# 503. *Amino-acids and Peptides. Part X.* The Nitrogenous Constituents of Some Marine Algae.*

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The chemical distribution of the nitrogen in samples of the brown seaweeds *Laminaria saccharina*, *Laminaria cloustoni*, *Ascophyllum nodosum*, and *Pelvetia canaliculata*, and in the red seaweed *Rhodymenia palmata*, has been determined. Peptides and proteins account for most of the organic nitrogen; there are small amounts of volatile bases, and in *L. cloustoni* and *R. palmata* appreciable quantities of free α-amino-acids (chiefly alanine, aspartic acid, and glutamic acid). The amino-acids obtained on hydrolysis of the *L. saccharina*, *A. nodosum*, *P. canaliculata*, and *R. palmata* samples have been identified by partition chromatography, and several have been determined quantitatively in the last two species. No amino-acid has been found which does not also occur in land plants. A preliminary investigation has been made of the complexity of the mixture of peptides and proteins in the *P. canaliculata* sample, by counter-current separation of their bromodinitrophenyl derivatives. The new reagent, 1-bromo-2-fluoro-3 : 5-dinitrobenzene, may prove useful for determining certain reactive groups in peptides, and, in special cases, their chain length.

The amino-acids and peptides of marine algae are of interest not only because of the salt-water environment of the plants but also because of the suggestion by Haas and Hill (*Ann. Bot.*, 1933, **47**, 55) that the low intensity of the available light, or in some cases the periods of desiccation, might result in a modification of protein metabolism. These authors initiated investigations in this field by the isolation (*Biochem. J.*, 1931, **25**, 1472) of what appeared to be an octapeptide of glutamic acid from *Pelvetia canaliculata*, of the Phaeophyceae class (brown algae); subsequently, glycine, alanine, arginine, and histidine were also detected in peptides isolated from other samples of this species (Haas, Hill, and Russell-Wells, *ibid.*, 1938, **32**, 2129; Haas, *ibid.*, 1950, **46**, 503). From certain Rhodophyceae (red algae) of the sub-class Florideae, peptides of low molecular weight were obtained, containing alanine, phenylalanine, arginine, and aspartic acid (Haas, Hill, and Russell-Wells, *loc. cit.*; Haas and Hill, *ibid.*, 1933, **27**, 1801); glycine and histidine were found in addition in peptides from other samples of Florideae (Haas, *loc. cit.*). From the brown marine alga *Eisenia bicyclis*, Ohira (*J. Agric. Chem. Soc. Japan*, 1939, **15**, 370) isolated a peptide ("eisenine") which he suggested was L-5-oxopyrrolidine-1-carbonyl-L-glutaminyl-L-alanine (*ibid.*, 1940, **16**, 1, 293). More recently, Dekker, Stone, and Fruton (*J. Biol. Chem.*, 1949, **181**, 719) obtained a peptide considered to be L-5-oxopyrrolidine-1-carbonyl-L-glutaminyl-L-glutamine from *P. fastigiata*; they pointed out that the ring closure may have occurred during extraction.

Mazur and Clarke (*ibid.*, 1938, **123**, 729; 1942, **143**, 39) have investigated formic acid

* Part IX, *J.*, 1952, 3937.

extracts of certain Phaeophyceae, Rhodophyceae, and Chlorophyceae (green algae). They identified the amino-acids formed on hydrolysis, and determined several (see also Lugg, *Adv. Protein Chem.*, 1949, **5**, 247). Lugg also (*Biochem. J.*, 1943, **37**, 132) has examined hydrolysates of the green marine alga *Ulva lactuca*. Simultaneously with the publication of a preliminary note of our results (*J. Soc. Chem. Ind.*, 1952, 519), Ericson and Sjöström (*Acta Chem. Scand.*, 1952, **6**, 805) reported the identification of a wide range of amino-acids in the hydrolysates of brown algae of three different orders, *Sphacelaria arctica*, *Laminaria saccharina*, and *Fucus vesiculosus*. Excepting di-iodotyrosine, which Roche and Lafon (*Compt. rend.*, 1949, **229**, 481) detected in the base of the thallus of *L. saccharina* and *L. flexicaulis*, all the amino-acids reported to be present in hydrolysates of marine algae are also constituents of land-plant proteins.

Aliphatic amine salts occur in marine algae, methylamine and trimethylamine having been found (together with ammonium salts) by Kapeller-Adler and his co-workers (*Biochem. Z.*, 1930, **224**, 378; 1931, **243**, 292) in species of *Fucus* and in red and green algae. Haas and Hill (*Biochem. J.*, 1931, **25**, 1472) similarly found trimethylamine in *P. canaliculata*.

In the present investigation, we have examined samples of the brown algae *L. saccharina*, *L. cloustoni*, *Ascophyllum nodosum*, and *P. canaliculata*; the first two belong to the family Laminariaceae (order Laminariales) and the last two to the family Fucaceae (order Fucales). We have also examined a sample of the red alga *Rhodymenia palmata* (sub-class Florideae, order Rhodymeniales). The place and date of harvesting of each sample are shown in Table 1. Volatile bases were determined by aeration of a suspension in saturated aqueous

TABLE 1.

| No. | Alga | Origin: When collected | Origin: Locality | Portion of plant |
|---|---|---|---|---|
| 1 | *Laminaria saccharina* | March, 1945 | Eilean Coltair, Loch Melfort, Argyllshire | Frond |
| 2 | *L. saccharina* | Sept., 1945 | ,, ,, ,, | ,, |
| 3 | *L. cloustoni* | July, 1948 | Luing Island, Cullipool, Argyllshire | Frond |
| 4 | | | | Stipe |
| 5 | | | | Holdfast |
| 6 | *L. cloustoni* | Oct., 1948 | ,, ,, ,, | Frond |
| 7 | *Pelvetia canaliculata* | June, 1948 | Atlantic Bridge, Clachan Sound, Argyllshire | Whole plant |
| 8 | *P. canaliculata* | March, 1949 | ,, ,, ,, | ,, |
| 9 | *Ascophyllum nodosum* | Dec., 1949 | North Berwick, East Lothian | ,, |
| 10 | *Rhodymenia palmata* | Jan., 1952 | ,, ,, | ,, |

| No. | Kjeldahl-N, % | Volatile base-N, % | Amide-N, % | Nitrate-N, % | Residual-N, % | Total org.-N, % | Residual-N, %, ×6·25 | α-Amino-acid-N found after hydrolysis, % | Free α-amino-acid-N, % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2·39 | 0·05 | 0·22 | 0·86 | 1·81 | 2·08 | 11·3 | 1·30 | — |
| 2 | 0·71 | 0·01 | 0·09 | 0·01 | 0·59 | 0·69 | 3·7 | 0·53 | — |
| 3 | 1·58 | 0·03 | 0·25 | 0·02 | 1·27 | 1·55 | 7·9 | — | 0·20 |
| 4 | 1·61 | 0·05 | 0·17 | 0·19 | 1·24 | 1·46 | 7·8 | — | 0·23 |
| 5 | 2·42 | 0·07 | 0·25 | 0·31 | 1·86 | 2·18 | 11·6 | — | 0·22 |
| 6 | 1·55 | 0·05 | 0·28 | 0·06 | 1·15 | 1·48 | 7·2 | 1·08 | — |
| 7 | 0·88 | 0·01 | 0·11 | 0·03 | 0·72 | 0·84 | 4·5 | 0·63 | — |
| 8 | 1·73 | 0·01 | 0·32 | 0·04 | 1·36 | 1·69 | 8·5 | 1·10 | 0·06 |
| 9 | 1·58 | 0·03 | 0·29 | 0·03 | 1·22 | 1·54 | 7·6 | 1·00 | — |
| 10 | 3·91 | 0·01 | 0·48 | 0·13 | 3·28 | 3·77 | 20·5 | 2·80 | 0·25 |

All figures have been corrected for moisture.

potassium carbonate. Steam-distillation from sodium hydroxide gave the total of volatile base and amide-ammonia; reduction of the residue with Devarda's alloy reduced any nitrate present to ammonia, which was again distilled. The residue was digested by the Kjeldahl method, and the remaining nitrogen so determined. The chemical distribution of the nitrogen is shown in Table 1. Only very small amounts of ammonium and amine salts were present in these samples (column headed " volatile base "); examination of the conditions used in the work reported earlier (Channing and Young, *loc. cit.*) has shown that the figures reported then for " volatile-base nitrogen " will include much, but not all, of the amide-nitrogen. In contrast, Haas and Hill (*Ann. Bot.*, 1933, **47**, 55) found considerable amounts of ammonium salts in *L. digitata* (January) and *Fucus vesiculosus* (April)

samples. It should be noted that direct Kjeldahl determinations (Table 1) give unreliable values for the samples containing nitrate (compare column headed " total org.-N "). Dr. W. A. P. Black (personal communication) has found that when nitrate is added to algae samples a portion is reduced to ammonia during Kjeldahl digestion, whilst we have found that the Kjeldahl-nitrogen value of a known mixture of glycine with potassium nitrate corresponded to less than half of the glycine-nitrogen.

The total α-amino-acid-nitrogen obtained on hydrolysis of the algae samples was determined by titration of the carbon dioxide evolved in the reaction with ninhydrin (Van Slyke, MacFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 671). Preliminary experiments with *Ascophyllum nodosum* showed that the optimum time of hydrolysis with boiling 6N-hydrochloric acid is *ca.* 72 hours, and this period was used for all the samples. It will be seen from Table 1 that in each case the α-amino-acid-nitrogen is lower than the " residual-nitrogen " determined as described above. Only small amounts of basic amino-acids have been found in the hydrolysates, and the most likely cause of this discrepancy is the destruction known to occur when proteins are hydrolysed in the presence of carbohydrate. The Table also gives an approximate figure for the percentage of peptide and protein in the dried seaweed; the high figure for *Rhodymenia palmata* is noteworthy.

Investigation of the bases distilled from a suspension of the *Pelvetia canaliculata* (March, 1949) sample in sodium hydroxide showed that the major constituent was ammonia (identified as toluene-*p*-sulphonamide). Extraction of the hydrochlorides with chloroform yielded trimethylamine hydrochloride (identified as the picrate). A trace of primary amine was detected in the chloroform-insoluble fraction, but the carbon content showed that an amine could not represent more than 5% of this fraction, which was chiefly ammonium chloride.

Preliminary work with A. Basu (unpublished) had shown that the holdfast of a *Laminaria cloustoni* sample (July, 1948) contained a significant amount of uncombined α-amino-acids. We now find that the frond and stipe of this plant also contain similar amounts, as does the *Rhodymenia palmata* sample (Table 1, last column). In contrast, the *Pelvetia canaliculata* sample contained little free α-amino-acid. Paper chromatography showed that in both *Laminaria cloustoni* and *Rhodymenia palmata* the main free α-amino-acids were alanine, aspartic acid, and glutamic acid; the latter species gave, in addition, spots corresponding to valine, the leucines, and proline. It is interesting that the first three amino-acids are those which in higher plants may be formed directly from the corresponding α-keto-acids, which are themselves known intermediates in plant metabolism.

Acid hydrolysates of the *L. saccharina* (Sept., 1945), *A. nodosum*, *P. canaliculata* (March, 1949), and *R. palmata* samples were then examined by partition chromatography in order to identify the constituent amino-acids. In the first two cases, a preliminary separation was effected by means of a column of powdered cellulose, with butanol–acetic acid–water as the developing solvent. The fractions so obtained were analysed by one-dimensional paper chromatography. In later work we effected the initial separation by partition of a " band " of the hydrolysate on paper (after removal of salts by electrodialysis). The position of the fractions so obtained was indicated by spraying the edges of the chromatogram with ninhydrin; the narrow strips were then cut out, and eluted, and the constituents identified by one-dimensional paper chromatography. By the use of a thick paper (Whatman No. 3), sufficient of each fraction could be provided for partition in several solvents.

The following amino-acids were identified in the hydrolysates of the *L. saccharina* and *A. nodosum* samples: glycine, alanine, valine, leucine, *iso*leucine, phenylalanine, serine, threonine, aspartic acid, glutamic acid, proline, lysine, and arginine. Acid hydrolysates of the *P. canaliculata* and *R. palmata* samples contained, in addition, methionine, tyrosine, and histidine. Ornithine was also found, but is likely to have been formed from arginine during electrodialysis (cf. Stein and Moore, *J. Biol. Chem.*, 1951, **190**, 103). In alkaline hydrolysates, tryptophan also was detected. We consider that these additional amino-acids may also have been present in the first two hydrolysates, the examination of which was less exhaustive. Our results agree with those of Ericson and Sjöström (*loc. cit.*), except that these authors found cystine but not tryptophan in their materials. All the amino-

acids reported are found in proteins from many other sources. Our results show little qualitative difference between the brown and the red samples.

Partition methods do not normally distinguish between enantiomorphs, and a preliminary experiment was performed to determine whether D-amino-acids were present in the hydrolysate of *P. canaliculata* (March, 1949). The volume of oxygen absorbed on treatment with a D-amino-acid oxidase preparation suggested that small amounts of D-amino-acids were present, but such quantities may well arise by racemisation during hydrolysis. This question deserves further investigation.

Certain of the main amino-acids in hydrolysates of *P. canaliculata* (March, 1949) and *R. palmata* have been determined quantitatively. The high accuracy of the isotope-dilution method did not appear to be required at this stage, and a convenient sub-micro-procedure was adopted. Partition on Whatman No. 3 paper separated a narrow band containing 0·4—2 mg. of the amino-acid to be determined, which was then eluted and estimated by the ninhydrin–carbon dioxide method. Known mixtures of amino-acids gave results between 98 and 107% of the theoretical (Table 4, p. 2489), and the analyses of the two seaweed samples are shown in Table 2; the separation of valine and tyrosine was incomplete and they were determined together. It will be seen that despite the difference in total α-amino-acid content, the brown and the red algae contain remarkably similar proportions of each of these amino-acids, except in the case of glutamic acid; it is interesting to recall the earlier isolation of a glutamyl-peptide from *P. canaliculata*. The aspartic acid and glutamic acid found in each case are together approximately equivalent to the amide-nitrogen (Table 1) and it seems that most of those carboxyl groups not in peptide linkage are present as amides. It must be emphasised that in these analyses no allowance has been made for the destruction of the amino-acids during hydrolysis.

TABLE 2.

| | Amino-acid found, | | | |
|---|---|---|---|---|
| | N, % of total α-amino-acid-N | | G. per 100 g. of dry sample | |
| Amino-acid | *P. canaliculata* | *R. palmata* | *P. canaliculata* | *R. palmata* |
| Alanine | 9·7 | 10·8 | 0·65 | 1·85 |
| Leucine | 2·6 | 2·8 | 0·25 | 0·71 |
| *iso*Leucine | 2·9 | 2·6 | 0·28 | 0·66 |
| Valine + tyrosine | 5·4 | 5·1 | 0·60 * | 1·46 * |
| Aspartic acid | 7·5 | 8·6 | 0·75 | 2·20 |
| Glutamic acid | 18·0 | 8·4 | 1·98 | 2·37 |

* On the assumption that the nitrogen is equally distributed between valine and tyrosine.

TABLE 3.

| | Bromodinitrophenyl-peptide fractions | | | | | |
|---|---|---|---|---|---|---|
| | EtOAc-soluble | | | BuOH-soluble | | Residual $H_2O$-soluble |
| Amino-acids | A1 | A2 | A3 | B1 | B2 | C |
| Aspartic acid | + | — | + | — | + | ++ |
| Glutamic acid | +++ | ++ | +++ | + | +++ | +++ |
| Glycine | ± | — | + | — | — | + |
| Alanine | + | + | + | — | + | + |
| Serine | — | — | — | — | — | ± |
| Valine | — | — | — | — | — | + |
| Leucine(s) | — | — | — | — | — | + |
| Phenylalanine | — | — | — | — | — | + |
| Histidine | ± | — | ± | — | — | ± |
| Other basic amino-acids | — | — | — | — | — | ± |
| *N*-Terminal amino-acids | — | Serine, glycine | Glycine | — | — | — |

An attempt was made to examine the complexity of the peptides and proteins in the *P. canaliculata* (March, 1949) sample. Separation of such materials from the polysaccharide present is difficult, but preliminary experiments showed that the separation was facilitated by conversion into the *N*-dinitrophenyl derivatives (Sanger, *Biochem. J.*, 1945, **39**, 507).

There appeared also to be advantages in introducing, for example, a bromine atom into the nitrophenyl group; determination of the bromine in the derivative would then assay the reactive groups (amino, imino, phenolic-hydroxyl, and thiol), and in certain cases the chain length could then be deduced. With this object in mind, 1 : 3-dibromo-2-chloro-5-nitrobenzene was prepared, but the chlorine could not be replaced by fluorine under the usual conditions. However, 1-bromo-2-chloro-3 : 5-dinitrobenzene reacted smoothly with potassium fluoride to give 1-bromo-2-fluoro-3 : 5-dinitrobenzene as a crystalline solid, which coupled with amino-acids and peptides to give yellow or orange bromodinitrophenyl derivatives. Although, as will be seen, this method of chain-length determination could not be applied in the present work, it may be useful in other cases.

The *P. canaliculata* sample was extracted with cold 0·05N-sodium hydroxide, and the solution was saturated with carbon dioxide and then treated with 1-bromo-2-fluoro-3 : 5-dinitrobenzene in ethanol. The bromodinitrophenyl-peptides were precipitated with mercuric acetate and liberated by hydrogen sulphide; the aqueous solution was extracted first with ethyl acetate (to give fraction A), and then with butanol (fraction B), leaving an aqueous solution (fraction C) still containing peptide or protein derivatives. Evaporation of fractions A and B in the presence of aqueous ammonia gave residues which were subjected to counter-current partition between butanol and 5% aqueous ammonia, giving three fractions from A and two from B. From the small amount and unsatisfactory nature of these materials, it was clear that bromine determinations could give no reliable indication of the chain length, and each fraction was therefore hydrolysed and the amino-acids were identified by paper chromatography. Ether-extracts of the hydrolysates from A1, A2, and A3 contained the bromodinitrophenyl derivatives of the *N*-terminal amino-acids, and these were hydrolysed by ammonia, to yield the *N*-terminal amino-acids. Glycine and serine were so obtained from A2, and glycine alone from A3. The failure to detect *N*-terminal residues in the remaining fractions may have been due in part to the instability of their bromodinitrophenyl derivatives and in part to the small amount present. From Table 3 it will be seen that each fraction, except C, contained few amino-acids, and in every case glutamic acid was the major component. Fraction C contained many amino-acids and is likely to have been of considerably higher molecular weight.

Finally, it should be pointed out that the above conclusions apply to samples of algae collected and dried as indicated; whether or not significant changes occur after harvesting is a matter for further investigation.

## Experimental

The dried, milled seaweed samples (see Table 1) were provided by the Institute of Seaweed Research, to whom we are indebted for the following details of preliminary treatment (cf. Black, *J. Soc. Chem. Ind.*, 1948, **67**, 165, 169, 172; 1949, **68**, 183; 1950, **69**, 161; *J. Marine Biol. Assoc.*, 1950, **29**, 45, 379): Drying was commenced within 3 hr. of collection as follows: *A. nodosum* and *R. palmata*, 24 hr. at 60—70°; others, 48 hr., *L. cloustoni* (July, 1948) at 60—70°, and *L. saccharina*, *L. cloustoni* (Oct., 1948), and *P. canaliculata* at 25—35°. The samples were then pulverised in a Christy and Norris No. 8 laboratory mill (64-mesh screen). We determined the moisture contents of the milled samples before use, by drying to constant weight at 60—62° *in vacuo* over phosphoric oxide.

*Determination of the Chemical Distribution of the Nitrogen of the Algae Samples.*—Ammonia-free air was bubbled for 2 hr. through an agitated suspension of the seaweed sample (0·6—1 g.) in saturated aqueous potassium carbonate (30 ml.). The volatilised bases were determined by absorption in aqueous boric acid and titration with standard hydrochloric acid (methyl red–bromocresol green indicator; Ma and Zuazaga, *Ind. Eng. Chem., Anal.*, 1942, **14**, 280).

A sample (0·3—0·5 g.) of the seaweed in water (10 ml.) was warmed for 5 min. with 40% aqueous sodium hydroxide (20 ml.), and the volatile bases (including the ammonia liberated from amide groups) were removed by steam-distillation, and absorbed and titrated as described above. Devarda's alloy (0·5 g.) was added to the residual alkaline solution to reduce nitrate (and nitrite if present) to ammonia, which after 1 hr. was steam-distilled, absorbed in boric acid, and titrated. The residual mixture was acidified with sulphuric acid and evaporated to small bulk, and its nitrogen content was determined by the Kjeldahl procedure of Campbell and Hanna (*J. Biol. Chem.*, 1937, **119**, 1).

Corresponding blank experiments were performed in each case. The procedures described gave satisfactory results with known synthetic mixtures.

*Determination of the α-Amino-acid Nitrogen obtained by Acid Hydrolysis of the Algae.*—Acid hydrolysis of the seaweed samples was by an excess of boiling 6N-hydrochloric acid under reflux. Initial experiments with *A. nodosum* indicated that hydrolysis for *ca.* 72 hr. gave the maximum yield of total α-amino-acid nitrogen, and this period was used in all subsequent hydrolyses. After removal of insoluble humin by filtration, the hydrolysate was evaporated below 50° *in vacuo* with repeated additions of water, to remove excess of hydrochloric acid. The residue was taken up in water, and the solution was filtered, if necessary, and made up to a known volume. The total α-amino-acid nitrogen was determined by the titrimetric ninhydrin–carbon dioxide method (Van Slyke, MacFadyen, and Hamilton, *loc. cit.*).

In several cases the insoluble humin was extracted with dilute aqueous ammonia, to dissolve any amino-acids precipitated with the humin. The α-amino-acid nitrogen content of these extracts was almost invariably less than 1% of that of the corresponding main hydrolysates.

*Identification of the Volatile Bases from* Pelvetia canaliculata (*March*, 1949).—The volatile bases obtained by steam-distillation of an alkaline suspension of the seaweed were treated with toluene-*p*-sulphonyl chloride; the only product identified was toluene-*p*-sulphonamide (m. p. and mixed m. p. with an authentic specimen, 136—137°). The volatile bases from a second portion of the seaweed were absorbed in dilute hydrochloric acid. The hydrochlorides obtained on evaporation were divided into (*a*) a chloroform-soluble portion in which trimethylamine was identified as its picrate (m. p. and mixed m. p. with an authentic specimen, 218°), and (*b*) a chloroform-insoluble portion consisting chiefly of ammonium chloride, in which a trace of primary amine was detected by the carbylamine reaction. Attempts to identify this amine by paper chromatography in butanol–acetic acid (Bremner and Kenten, *Biochem. J.*, 1951, **49**, 651) were unsuccessful. Fraction (*b*) contained 0·78% of carbon, corresponding to the presence of less than 5% of its weight of primary amine (calc. as $CH_3 \cdot NH_2$,HCl).

*Determination and Identification of the Free α-Amino-acids.*—The seaweed sample (*ca.* 1 g.) was shaken mechanically for 1 hr. with warm 75% (v/v) aqueous ethanol (3 × 30 ml.). After each treatment the extract was separated by centrifuging. The combined extracts were evaporated below 50° *in vacuo*, the residue was taken up in very dilute aqueous ammonia, the mixture was centrifuged, and the liquid was made up to a known volume for the estimation of the total α-amino-acid nitrogen (Table 1, last column).

The amino-acids present in the extracts were identified by one-dimensional paper chromatography in phenol (with ammonia vapour) and in butanol–acetic acid (see the section on paper chromatography). In all the cases examined, the main spots obtained corresponded to aspartic acid, glutamic acid, and alanine; the extract from *R. palmata* gave, in addition, spots corresponding to proline, valine, and leucine(s). Asparagine was not detected, but the possibility of the presence of glutamine could not be excluded as this overlaps alanine when chromatograms are run in phenol, and overlaps aspartic acid when butanol–acetic acid is used.

*Qualitative Chromatographic Techniques.*—(a) *Partition chromatography on cellulose columns.* The columns were prepared (from " Solka-Floc " powdered cellulose) and operated as described by Hough, Jones, and Wadman (*J.*, 1949, 2511), their automatic fraction-collector being used; we are indebted to Dr. J. K. N. Jones for guidance in the construction and operation of this apparatus. The columns were developed with butanol–acetic acid–water (40 : 11 : 19 vol.); this mixture is homogeneous, and gives $R_F$ values similar to those given by the butanol–acetic acid mixture used in paper chromatography (see below). A satisfactory separation of a synthetic mixture of leucine, valine, alanine, glutamic acid, glycine, and aspartic acid (30 mg. of each) was obtained by using a cellulose column (31·7 × 3·0 cm.; cellulose content 115·5 g.), the only " overlaps " being between alanine and glutamic acid, and between glycine and aspartic acid.

(b) *Paper chromatography.* One-dimensional chromatography by the " descending " technique was used. Whatman No. 1 paper was employed, except where the separation of larger quantities of amino-acids was desired, for which Whatman No. 3 paper was used (see below). The chromatographic solvents chiefly used were: (*a*) aqueous phenol (containing 73·85% by wt. of phenol) in an atmosphere containing ammonia and hydrogen cyanide (Consden, Gordon, and Martin, *Biochem. J.*, 1944, **38**, 224); (*b*) the upper layer of butanol–acetic acid–water (4 : 1 : 5 vol.) (Partridge, *Biochem. J.*, 1948, **42**, 238); and (*c*) *tert.*-amyl alcohol in an atmosphere containing diethylamine (Work, *Biochim. Biophys. Acta*, 1949, **3**, 400). Each of these solvents was nearly but not quite saturated with water at 15—25°, so that a slight fall in temperature should not cause separation into two phases. The chromatograms were allowed to hang in the chromatographic tank for several hours before addition of the solvent to the glass trough

(Hanes and Isherwood, *Nature*, 1949, **164**, 1107). Pads of filter paper were clipped to the bottoms of chromatograms which were to be run for long periods (Hanes and Isherwood, *loc. cit.*). After development, chromatograms which were required for quantitative work (see later), or for which phenol had been used as solvent, were dried at room temperature (Fowden and Penney, *Nature*, 1950, **165**, 846; Novellie, *ibid.*, 1950, **166**, 1000; Brush, Boutwell, Barton, and Heidelberger, *Science*, 1951, **113**, 4); other chromatograms were usually dried at 50—80°. The spray reagent normally used to reveal the positions of the amino-acids was the acidified ninhydrin solution of Consden, Gordon, and Martin (*Nature*, 1948, **162**, 180). The Pauly reagent was used to confirm the presence of tyrosine and of histidine on the chromatograms, and Ehrlich's reagent for tryptophan. To detect cystine and cysteine, a portion of the solution was oxidised with hydrogen peroxide; the cysteic acid so formed is readily identified by its low $R_F$ value in phenol (Dent, *Biochem. J.*, 1947, **41**, 240). Methionine is oxidised by hydrogen peroxide to its sulphone, which may be identified by chromatography with butanol–acetic acid ($R_F$ 0·27).

For a more exhaustive examination than one-dimensional chromatograms can provide, we preferred the following technique to the use of two-dimensional chromatograms. A narrow band of the concentrated hydrolysate was developed (18 hr.) on Whatman No. 3 paper with butanol–acetic acid. Narrow strips, cut from the sides of the developed chromatogram, were sprayed with the ninhydrin reagent, and by reference to these " guide-strips " the chromatogram was cut transversely into strips. The amino-acids were eluted from each strip with distilled water by Dent's method (*loc. cit.*); subsequent treatment of the strips with ninhydrin showed that elution had been complete. Each eluate was separately concentrated and the amino-acids present were identified by one-dimensional chromatography. In this way the behaviour of the components in a variety of solvents and with varied reagents can be observed. The modifications required for quantitative work are described below.

*Identification of the Amino-acids obtained by Acid Hydrolysis of* Ascophyllum nodosum *and* Laminaria saccharina.—A portion of an acid hydrolysate of *A. nodosum* (prepared as previously described, and containing 0·046 g. of α-amino-acid nitrogen) was evaporated *in vacuo* below 50° and the residue was shaken with warm glacial acetic acid (4 × 25 ml.), the solution being separated each time by filtration. The combined filtrates were concentrated *in vacuo* and again filtered. The filtrate (9·6 ml.) was chromatographed on a cellulose column (27·7 × 3·7 cm.; cellulose content 128 g.) with the butanol–acetic acid mixture described above. The eluate was collected in fractions (3—4 ml.), each of which was examined for amino-acids by one-dimensional paper chromatography. Those identified were glycine, alanine, valine, leucine, *iso*leucine, phenylalanine, serine, threonine, aspartic acid, glutamic acid, proline, lysine, and arginine. The degree of separation of the amino-acids was poor, probably owing to the effect of contaminants in the extract.

An acid hydrolysate of *L. saccharina* (Sept., 1945) was treated as described above for *A. nodosum*, the only major difference being in a preliminary shaking of the *L. saccharina* hydrolysate with a small amount of activated charcoal (Partridge, *Biochem. J.*, 1949, **44**, 521) in an attempt to remove substances (*e.g.*, soluble humin) which might interfere with the chromatography. This treatment effected a slightly improved separation of the amino-acids. The amino-acids identified were the same as before. An attempt to detect tyrosine and phenylalanine in an extract of the charcoal (Partridge, *loc. cit.*) gave an indecisive result.

*Electrodialysis of Hydrolysates.*—The above hydrolysates of seaweed samples gave poor paper chromatograms. It was found that satisfactory chromatograms could be produced if the hydrolysates were subjected to preliminary treatment with charcoal (see above) followed by electrodialysis to remove electrolytes, and this procedure was followed in subsequent work. Electrodialysis was effected by Dent's modification (apparently unpublished) of the original apparatus of Consden, Gordon, and Martin (*Biochem. J.*, 1947, **41**, 590). The losses of amino-acids during the charcoal treatment and electrodialysis were evaluated by determination of the total α-amino-acid nitrogen of the hydrolysates before and after treatment; it was found possible to reduce the loss of α-amino-acid nitrogen in the combined procedures below 5% (see below).

*Identification of the Amino-acids obtained by Acid Hydrolysis of* Pelvetia canaliculata and Rhodymenia palmata.—An acid hydrolysate of the *P. canaliculata* (March, 1949) sample was prepared as already described. A portion of the crude hydrolysate (50 ml., containing 0·030 g. of α-amino-acid nitrogen) was gently shaken for 30 min. with activated charcoal (0·1 g.). The charcoal was removed by filtration and washed with water. The combined filtrates were concentrated *in vacuo* below 45°, and brought to pH 10—11 by aqueous sodium hydroxide. The solution was aerated vigorously for 2 hr. to remove ammonia (which interferes with electrodialysis), made just acid to litmus with hydrochloric acid, and then electrodialysed. The

current, initially 1 amp., decreased during 6·5 hr. to 0·085 amp., whereupon electrodialysis was discontinued. The solution was filtered from a very small residue, concentrated *in vacuo*, and made up to 50 ml. Ninhydrin–carbon dioxide determinations showed that the electrodialysed solution contained 95·1% of the α-amino-acid nitrogen originally present in the crude hydrolysate.

A portion of the electrodialysed hydrolysate was concentrated *in vacuo* at room temperature, and the amino-acids present were identified by the chromatographic procedures already described. In addition to those found in the acid hydrolysates of *A. nodosum* and *L. saccharina*, methionine, histidine, tyrosine, and ornithine were identified. Cystine was not found. Application of Crumper and Dent's technique (*Nature*, 1949, **164**, 441) showed that in each case the amino-groups were in the α-position.

An acid hydrolysate of *R. palmata* was treated as described above for *P. canaliculata*, the only major difference being that preliminary treatment of the crude hydrolysate with charcoal was unnecessary, the hydrolysate being much lighter in colour than that from *P. canaliculata*. The electrodialysed hydrolysate contained 96·1% of the total α-amino-acid nitrogen of the crude hydrolysate. The amino-acids identified were the same as those found in the *P. canaliculata* hydrolysate.

*Alkaline Hydrolysis: Tests for Tryptophan and Di-iodotyrosine.*—A portion (10 g.) of *P. canaliculata* (March, 1949) was heated under reflux with 5N-sodium hydroxide (200 ml.) for 6·5 hr. at 120—130°. The hydrolysate (a dark, opaque liquid) was brought to pH *ca.* 6 by the addition of hydrochloric acid, and the mixture was cooled. The gelatinous solid which separated on centrifugation was shaken with very dilute hydrochloric acid and again centrifuged. The combined extracts were made up to 500 ml. A portion (20 ml.) of this solution was freed from ammonium ions (see above) and electrodialysed; the filtered residual solution was evaporated *in vacuo* below 40° and the residue was taken up in a small volume of very dilute aqueous ammonia. A one-dimensional chromatogram of this solution was developed with butanol–acetic acid and sprayed with Ehrlich's reagent; a bright pink spot ($R_F$ 0·55) revealed the presence of tryptophan. Di-iodotyrosine could not be detected on a similar chromatogram sprayed with the Pauly reagent, but this test was not regarded as conclusive, because a concentrated standard solution of di-iodotyrosine gave only faint spots under the same conditions.

An alkaline hydrolysate of *R. palmata* was similarly prepared and examined, with similar results.

*Detection of* D-*Amino-acids by Means of an Oxidase.*—We are grateful to Dr. H. Blaschko, of the Department of Pharmacology, for examining a solution which was obtained by acid hydrolysis of *P. canaliculata* (March, 1949); salts were removed by electrodialysis, and a few drops of toluene were added as a preservative. The oxidase was prepared from pig kidney (see, for example, Blaschko, *Biochem. J.*, 1949, **44**, 268). The hydrolysate (2·0 ml., containing 1·14 mg. of α-amino-acid nitrogen) was allowed to react in a Warburg apparatus at 37·5° with 0·6 ml. of the oxidase preparation, with the addition of 0·2M-sodium phosphate buffer (pH 7·4; 0·4 ml.). Two control experiments were run simultaneously; in these, the hydrolysate was replaced (*a*) by water (2·0 ml.) saturated with toluene, and (*b*) by 0·4M-DL-methionine (2·0 ml.) saturated with toluene. After 12 min., control (*b*) had absorbed 249 μl. of oxygen; after 150 min., the hydrolysate had absorbed 69 μl., and control (*a*) 31 μl., of oxygen. These results suggest that the hydrolysate contained small amounts of D-amino-acids, but the considerable variation in the rates of oxidation of D-amino-acids makes it impossible to draw quantitative conclusions. Under similar conditions, with 2·0 ml. of the following amino-acid solutions, the volumes of oxygen absorbed after 90 min. were: 0·008M-DL-aspartic acid, 86; 0·016M-DL-glutamic acid, 13; 0·004M-D-alanine, 79; 0·004M-D-valine, 117; 0·004M-D-leucine, 92; 0·004M-D-*iso*leucine, 97 μl.

*Quantitative Determination of Amino-acids obtained by Hydrolysis of the Algae.*—(a) *Procedure.* Before use in quantitative work, the Whatman No. 3 paper was thoroughly washed with a 0·1% (w/v) solution of 8-hydroxyquinoline in aqueous ethanol, then with 2N-acetic acid, and finally with distilled water. An apparatus of the type described by Hanes and Isherwood (*loc. cit.*) enabled a number of paper sheets to be treated simultaneously.

The quantitative separations were carried out on sheets *ca.* 50 cm. long and 23 cm. wide. An "Agla"-brand micro-syringe was used to apply an accurately known volume of the amino-acid solution to the central portion (13 cm.) of the "starting-line"; the width of the amino-acid band was kept small (*ca.* 0·5 cm.) by applying the solution in successive small portions (0·03—0·05 ml.), each portion being dried by gentle warming before application of the next. The total volume of solution applied was chosen to correspond to *ca.* 0·04—0·2 mg. of α-amino-acid nitrogen of each amino-acid to be determined. "Guide-spots" of the same solution were

applied 1—1·5 cm. from the extremities of the starting-line. After development, the chromatogram was allowed to dry, and washed if necessary (with an organic solvent) to remove traces of the developing solvent. A longitudinal strip of width 2—2·5 cm. was cut from each side of the chromatogram; these strips, on treatment with ninhydrin (or other suitable reagent) indicated the positions of the amino-acid bands on the main portion of the chromatogram. Transverse strips were cut from the latter to isolate the bands of the amino-acids which were to be determined, and these amino-acids were eluted from the strips by distilled water (Dent, *loc. cit.*). Subsequent treatment of the strips with ninhydrin indicated whether elution had been complete. The eluate from each strip was collected in the reaction-flask to be used for the subsequent determination (Van Slyke, MacFadyen, and Hamilton, *loc. cit.*); the solvent was removed *in vacuo* at room temperature, water (2 ml.) was added to the residue, the solution was brought to the appropriate pH, and the amino-acid was determined by the ninhydrin–carbon dioxide method. Strips cut from a blank chromatogram which had been developed and treated in the same way were also eluted; the extracts were submitted to the ninhydrin determination, and these blank experiments gave small corrections which were applied to each analysis.

(b) *Analysis of synthetic mixtures.* The accuracy was tested by experiments with known synthetic mixtures of (A) glutamic acid, glycine, and valine; and (B) glycine, valine, and phenylalanine, the chromatographic solvents used being respectively phenol (in the presence of ammonia) and butanol–acetic acid (see above). Development for 18 hr. was found to effect complete separation in both cases. The phenol chromatogram, when dry, was washed with dry ether; no washing was considered necessary in the other case. The blanks were found to be small and relatively constant. The results of the determinations are given in Table 4.

TABLE 4.

| Synthetic mixture (A) | α-Amino-acid nitrogen | | | Synthetic mixture (B) | α-Amino-acid nitrogen | | |
|---|---|---|---|---|---|---|---|
| | Found (mg.) | Calc. (mg.) | Found, as % of calc. | | Found (mg.) | Calc. (mg.) | Found, as % of calc. |
| Glutamic acid ... | 0·110 | 0·110 | 100 | Glycine ..... | 0·093 | 0·095 | 98 |
| Glycine ..... | 0·093 | 0·093 | 100 | Valine ..... | 0·102 | 0·104 | 98 |
| Valine ..... | 0·106 | 0·099 | 107 | Phenylalanine | 0·090 | 0·089 | 101 |

(c) *Analysis of hydrolysates of algae.* Certain amino-acids were determined in the electrodialysed solutions obtained by acid hydrolysis of *P. canaliculata* (March, 1949) and *R. palmata* (these solutions had been protected from micro-organisms by the addition of a few drops of toluene). Aspartic acid, glutamic, and alanine were separated by development (24 hr.) in phenol–ammonia; valine, leucine, and *iso*leucine by development (65 hr.) in *tert.*-amyl alcohol–diethylamine. The separation of valine and tyrosine was incomplete, and these two amino-acids were eluted and determined together. The results are given in Table 2, the values being the means of duplicate analyses.

1 : 3-*Dibromo*-2-*chloro*-5-*nitrobenzene*.—Bromination of *p*-nitroaniline (Shepherd, *J. Org. Chem.*, 1947, **12**, 275) gave 2 : 6-dibromo-4-nitroaniline (m. p. 200—202°; 96%), which was diazotised (Hodgson and Walker, *J.*, 1933, 1620) and converted into the chloro-compound (m. p. 89·5—91°; 70%) by the Sandmeyer method. The last compound failed to react with potassium fluoride under the conditions of Cook and Saunders (*Biochem. J.*, 1947, **41**, 558).

1-*Bromo*-2-*fluoro*-3 : 5-*dinitrobenzene*.—Bromination of 2 : 4-dinitroaniline (I. G. Farbenind. A.-G., B.P. 399,769/1933) gave 2-bromo-4 : 6-dinitroaniline (m. p. 145—148°; 57%) which was diazotised (Hodgson and Walker, *loc. cit.*) and converted into 1-bromo-2-chloro-3 : 5-dinitrobenzene (m. p. 58—59°; 66%) by the Sandmeyer method. The chloro-compound reacted with potassium fluoride under the conditions of Cook and Saunders (*loc. cit.*) to give 1-*bromo*-2-*fluoro*-3 : 5-*dinitrobenzene*. Recrystallisation from ether and then from aqueous ethanol gave pale yellow needles, m. p. 69° (46%) (Found: C, 27·6; H, 0·8; N, 10·6; Br, 30·2. $C_6H_2O_4N_2BrF$ requires C, 27·2; H, 0·8; N, 10·6; Br, 30·2%).

2-*Bromo*-4 : 6-*dinitrophenyl Derivatives of Glycine and of Glycyl-peptides*.—The amino-acid or peptide was shaken at room temperature with an excess of 1-bromo-2-fluoro-3 : 5-dinitrobenzene in aqueous-ethanolic solution containing sodium hydrogen carbonate. After removal of ethanol *in vacuo* below 35°, the solution was shaken with ether to remove excess of reagent. Acidification of the aqueous layer yielded the desired derivative as a yellow or orange solid, which was recrystallised from methanol or aqueous methanol below 50°. Thus were prepared 2-*bromo*-4 : 6-*dinitrophenyl-glycine*, m. p. 181·5° with previous sintering (Found: C, 30·2;

H, 2·2; Br, 25·1. $C_8H_6O_6N_3Br$ requires C, 30·0; H, 1·9; Br, 25·0%), and *-glycylglycine*, m. p. 191° with previous sintering (Found: C, 31·7; H, 2·7; N, 14·5; Br, 20·8. $C_{10}H_9O_7N_4Br$ requires C, 31·85; H, 2·4; N, 14·9; Br, 21·2%). Crystalline solids corresponding to the 2-bromo-4 : 6-dinitrophenyl derivatives of triglycine, tetraglycine, and leucylglycine (m. p.s respectively 187°, 214°, and 80°, all with previous sintering) were prepared in small quantity, but purification was difficult and analytically pure samples were not obtained.

Paper chromatography of the above compounds, with butanol–acetic acid, phenol (with and without ammonia), and butanol as solvents, gave unsatisfactory results.

*2-Bromo-4 : 6-dinitrophenyl-peptides from* Pelvetia canaliculata.—(*Note:* In this section, the prefix " 2-bromo-4 : 6-dinitrophenyl- " has been abbreviated to " BDNP-"). The seaweed sample (March, 1949; 280 g.) was mechanically shaken for 25 hr. at room temperature with 0·05N-sodium hydroxide (1·5 l.). The residue obtained on centrifugation and filtration through cloth was shaken for a further 4 hr. with fresh solvent (0·5 l.), and the mixture was again centrifuged and filtered through cloth. The combined filtrates (*ca.* 1 l.) were shaken with ethanol (250 ml.) and centrifuged, and the liquid was filtered., The filtrate was saturated with solid carbon dioxide and sodium hydrogen carbonate (10 g.) was added. Determinations on aliquot portions of the solution indicated that it contained 13% of the total nitrogen of the original seaweed. A solution of 1-bromo-2-fluoro-3 : 5-dinitrobenzene (5 g.) in ethanol (50 ml.) was added, and the mixture was kept at 40—50° for 23 hr., with mechanical stirring for the first 11 hr. Evaporation *in vacuo* at 40—50° gave a solution (*ca.* 250 ml.) which was centrifuged, a small residue being rejected. The yellow turbid supernatant liquid was diluted with water (250 ml.) and shaken with ether (3 × 500 ml.) to remove excess of bromofluorodinitrobenzene. The aqueous layer (which separated with difficulty, owing to the tendency to form an emulsion) was concentrated *in vacuo* at 40—50°, and after centrifuging a very small residue was rejected. The turbid liquid was acidified with hydrochloric acid, and centrifuged again, a gelatinous solid separating in small amount (this was later investigated by the methods described below, and was found to contain only very small amounts of BDNP-peptides). Almost one-half of the supernatant liquid was used in attempts to isolate BDNP-peptides, and eventually the following method was adopted. The remainder of the liquid (which was acid to litmus but not to Congo-red) was shaken with a solution of mercuric acetate (50 g.) in water (250 ml.); centrifugation of the resulting mixture yielded a solid, probably consisting largely of mercuric salts of BDNP-peptides. (On neutralisation with sodium carbonate the supernatant liquid yielded further solid, but this was later found to contain only very small amounts of BDNP-peptides.) The solid was washed twice with water by decantation; its aqueous suspension was then saturated with hydrogen sulphide, and the mercuric sulphide was removed by filtration; excess of hydrogen sulphide was removed from the filtrate by evaporation *in vacuo* below 35°. The mixture of BDNP-peptides contained in the resulting solution was fractionated by successive extraction with ethyl acetate and butanol, followed by counter-current partition between 5% aqueous ammonia and butanol (cf. Woolley, *J. Biol. Chem.*, 1949, **179**, 593). The partition was carried out in a series of centrifuge-tubes, as centrifugation was necessary at each stage to separate the liquid layers. 15 Ml. of each solvent were used in each tube, and the lower layers were transferred each time by means of a suitable pipette. The colour peaks were judged visually against a white background. The ethyl acetate extract (A), after partition in a system of 20 tubes, gave colour peaks in tubes 1—3 (upper layers), 11—14 (both layers) and 18—20 (lower layers), and from these fractions were obtained, respectively, the sticky yellow-brown solids A1 (13·5 mg.), A2 (22 mg.), and A3 (35·5 mg.). The butanol extract (B) was partitioned in a system of 10 tubes; colour peaks were observed in tubes 1—3 (upper layers) and 9—10 (lower layers) which yielded, respectively, the sticky brown solids B1 (11 mg.) and B2 (89 mg., probably containing a high proportion of inorganic salt). The fractions A1, A2, A3, B1, B2, and also the fraction C (obtained on evaporation of the faintly yellow aqueous solution left after extraction with ethyl acetate and butanol), were hydrolysed by boiling them under reflux with 6N-hydrochloric acid for 12 hr. The hydrolysates were shaken with ether. All *N*-terminal amino-acids present in the original BDNP-peptides should now have been present in the ethereal layers as the BDNP derivatives; the remaining amino-acids, including the mono-BDNP derivatives of diamino-acids, should have been retained in the aqueous layer. Each ethereal layer was evaporated, and the residues (if any) were hydrolysed by aqueous ammonia (*d* 0·88) at 100—110° for 4 hr.; this procedure (Lowther, *Nature*, 1951, **167**, 767) has been reported to liberate the free amino-acids (except cystine and arginine) from their DNP derivatives, and it seemed likely that the BDNP derivatives would behave in the same way. The hydrolysates were examined for amino-acids by paper chromatography; the results were negative except in the solutions

from fractions A2 and A3, for which serine and glycine, and glycine, respectively, were identified as *N*-terminal amino-acids.

The amino-acids in the aqueous layers of the acid hydrolysates of the BDNP-peptide fractions were identified by paper chromatography; the hydrolysates were then evaporated and the residues hydrolysed with concentrated aqueous ammonia as described above, to hydrolyse any mono-BDNP derivative of a diamino-acid. The products were then examined chromatographically for basic amino-acids. The results are given in Table 3.

This work is part of the programme of research sponsored by the Institute of Seaweed Research, whom we thank for permission to publish, for a maintenance allowance (to D. M. C.), for a grant for materials, and for continued co-operation. It is regretted that in Part IX (*J.*, 1952, 3937) an acknowledgment of financial assistance from the Medical Research Council was inadvertently omitted.

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