8. Fumarase is progressively inactivated by suspensions of *Lactobacillus arabinosus* at pH 5·0 and 30°.

9. Animal tissues were found to contain between 1 and 13 μmol./g. wet weight malate plus fumarate. All plant materials tested contained malate (between 10 and 200 μmol./g. wet weight), whilst fumarate was absent from most materials. The occurrence of fumarate in *Myrrhis odorata* and *Glaucium flavum* (13 and 99 μmol./g. wet weight respectively) has been confirmed.

10. The haemolymph of *Gastrophilus intestinalis* larvae contained exceptionally high concentrations of malate and fumarate (24–38 μmol./ml.); these acids account largely for the anion deficit reported by Levenbook (1950).

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The Composition of the Bulk Proteins of *Chlorella*

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Little information is at present available regarding the nature of the algal proteins. Mazur & Clark (1938, 1942) have given the amino-acid composition of the proteins of several species of marine algae, and one fresh-water alga. However, Lugg (1949) has recently questioned the accuracy of the values obtained, and doubted the possible phylogenetic implications. Hot formic acid was used to extract the proteins from the defatted algae, and since this procedure was only 70–80 % efficient, the resulting protein fractions may not have been representative of the whole. Analyses of the acid hydrolysates of the extracted material accounted for 76–88 % of the total protein nitrogen, though of this nitrogen as much as 20 % was combined in the form of humin. No other complete analyses of algal proteins appear to have been published.

This paper presents the results of an investigation of the amino-acid composition of the bulk protein of the unicellular alga, *Chlorella vulgaris*, and is a continuation of the qualitative work reported earlier (Fowden, 1951 *b*). The analyses were made on a protein fraction believed to be representative of the whole, using the technique of paper chromatography.

EXPERIMENTAL

Material. *Chlorella vulgaris* was grown on the medium of Pearsall & Loose (1936) in penicillin culture flasks maintained at 25° under continuous illumination; the flasks were occasionally gently shaken. When nearing the end of its phase of exponential growth (6–7 days), the *Chlorella* was harvested by centrifugation at 500 *g*, and then twice washed with distilled water.

Protein extraction. The method used was based on the borate-ether-ethanol method of Lugg (1939). The freshly harvested *Chlorella* cells (about 2 g. on a dry-wt. basis) were suspended in 35 ml. of sodium borate solution (1·1 g./l.), and then the cell walls were broken, releasing the cytoplasmic proteins, by applying a shearing stress of 20,000 lb./sq.in. (Milner, Lawrence & French, 1950). Haemocytometer counts showed that 8–10 % of the cells remained unbroken after this treatment. A sample of the suspension was taken for total-nitrogen assay. The bulk of the suspension was diluted to 250 ml. with more sodium borate solution and 250 ml. of an ether-ethanol mixture (1 vol. ether to 4 vol.

ethanol) at about 5° were then slowly added with good stirring. This treatment disrupted the chloroplasts, and their protein passed into solution. After standing for 1 hr., the mixture was centrifuged for 30 min. at 500 *g*. The supernatant was poured off and the separated solid material (fraction *A*) was washed, dried and stored for nitrogen assay. The supernatant and washings were then adjusted to pH 4·5 with acetic acid, and warmed to 70° to flocculate the protein. The protein was separated after cooling by centrifugation, and the supernatant (fraction *B*) was set aside for nitrogen assay. The protein was washed in turn with dilute acetic acid (pH 4·5), boiling ethanol (twice), dilute citric acid, boiling ethanol (twice), and finally ether. It was then air dried, weighed and finely ground to a light buff-coloured powder (fraction *C*). Nitrogen determinations were made on a sample of the protein, and upon the combined washings (fraction *D*).

Protein hydrolysis. The protein was hydrolysed for 24 hr. at 105° in a sealed ampoule using 5 ml. of a mixture of equal vol. of 10 N-HCl and glacial acetic acid, and following a suggestion of Kofranyi (1948), $SnCl_2.2H_2O$ (4% w/v) was added. The protein concentration during the hydrolysis was about 10 mg./ml. The addition of the $SnCl_2$ completely prevented the formation of insoluble humin, and the final hydrolysate had a pale-yellow colour. The mixture was evaporated to dryness *in vacuo* at laboratory temperature.

The residue was dissolved in about 5 ml. of distilled water, and then desalted by the method of Consden, Gordon & Martin (1947). The pH of the solution rapidly increased during the desalting, and the remaining soluble tin was precipitated as $Sn(OH)_2$. This was removed by centrifugation, and washed several times with small volumes of distilled water. The supernatant and washings were combined, and evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. water; 100 µl. of the solution were used for each chromatogram.

Rees (1946) has reported some loss of pure threonine and serine under the normal conditions of hydrolysis of proteins in 6 N-HCl, but under the hydrolysis conditions used here, there was no detectable loss of threonine, and a smaller (6·0%) loss of serine than that reported by Rees. The values given later for serine have been corrected for this loss.

Since some insoluble $Sn(OH)_2$ was produced during desalting, it seemed quite probable that some of the dicarboxylic amino-acids present in the hydrolysate may have been absorbed by this basic precipitate, resulting in estimates that were too low for these amino-acids. In order to check this point they were also determined after hydrolysing the protein in 6 N-HCl for 24 hr. at 105°. Though in this case 4% of the total protein nitrogen was lost as insoluble humin nitrogen, the values obtained for the aspartic and glutamic acid contents of the hydrolysate were accepted as true estimates of the amounts present in the protein, since these two amino-acids are thought to play no significant role in humin production. The aspartic and glutamic acid contents found by this procedure were approximately 50% higher than those determined after desalting in the presence of $SnCl_2$.

Stein & Moore (1951) have reported an appreciable conversion of arginine to ornithine during prolonged desalting of amino-acid-salt mixtures, but the conversion occurring under the conditions described above must have been less than 5%, as demonstrated by chromatographic analysis of arginine solutions desalted under the same conditions, and by an independent assay of arginine by the Sakaguchi method described by Brand & Kassell (1942). No losses of the other amino-acids present in the hydrolysate were encountered during desalting.

Nitrogen determinations. Total N determinations were obtained using the micro-Kjeldahl method of Chibnall, Rees & Williams (1943). Amide N values were obtained by estimating the NH_3 formed during hydrolysis with N-H_2SO_4 for 3 hr. at 100°. α-NH_2-N determinations were made by the colorimetric copper method of Woiwod (1949).

Amino-acid assays. These were performed as described by Fowden (1951 *a*) after the complete separation of each amino-acid had been achieved on Whatman no. 4 paper chromatograms.

Chromatographic technique. This was essentially that of Consden, Gordon & Martin (1944). The separation of the amino-acids was achieved entirely on one-dimensional chromatograms using three solvent mixtures. Since a suitable source of ultraviolet light was not available, all spots were located and cut out by comparison with an identical parallel chromatogram (Martin & Mittelmann, 1948). The recovery of the amino-acids was checked at regular intervals by running known control mixtures on the same sheet of paper and, in the case of amino-acids such as methionine, where the recovery of these standards was constant but below the theoretical amount, the values given for the protein were based on these estimated recoveries.

Solvents. Aspartic acid, glutamic acid, serine, glycine and threonine were separated as well defined spots after running for 60 hr. in phenol saturated with 0·1% NH_3 (w/v) solution. Any cystine present in the hydrolysate was oxidized to cysteic acid by H_2O_2 (Dent, 1947) before the commencement of the run. The NH_3 concentration in the water phase placed in the bottom of the tank was increased to 0·6% (w/v) to give better separations of these amino-acids.

Alanine, tyrosine and valine were separated by running for 30 hr. in the *n*-butanol-acetic acid mixture of Partridge (1948). Methionine, which would otherwise tend to overlap valine, was oxidized by H_2O_2 to the slower moving sulphone.

Lysine, histidine and arginine were also separated using this *n*-butanol-acetic acid mixture. It was found advisable to use a mixture which had been equilibrated for 5–6 days before separating the two phases. The R_F values were then lower than in the freshly prepared solvent and the resolution of the three amino-acids was much better. Good separation was achieved at the end of a 5-day run.

Proline, methionine, phenylalanine, isoleucine and leucine were separated using water-saturated *tert.*-amyl alcohol (technical). 1% (v/v) diethylamine was added to the aqueous phase in the bottom of the tank and each chromatogram was developed for 5 days. The amino-acids are mentioned above in the order of their increasing R_F values for the respective solvents. It was noticed that phenylalanine did not occupy the same relative position in *tert.*-amyl alcohol as that described by Work (1949), but this was probably due to differences in the purity of the alcohol used. The dilute alkali treatment used to remove NH_3 from the paper before amino-acid assay (Fowden, 1951 *a*), was found to remove efficiently all of the diethylamine absorbed on the paper. Proline was estimated after chromatographic separation by the method of Woiwod (1949), since the ninhydrin method of estimation is very insensitive for this amino-acid.

Cystine could not be estimated as cysteic acid on chromatograms without overloading the paper because of the relatively small amount of this amino-acid present in the protein. It was estimated in duplicate 1 ml. samples of the

hydrolysate by the method of Sullivan (1929). Tryptophan was estimated using *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) after 5 N-NaOH hydrolysis of the protein for 30 hr. at 100°. A correction was applied for an estimated loss of 5 % during the course of the hydrolysis (Lugg, 1938).

RESULTS

The sample of *Chlorella* used in this investigation contained 4·3 % nitrogen on a dry-weight basis. Repeated extraction of the dried cells with 70 % (v/v) aqueous ethanol at room temperature showed 10·3 % of this to be soluble non-protein nitrogen.

Table 1 shows the distribution of total nitrogen between the fractions *A*, *B*, *C* and *D* obtained during the protein extraction, together with the probable nature of the fractions.

Table 1. *The distribution of total nitrogen between the fractions obtained during the protein extraction*

Fraction	Percentage total nitrogen	Composition of fraction
A	10·2	Broken cell wall material and 8–10 % unbroken cells
B	12·4	Soluble nitrogenous compounds, carbohydrates, lipids, etc.
C	75·8	Protein with carbohydrate impurities
D	1·4	Chlorophyll eluted from protein, some carbohydrate, etc.

The nitrogen in fraction *A* was probably all present in the unbroken cells, from which no protein would be released. Of the nitrogen released from the broken cells (nitrogen appearing in fractions *B–D*), 85 % appeared as protein nitrogen in fraction *C*. The extraction of the dried cells with 70 % ethanol had indicated that 89·7 % of the total nitrogen was present as protein nitrogen. Since good agreement was found between these two figures the extracted protein was considered to be representative of the proteins of the whole cells. The nitrogen content of this protein fraction was 11·5 %.

Table 2 gives the results of the amino-acid analyses performed on hydrolysates of this protein fraction. The figures obtained using paper chromatography represent the means of six determinations. Cystine and tryptophan values are the means of duplicate estimations. The probable errors of the values quoted are given in parentheses. The table also includes for comparison the amino-acid composition of the bulk proteins obtained from leaves of the Gramineae family (from Lugg, 1949), and the composition of an acid hydrolysate of the nitrogen fraction of a brewer's yeast which was insoluble in 75 % ethanol (Lindan & Work, 1951).

The arginine-nitrogen figure obtained by the Sakaguchi method was 15·5 %. The figures in Table 2 show that 73·9 % of the total protein nitrogen is present as α-amino nitrogen after hydrolysis. A direct estimate of the α-amino nitrogen in the desalted acid hydrolysate made by the method of Woiwod (1949) gave a value of 74·4 % after correction for the losses of aspartic and glutamic acids occurring during the desalting.

Table 2. *The amino-acid composition of the bulk protein of* Chlorella vulgaris *compared with similar data for brewer's yeast and higher plants*

(Figures in brackets indicate probable error.)

Amino-acid	g. amino-acid N/100 g. protein N: *Chlorella vulgaris*	Brewer's yeast (Lindan & Work, 1951)	Gramineae (Lugg, 1949)
Aspartic acid	6·4 (0·3)	8	4·9–5·4
Glutamic acid	7·8 (0·3)	10	6·6–7·8
Serine	3·3 (0·2)	4	—
Threonine	2·9 (0·15)	7	3·0
Glycine	6·2 (0·3)	8	0·4
Alanine	7·7 (0·3)	10	4·4–5·1
Valine	5·5 (0·2)	8	3·3–4·2
Leucine	6·1 (0·3)	6	7·1–8·8 (Leucine + Isoleucine)
Isoleucine	3·5 (0·2)	4	
Phenylalanine	2·8 (0·1)	1·6	2·5–2·6
Tyrosine	2·8 (0·1)	2	2·3–2·5
Proline	7·2 (0·3)	3	3·1
Tryptophan	2·1	0	1·8–2·1
Methionine	1·4 (0·1)	0·5	1·4–1·6
Cystine	0·2	0·9	1·3–1·5
Arginine	15·8 (0·8)	10	13·7–14·3
Histidine	3·3 (0·15)	8	3·6–3·7
Lysine	10·2 (0·35)	9	6·3–6·6
Amide N	6·1	—	4·7–5·3
Total N	101·3	100	**74·4** (mean)

DISCUSSION

The results presented in Table 2 give a very complete analysis of the protein of *Chlorella*, the probable errors of the values obtained by the paper-chromatographic technique being about 5 % of the individual mean values. The protein analysed was extracted from rapidly growing cells, and is marked by its high content of basic amino-acids. In contrast to the findings of Mazur & Clark (1938) for other algae, all the usual amino-acids were found to be present.

When the composition of the *Chlorella* protein is compared with that of the proteins in the leaves of higher plants, it is seen that a close similarity exists between the values for many amino-acids, the major differences occurring in the values given for the monoamino-monocarboxylic acids and proline. Since the analyses of the leaf proteins are only 75 % complete, it seems possible that the values given by Lugg (1949) for these amino-acids were underestimated by the rather unspecific methods

available prior to partition chromatography. If this were the case, the only outstanding difference between the composition of leaf proteins and the algal protein reported here is in the very low cystine content of the latter. The composition of the yeast protein also shows many similarities with that of *Chlorella*, the values for the monoamino-monocarboxylic acids again being much higher than those reported for the leaf proteins.

SUMMARY

1. A representative sample of the proteins of the alga *Chlorella vulgaris* was prepared.

2. The amino-acid composition of this protein fraction was investigated by the technique of paper-partition chromatography after an acid hydrolysis during which no humin formation occurred.

3. The analysis accounted for 101·3% of the total protein nitrogen.

4. The amino-acid composition of this algal protein fraction is compared with figures available for the composition of the proteins obtained from the leaves of the higher plants and of the proteins found in a sample of a brewer's yeast.

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The Branched-chain Fatty Acids of Butterfat

2. THE ISOLATION OF A MULTI-BRANCHED C_{20} SATURATED FATTY ACID FRACTION

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In Part 1 of this series (Hansen & Shorland, 1951) the occurrence of small amounts of branched-chain acids in butterfat was indicated and the isolation of two C_{17}-methyl branched-chain acids, isomeric with normal heptadecanoic acid, was reported.

Further investigation of the acetone-soluble components separated at low temperature from hydrogenated methyl esters of the C_{18} acids of butterfat, has revealed the presence of a multi-branched C_{20} acid fraction.

EXPERIMENTAL

As reported earlier (Hansen & Shorland, 1951) a concentrate (4·4 kg.) of methyl esters of C_{18} acids prepared from butterfat was crystallized from acetone at $-30°$. The soluble portion after hydrogenation was again crystallized from acetone at $-30°$ yielding 87·6 g. of acetone-soluble material with iodine value 8·3. This latter fraction was distilled *in vacuo* in an 8 ft. Vigreux column yielding eight fractions, EL1 to EL8, together with a viscous residue ELR (compare Table 1, Hansen & Shorland, 1951). From these eight fractions were derived the C_{17} branched-chain acids previously reported, together with all subsequent fractions referred to in this paper. By repetition of the processes of selective bulking of those fractions with similar molecular weight, followed by fractionation, a series of fractions was obtained which could not be further resolved by the columns at our disposal and which were combined to constitute E4 (9·6 g. methyl esters, mean saponification equivalent 303·4).

The methyl esters of E4 were crystallized from 20 vol. of methanol at $-37°$ yielding: (*a*) E4L 5·56 g., soluble