enzyme dealing with these substances is so widespread. Possibly there are animals in which D-amino-acids are metabolized either as constituents of the diet or as products of intermediate metabolism.

SUMMARY

1. The enzyme D-amino-acid oxidase occurs in the liver of two cephalopods, *Sepia officinalis* and *Octopus vulgaris*. The substrate specificity of the enzyme has been studied in tissue homogenates. The

Octopus enzyme is also present in the extract of an acetone-dried powder of the liver.

2. The enzyme was also found in the 'liver' of a gastropod species, *Helix*.

3. The 'livers' of two lamellibranchs were examined. The enzyme was found in *Mytilus edulis*, but it was not found in *Anodonta*.

The authors are grateful to the Director and Staff of the Marine Biological Laboratory for help and facilities offered. They also wish to thank Miss Jean Himms for technical assistance.

REFERENCES

- Bender, E. A. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
 Blaschko, H. & Hawkins, J. (1951). *Biochem. J.* **49**, xlv.
 Duchâteau, G., Sarlet, H., Camien, M. N. & Florkin, M. (1952). *Arch. int. Physiol.* (in the Press).
 Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **219**, 191.
 Krebs, H. A. (1948). *Biochem. Soc. Symp.* **1**, 2.
 Warburg, O. (1948). *Wasserstoffuebertragende Fermente*. Berlin: W. Saenger.
 Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
 Yonge, C. M. (1937). *Biol. Rev.* **12**, 87.

The Effect of Age on the Bulk Protein Composition of *Chlorella vulgaris*

By L. FOWDEN

Department of Botany, University College, Gower Street, London, W.C. 1

(Received 30 January 1952)

The effect of time of harvesting upon the proportions of the total protein nitrogen found as amide N, 'basic N' and monoamino N in the leaves of higher plants has been studied by Chibnall (1922, 1926) and Pearsall (1931). Such investigations have been continued by Lugg & Weller (1941, 1948) who determined the percentages of certain amino-acids (tryptophan, methionine and cystine-cysteine) in leaf-protein fractions. Methionine and cystine contents of the proteins of pasture plants harvested at different times have also been determined by Smith & Wang (1941).

The variation of protein composition with age in members of the lower plant orders has been little investigated. Several workers (Camien, Salle & Dunn, 1945; Freeland & Gale, 1947) have studied the protein composition of various micro-organisms, and the effects produced by variations in the culture media. The effect of the age of the culture upon the protein composition was not, however, investigated, the cultures normally being harvested during their exponential phase of growth. Fowden (1951a) has reported the composition of the bulk protein of the alga *Chlorella vulgaris* during its phase of exponential growth; Mazur & Clark (1938, 1942) earlier reported the protein composition of several algae at undefined stages of their growth.

The present paper presents the results of an investigation of the protein composition of *Chlorella* and includes complete analyses of protein fractions obtained from cultures of widely differing ages. In addition to contributing to knowledge of the nitrogen metabolism of this alga, the analyses can be expected to provide data for assessing the merits of *Chlorella* cultures of different ages if used as a source of food, as envisaged by several authors (Spoehr & Milner, 1948; Meier, 1949; Pearsall & Fogg, 1951; Geoghegan, 1951).

EXPERIMENTAL

Growth of alga. *C. vulgaris* was grown on the medium of Pearsall & Loose (1936) to which was added 1 ml./l. of trace-element solution A4 (Arnon, 1938) containing B, Mn, Cu and Zn. Into each one of a batch of penicillin-culture flasks were introduced 500 ml. of the medium; they were then autoclaved for 20 min. at a pressure of 12 lb./sq. in. The medium, which was heavily buffered with phosphate, usually had a pH of 6.1–6.2 at this stage. Each flask was then inoculated with 1 ml. of an actively growing *Chlorella* culture containing about 5000 cells/cu.mm. The flasks, now containing about 10 cells/cu.mm., were maintained at $20 \pm 2^\circ$ under a continuous uniform illumination of 150 ft.c. Growth was allowed to proceed for various periods of time, the flasks being gently shaken twice per day.

Harvesting. At selected intervals of time, the contents of one flask were harvested. The flask was gently shaken to obtain a uniform suspension and a few ml. of the culture were removed for haemocytometer cell counts. The remainder of the culture was centrifuged at 500 *g* for 5 min., after which the supernatant medium could be decanted without disturbing the packed cells. The supernatants obtained in this way were preserved under toluene at 3° until the necessary analyses could be performed. The cells were washed twice by centrifugation with distilled water. A small sample was then removed, dried at 95°, ground to a fine powder, and then stored for determination of total N. The remaining cells were immediately treated as described later for protein extraction. During the first few days of growth, the contents of several flasks had to be combined in order to obtain sufficient material for a successful protein extraction to be performed. The maximum age of any culture examined was 5 weeks. Additional culture flasks were harvested at intervals to determine more accurately the rate of growth of the *Chlorella* and the rate of absorption of N from the medium by the cells. No attempt was made to extract the protein from the cells in these additional cultures.

Nitrogen absorption from the medium. Equal amounts of $\text{NH}_3\text{-N}$ and $\text{NO}_3^-\text{-N}$ were supplied as NH_4NO_3 in the medium, this being the only source of N. The assimilation of each form of N by the *Chlorella* was determined as the difference between the amounts originally present in the media and those remaining after harvesting the cells.

The $\text{NH}_3\text{-N}$ present in 5 ml. of each medium was removed by distillation with MgO in a Markham still, the NH_3 being collected in the boric acid-indicator mixture of Conway (1947), and determined by titration against 0.01 *N*-HCl.

The $\text{NO}_3^-\text{-N}$ present in 0.25 ml. of each medium was determined colorimetrically using phenoldisulphonic acid (Snell & Snell, 1949). After making alkaline, the volumes were made up to 25 ml., and the absorption measured at $\lambda=440\text{ m}\mu$. in a Unicam spectrophotometer. It was observed that the optical density of the colour produced in the presence of the other constituents of the medium was less than that produced from the same amount of $\text{NO}_3^-\text{-N}$ when NH_4NO_3 was used in pure solution. Thus the optical densities corresponding to known amounts of nitrate had to be determined by difference between the intensities of the colours produced from 0.25 ml. of each medium, and those obtained after the addition of standard amounts of $\text{NO}_3^-\text{-N}$ to further 0.25 ml. samples of the media.

Protein extraction, hydrolysis and analysis. The procedures employed for the extraction, hydrolysis and subsequent chromatographic amino-acid analysis of the protein fractions from the harvested cells were those described in an earlier paper (Fowden, 1951*a*) with the following slight modifications and additions. In the case of cultures having a cell count greater than 35 000/cu.mm., the freshly harvested cells were suspended in a volume of borax solution somewhat greater than the 35 ml. used previously, in order to maintain the high percentage of cell breakdown during the shearing process. Total N determinations were performed on each of the protein fractions isolated.

All the protein fractions were hydrolysed without any trace of humin formation, with the exception of one fraction which had a very low N content of 8.6%. In this case a small amount of humin was precipitated during the course of the hydrolysis, but this resulted in a loss of less than 1% of the total protein nitrogen.

In order to confirm the histidine contents of the different protein fractions obtained using the paper-chromatographic method of assay, additional estimates were obtained using the modified Pauly colorimetric method of Macpherson (1946). The method was subject to interference from tyrosine, which produced a colour similar to that given by histidine with the Pauly reagent. The sensitivity for tyrosine was found to be only 28% of that obtained with histidine, and so accurate corrections could be applied to the histidine values for the known amounts of tyrosine in each protein hydrolysate.

Proline estimations were carried out after the chromatographic separations using the colorimetric method of Fowden (1951*b*), the final colours being read at $\lambda=440\text{ m}\mu$. Although the sensitivity of this method for proline was only one-third of that obtained for the α -amino-acids, the method was found to be more reliable than the copper colorimetric method used previously for proline assay (Fowden, 1951*a*).

Total N determinations. These were done by a micro-Kjeldahl method.

Amide N. Values were obtained by estimating the NH_3 produced during hydrolysis with *N*- H_2SO_4 for 3 hr. at 100° by the nesslerization procedure of Umbreit, Burris & Stauffer (1945).

RESULTS

Growth of *Chlorella*. No significant lag phase in the growth of the *Chlorella* was observed after the initial inoculation of the culture flasks. The cell count increased in an exponential manner during the first 7 days of growth, when the cell concentration was about 15 000/cu.mm. After this time, the frequency of cell division decreased, maximum growth being recorded at about 20 days when the cell count had reached approximately 68 000/cu.mm. In older cultures, cell breakdown had commenced with a consequent reduction in the cell counts, which at the end of the 5-week growth period had fallen to 40 000/cu.mm.

The nitrogen available in the medium appeared to determine, at least in part, the amount of growth possible; virtually all the nitrogen present in the medium had been assimilated by the cells in the first 13 days of growth, when the cell count had reached 58 000/cu.mm. The further growth beyond this point must have occurred by a process in which some of the limited store of soluble nitrogenous compounds within the cells was utilized for new protein synthesis.

During the early stages of growth, the $\text{NH}_3\text{-N}$ was used much more rapidly than the $\text{NO}_3^-\text{-N}$. At the end of the phase of exponential growth (7 days) one-half of the $\text{NH}_3\text{-N}$ supplied had been absorbed, whilst only about one-eighth of the total $\text{NO}_3^-\text{-N}$ had left the medium. The remaining $\text{NH}_3\text{-N}$ was absorbed by the end of the 10th day of growth. The rate of $\text{NO}_3^-\text{-N}$ utilization then increased markedly until nitrate also was completely removed from the medium. The preferential absorption by the cells of $\text{NH}_3\text{-N}$ resulted in a decrease in the pH of the medium of about 0.5 unit, the fall being following

a slight rise of pH as the nitrate concentration decreased.

Table 1 presents the data obtained during the growth of the *Chlorella* showing the numbers of cells and the absorption of $\text{NH}_3\text{-N}$ and $\text{NO}_3^-\text{-N}$ from the medium.

Total nitrogen determinations on whole dried

analyses on whole dried cells and on the protein fractions isolated.

Composition of bulk protein fractions. Four of the protein fractions were subjected to amino-acid analysis. These fractions had been isolated from *Chlorella* cells representative of the four main stages of growth observed, namely from cells

Table 1. *The growth rate of Chlorella vulgaris, the levels of combined nitrogen remaining in the medium, and the changes in pH of medium*

Time of growth (days)	Cell count (per cu.mm.)	$\text{NH}_3\text{-N}$ remaining in medium ($\mu\text{g. N/ml.}$)	$\text{NO}_3^-\text{-N}$ remaining in medium ($\mu\text{g. N/ml.}$)	pH of medium
0	12	131	131	6.20
3	525	129	129	6.09
4	1 250	—	—	—
5	2 700	116	127	6.05
6.7	14 560	66	117	5.86
8.8	34 000	5	91	—
9.9	40 400	0	80	5.66
12.9	58 000	0	8	5.90
16.7	66 000	0	0	5.89
21	68 000	0	0	5.86
28	52 000	0	0	—
35	40 000	0	0	—

cells indicated that protein formed 30% of the cell dry weight during the phase of exponential growth. During the later stages of growth the total cell nitrogen content decreased to about two-thirds of its former value. Soluble cell nitrogen extracted with 70% (v/v) aqueous ethanol, when expressed as a percentage of the total cell nitrogen, remained fairly constant during the whole period of growth studied.

Extraction of protein fractions. Protein was extracted from freshly harvested *Chlorella* cells at six different stages during their growth. The protein

harvested during the exponential phase of growth (culture A), at the end of this phase (culture B), at the time of maximum growth (culture C), and during the senescent breakdown stage (culture D). In cultures A and B the cells had used ammonia as their main nitrogen source, whilst both the ammonia and the nitrate had been completely absorbed at the stage of maximum growth.

Table 3 presents the results of the amino-acid analyses performed on the hydrolysates of the protein fractions. With the exception of the values given for cystine and tryptophan, all estimates were obtained by the method of quantitative amino-acid paper chromatography. Cystine and tryptophan determinations were made, using specific colorimetric methods. Histidine values obtained by the chromatographic and modified Pauly methods are given separately in the Table. The figures presented are the mean values of four determinations in the case of amino-acids estimated by the chromatographic method, whilst cystine, tryptophan, histidine (by modified Pauly) and amide N values are the means of duplicate determinations. The standard deviations from the mean values are given in parentheses.

Table 2. *The total and soluble nitrogen contents of Chlorella vulgaris, and nitrogen contents of protein fractions isolated*

Age of culture (days)	Total N of dried cells (g. N/100 g.)	Soluble N of dried cells (g./100 g. total N)	N in protein fractions isolated (g. N/100 g.)
5	5.8	10.1	10.3
8.8	3.9	—	8.6
12.9	3.6	10.3	10.5
21	3.8	—	10.0
28	3.6	13.1	10.5
35	3.7	—	10.1

fractions isolated after the cells had been broken by shearing contained 80–85% of the total nitrogen released from the cells which had been broken. Since 10–15% of the total nitrogen of the cells was present as soluble nitrogenous constituents, the fractions isolated were considered to be representative of the whole cell proteins. The nitrogen contents of the protein fractions isolated were generally slightly greater than 10% of their dry weight.

Table 2 contains the data obtained from nitrogen

DISCUSSION

In form, the growth curve of the *Chlorella*, which reached a maximum value at about the 20th day, resembled that obtained by Pearsall & Loose (1936) for the same species. The maximum cell concentration observed was, however, over twice that found by the earlier workers. Temperature, light intensity

and the amounts of available nitrogen and other inorganic salts are probably the factors controlling the rate and total amount of growth under the conditions used. In the present investigation, all the nitrate supplied in the medium was absorbed by the *Chlorella* cells during their growth, whereas Pearsall & Loose (1936) found that only one-third of the nitrate had been absorbed from the medium by the time growth ceased. This difference in the utilization of nitrate by the cells was probably the most important factor in determining the differences in the cell concentrations at the time of maximum growth.

determinations. The histidine contents, however, increased steadily to a value in culture *D* more than 1.5 times that in culture *A*; smaller increases in the percentages of alanine and lysine similarly occurred. In addition, ageing of the cultures was marked by a decrease of one-fifth in the arginine values, and a slight fall in the amide N figures.

The fact that the amino-acid compositions of the bulk proteins of *Chlorella* remained virtually unchanged with increasing age cannot be regarded as proof that the relative amounts of the individual proteins present within the cells was unaltered.

Table 3. *The amino-acid compositions of bulk protein fractions isolated from cultures of Chlorella vulgaris during different phases of growth*

(Figures in brackets are standard deviations.)

Culture	A	B	C	D
Age of culture (days) ...	5	8.8	21	35
Cell count (per cu.mm.)	2700	34 800	68 000	40 000
	g. amino-acid N/100 g. protein N			
Aspartic acid	6.0 (0.3)	5.7 (0.4)	6.5 (0.1)	6.0 (0.3)
Glutamic acid	7.9 (0.5)	6.9 (0.3)	7.5 (0.2)	7.8 (0.4)
Serine	3.4 (0.2)	3.0 (0.2)	2.9 (0.2)	3.6 (0.1)
Threonine	3.0 (0.2)	3.5 (0.2)	3.5 (0.2)	2.7 (0.2)
Glycine	6.3 (0.1)	5.9 (0.4)	5.8 (0.3)	5.7 (0.3)
Alanine	7.9 (0.2)	8.8 (0.3)	9.0 (0.4)	9.2 (0.3)
Valine	5.6 (0.3)	5.2 (0.3)	5.4 (0.3)	5.9 (0.1)
Leucine	6.1 (0.1)	5.6 (0.4)	5.6 (0.4)	6.0 (0.2)
Isoleucine	3.1 (0.1)	3.4 (0.2)	3.2 (0.2)	3.5 (0.2)
Phenylalanine	2.3 (0.1)	2.3 (0.1)	2.3 (0.1)	2.2 (0.1)
Tyrosine	2.6 (0.1)	2.6 (0.1)	2.8 (0.1)	2.7 (0.1)
Proline	4.6 (0.2)	4.6 (0.3)	4.5 (0.2)	4.7 (0.2)
Tryptophan	2.1	2.1	2.0	1.8
Methionine	1.4 (0.1)	1.4 (0.1)	1.3 (0.1)	1.2 (0.1)
Cystine	0.15	0.2	0.2	0.2
Arginine	17.8 (1.0)	16.0 (0.7)	16.6 (1.1)	14.0 (1.0)
Histidine	3.6 (0.1)	4.1 (0.3)	4.9 (0.2)	6.0 (0.3)*
	3.5	3.6	4.8	5.2 †
Lysine	8.3 (0.4)	8.3 (0.3)	8.9 (0.4)	9.1 (0.3)
Amide N	6.4	6.8	6.1	5.8
Total N	98.6	96.2	99.0	97.7

* Chromatographic values.

† Modified Pauly values.

According to current theories, an absence of manganese from plant tissues results in a number of abnormalities, one of which is an impaired efficiency of nitrate reduction. It is therefore possible that in the earlier experiments, where no trace elements were added to the culture medium, the incomplete utilization of nitrate may have been due to a deficiency of manganese within the cells.

The analyses presented in Table 3 show good agreement with those published earlier (Fowden, 1951a) for the protein extracted from *Chlorella* cells during the exponential growth phase, although the proline value then reported is now considered to have been overestimated. Table 3 also shows that there was little change in the composition of the bulk protein with the age of the various cultures, the percentages of most amino-acids remaining constant within the limits of the probable errors of the

Changes may have occurred in the concentrations of proteins of similar amino-acid composition, whilst the possibility of a more general change in the protein balance within the cells cannot be completely overlooked. The increasing histidine contents may then be interpreted as arising from a synthesis of one or more proteins relatively rich in this amino-acid during the later stages of growth.

The investigations have so far studied only the amino-acids bound in the form of protein, which accounts for about 85% of the total cell nitrogen. Whether the age of the culture has any more marked effect on the concentrations of the soluble nitrogenous compounds, particularly the free amino-acids and peptides within the cells, awaits further investigation.

Several papers have already been mentioned in

which *Chlorella* has been envisaged as a possible source of dietary protein. When the results in Table 3 are examined from this angle, it is evident that the quality of the protein is nearly independent of culture age. Thus harvesting of the cells could be arranged at a time which from an industrial viewpoint would be most economical. The ten 'essential' amino-acids of Rose account for 53 % of the total protein nitrogen, or 50 % of the total weight of all amino-acids released during protein hydrolysis. Therefore, although the content of cystine is low, the remaining essential amino-acids are present in *Chlorella* protein in proportions comparing very favourably with those for other plant proteins.

SUMMARY

1. The unicellular alga *Chlorella vulgaris* was grown in pure culture. The utilization by the cells of

$\text{NH}_3\text{-N}$ and $\text{NO}_3^-\text{-N}$ supplied in the medium was examined, growth being observed to continue until the supplies of nitrogen became exhausted.

2. Bulk protein samples representative of the whole cell proteins were isolated from cultures of different ages, and their amino-acid compositions determined by a paper-chromatographic technique. It was found that culture age had little effect on the protein composition except for a significant increase in the histidine contents with increasing age of the cells, and slight changes in alanine, lysine, arginine and amide N contents.

3. The 'essential' amino-acids accounted for 50 % by weight of the total amino-acids released during hydrolysis of the proteins, although the cystine contents were uniformly low.

My thanks are due to Mr P. J. Syrett for providing actively growing cultures of *Chlorella vulgaris*, and to Prof. W. H. Pearsall, F.R.S., for his interest in this work.

REFERENCES

- Arnon, D. I. (1938). *Amer. J. Bot.* **25**, 322.
 Camien, M. N., Salle, A. J. & Dunn, M. S. (1945). *Arch. Biochem.* **8**, 67.
 Chibnall, A. C. (1922). *Biochem. J.* **16**, 344.
 Chibnall, A. C. (1926). *Biochem. J.* **20**, 108.
 Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, p. 86. London: Crosby Lockwood.
 Fowden, L. (1951a). *Biochem. J.* **50**, 355.
 Fowden, L. (1951b). *Biochem. J.* **48**, 327.
 Freeland, J. C. & Gale, E. F. (1947). *Biochem. J.* **41**, 135.
 Geoghegan, M. J. (1951). *Nature, Lond.*, **168**, 426.
 Lugg, J. W. H. & Weller, R. A. (1941). *Biochem. J.* **35**, 1099.
 Lugg, J. W. H. & Weller, R. A. (1948). *Biochem. J.* **42**, 412.
 Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.
 Mazur, A. & Clark, H. T. (1938). *J. biol. Chem.* **123**, 729.
 Mazur, A. & Clark, H. T. (1942). *J. biol. Chem.* **143**, 39.
 Meier, R. L. (1949). *Chem. Engng News*, **27**, 3112.
 Pearsall, W. H. (1931). *J. exp. Biol.* **8**, 279.
 Pearsall, W. H. & Fogg, G. E. (1951). *Department of Scientific and Industrial Research Food Science Abstracts*, **23**, 1.
 Pearsall, W. H. & Loose, L. (1936). *Proc. roy. Soc. B*, **121**, 451.
 Smith, A. M. & Wang, T. (1941). *Biochem. J.* **35**, 404.
 Snell, F. D. & Snell, C. T. (1949). *Colorimetric Methods of Analysis*, 3rd ed., vol. 2, p. 792. New York: Van Nostrand.
 Spoehr, H. A. & Milner, H. W. (1948). *Rep. Dir. Div. Plant Biol., Carneg. Instn. Wash. Yearb.* **47**, 100.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Methods*, p. 103. Minneapolis: Burgess.

The Biosynthesis of L-Cystine and L-Methionine Labelled with Radioactive Sulphur (^{35}S)

By R. B. WILLIAMS AND R. M. C. DAWSON

Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(Received 20 February 1952)

The sulphur-containing amino-acids labelled with radioactive sulphur (^{35}S) are usually administered in biological experiments as a racemic mixture of their optical isomers. As the metabolism of L-amino-acids in animals generally differs from that of the D forms (Neuberg, 1948; Graham *et al.* 1950; Camien, Malin & Dunn, 1951), it may be desirable in certain experiments to use the labelled amino-acids as their naturally occurring isomers. The chemical synthesis of cystine or methionine from labelled sulphate ions followed by the resolution of the isomers is a some-

what tedious procedure and a biological synthesis may be preferred.

During the growth of baker's yeast, inorganic sulphate is easily assimilated and is a better sulphur source than organically combined sulphur (Sugata & Koch, 1935; Schultz & McManus, 1950). It seemed likely therefore that the proteins of yeast grown in a medium containing labelled sulphate ions would serve as a convenient source for the isolation of labelled L-cystine and L-methionine. This paper gives details of the preparation of these