cells, and suggesting that "cells with a high lipid content carry on photosynthesis with a chlorophyll content 1/500 to 1/2000 that of cells with low lipid content" (2). It has been stressed

TABLE IV.—A, DRY WEIGHT, PERCENTAGE PROTEIN, AND LIPID YIELDS FROM *C. vulgaris* AND *C. pyrenoidosa* EXPOSED TO HIGH LIGHT INTENSITY (1250 F.-C.). B, YIELD OF PROTEIN AND LIPID EXPRESSED IN ABSOLUTE TERMS (MG./ML. OF CULTURE)

	C. vulgaris				*C. pyrenoidosa*			
	Std. Medium		SM Medium		Std. Medium		SM Medium	
	2 wks. (*1*)	6 wks. (*2*)	2 wks. (*3*)	6 wks. (*4*)	2 wks. (*5*)	6 wks. (*6*)	2 wks. (*7*)	6 wks. (*8*)
A								
Dry weight (mg./ml. of culture)	2.373	5.009	1.173	1.216	2.276	5.573	1.051	1.271
Protein (% dry wt.)	50.5	32.2	13.6	12.5	56.6	27.8	15.2	10.5
Lipid (% dry wt.)	7.5	19.7	16.8	29.4	13.3	26.2	24.3	43.0
B								
Protein (mg./ml. of culture)	1.198	1.613	0.160	0.152	1.276	1.549	0.160	0.133
Lipid (mg./ml. of culture)	0.178	0.987	0.197	0.358	0.303	1.460	0.255	0.547

that to induce development of high-lipid-cells a long growth period and high light intensity are required in addition to low nitrogen concentration in the medium (2). A precise quantitative definition of high light intensity was not given, but calculations based on informed estimates suggested that it was approximately comparable to the 600 f.-c. level adopted for the present work. However, since the maximum lipid value found in the present investigation was 20 to 30% lower than the values reported by the previous workers for a similar medium, it seemed desirable to extend the culture period and to increase the incident light.

Replicate cultures of *C. vulgaris* and of *C. pyrenoidosa* were started in standard and in SM media and were exposed to 1250 f.-c. The column culture vessels were replaced by Fernbach flasks illuminated from below by a water-screened bank of thirty 100-watt Mazda lamps and by Florence flasks placed around a central, water-jacketed 500-watt Mazda lamp. These conditions more nearly simulated those provided by the earlier investigators. The conventional 5% CO_2 in air was provided. The temperature was 22.5 ± 2°.

All cultures in SM medium became progressively more unhealthy, flocculent, and "scummy" looking after the second week. It was mentioned above that (column) cultures with 600 f.-c. illumination acquire a yellowish cast after 5 to 7 days. In the (flask) cultures provided with higher light intensity, the cells become distinctly yellow—so much so that the pellet remaining after cultures are spun down in the centrifuge is clearly yellow with no trace of green. Microscopic examination revealed no contamination. Results from the cultures harvested after 2 weeks and 6 weeks are shown in Table IV.

The dry weight of *C. pyrenoidosa* in SM medium increased only 20% between the end of the second and sixth weeks (Table IV, part A, column *8* ÷ column *7*). In standard medium it increases approximately 145% in the same period (column *6* ÷ column *5*). In both media, the lipid content of the organisms increases considerably during this period, regardless of whether the yields are expressed in terms of per cent of dry weight (Table IV part A, columns *5–8*) or in terms of absolute values (Table IV, part B). However, in terms of the actual amount of lipid formed between the second and sixth weeks, the increase was almost four times greater in the standard medium—1.157 *versus* 0.292 mg./ml. of culture. It was greater on a relative basis also—4.8-fold *versus* 2.1-fold (compare Table IV, part B, column *6* ÷ column *5* with column *8* ÷ column *7*).

C. vulgaris is far less prone than *C. pyrenoidosa* to produce lipid in either medium (Table IV, parts A and B) but, even so, with *C. vulgaris* also the absolute increase in lipid was greater in the standard medium than in SM—0.809 *versus* 0.161 mg./ml. of culture (compare Table IV, part B, column *2* minus column *1* and column *4* minus column *3*). The ratio of the increases in the two media is approximately 5 to 1 in favor of the standard, a figure remarkably close to the 4.8 to 1 ratio found with *C. pyrenoidosa*.

Both organisms, regardless of the medium, give substantially lower protein yields (as per cent of dry weight) after 6 weeks than after 2 weeks (Table IV, part A). However, in terms of the actual amount of protein recoverable from the cells, both organisms make a slight gain during the extended growth period in the standard medium (Table IV, part B). But in SM medium, despite the smaller percentage decreases (Table IV, part A), there is an actual loss of protein (Table IV, part B). This emphasizes the necessity of considering absolute as well as relative yields in experiments of this kind.

The increase in protein, expressed in absolute terms of mg./ml. of culture, between the end of the second and sixth weeks in standard medium (Table IV, part B), accounted for approximately 16% of the total increase in dry weight (Table IV, part A) of *C. vulgaris* and about 8% of the total increase in dry weight of *C. pyrenoidosa*. But increase in lipid calculated in the same way accounted for 31% of the increase in total dry weight of *C. vulgaris* and about 35% of the increase in total dry weight of *C. pyrenoidosa*. Stated another way, the lipid yield from *C. vulgaris* in standard medium doubled with respect to protein yield between the end of the second and sixth weeks and the yield from *C. pyrenoidosa* quadrupled.

DISCUSSION

The experiments described above confirm the observation that *Chlorella* has a higher lipid and lower protein content (both expressed as per cent of dry weight) when cultured in a nitrogen-deficient medium than when provided with an adequate amount and form of nitrogen. However, the experiments show clearly that, important as percentages may be in terms of fundamental biology or biochemistry, values expressed in percentages provide only a partial and sometimes misleading picture. Two examples will be cited. Others have been pointed out

above, and still others can be found by making appropriate calculations from the tabulated data.

Percentage data in Table I indicate less than 4% difference in per cent protein of *C. pyrenoidosa* and of *C. vulgaris* after 2 weeks in column cultures with standard medium and 600 f.-c. illumination. But in terms of actual amount of protein (mg./ml. of culture), the yield from the former was about 35% greater than from the latter. The discrepancies become even greater when performance in different media is considered. After 6 weeks in SM medium in flask cultures with twice as much light, the lipid content of *C. pyrenoidosa* was 43% *versus* 26.2% for the same organism in standard medium—a difference of 16.8% in favor of SM medium. But the actual amount of lipid present in the cultures in standard medium was more than 2.5 times the amount present in cultures reared in the nitrogen-deficient medium, and, as was pointed out above, the actual production of lipid during the last 4 weeks of cultivation was nearly five times as great in the standard as in the nitrogen-deficient medium. It is evident that if one wished to produce fats microbiologically using *Chlorella* as the biosynthetic mediator, much greater yields could be obtained in less time from healthy cells cultured in the standard medium than from the unhealthy cells produced in the SM medium—the higher percentage yields from the latter notwithstanding. It should be pointed out that the composition of *Chlorella* lipids may vary depending on the medium in which the cells develop. Milner (12) noted that the fatty acid content of lipid from high-lipid cells was about three times that of the lipid from low-lipid cells but that the unsaponifiable fraction of the latter was more than three and one-half times greater than that of the former. Even so, in view of the very poor development in SM medium, it is likely that normal cells in standard medium would provide higher absolute yields of any of the lipid fractions than would cells produced in the inferior medium.

Although confirming that *Chlorella* produced in SM medium has a higher percentage of total lipid than those produced in the standard medium, we have been unable to obtain percentage values as high as those reported by Spoehr and Milner (2). An unequivocal explanation for the apparent discrepancy cannot be offered because the experimental conditions used by them cannot be duplicated due to the lack of specific details with respect to light intensity, concentration of trace elements in the medium, and other important factors. It is not possible to obtain a culture of *C. pyrenoidosa* that can be stated authoritatively to be the same Emerson strain as the one with which they worked. It is not clearly stated in the Spoehr and Milner paper that most of their cultures were "aerated" with a carbon dioxide–nitrogen mixture, and we learned this inadvertently only after the present work (employing the conventional carbon dioxide–air mixture, was completed. Whether substitution of nitrogen for air in the "aeration" mixture has a significant effect on the formation of lipid and protein and on the ratio of one to the other is under investigation.

It has been stated that "a culture with any previously obtained R-value [which may be taken as an indicator of per cent lipid] could be grown again at will" (2). In the light of our experiments, we interpret that statement to mean that a given strain of *Chlorella* tends to be genetically stable and remarkably uniform in its response to alterations in the environment. One such response to deficient nitrogen supply is an increased percentage of lipid in the cells—a phenomenon that is a concomitant of senescence and waning vigor and occurrence of which is accelerated in the unfavorable high-lipid-inducing medium.

SUMMARY

Two species of unicellular green algae, *C. vulgaris* and *C. pyrenoidosa*, have been compared with respect to production of protein and of lipid in two different media—one reputed to favor development of high protein content and the other to favor high yields of lipid. The two species were found to differ in their rates of achieving full development and in their tendency to produce protein and lipid.

The standard medium supports healthy, vigorous growth of both species. In this medium *C. pyrenoidosa* attains full development more rapidly than *C. vulgaris*, but the latter has a higher protein content, both in terms of percentage of dry weight and in terms of absolute amount. *C. pyrenoidosa* yields more lipid, both in terms of percentage and of absolute amount. The yield of protein, as per cent of dry weight but not in absolute terms of mg./ml., decreases as cultures of either organism approach full development. Concurrently, the lipid content increases in both species.

When the source of nitrogen is sufficiently limited (situation reputed to favor development of high lipid content) the cultures soon become yellow and sickly. Under these conditions, the lipid content of both organisms (expressed as per cent of dry weight) is approximately two or more times that found in normal healthy cultures of the same age. But the absolute amount of lipid present at full development is markedly less than in normal cultures because of the very much lower dry weight produced under these conditions.

The lipid content of *C. pyrenoidosa* was consistently higher than that of *C. vulgaris* regardless of the culture medium used and whether the content is expressed as percentage of dry weight or in terms of absolute yield.

All the experiments indicated that substantial increase in the proportion of total dry weight accounted for as lipid accompanies aging of the cultures of both species. When the lipid content, expressed as percentage of total dry weight, closely approaches, equals, or exceeds the protein content, the cells are extremely senescent, if not actually moribund.

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