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AMINO ACIDS AND SOME OTHER NITROGENOUS COMPOUNDS IN *CALANUS FINMARCHICUS*

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In the summer of 1960 we carried out some preliminary analyses of the total amino acid content of the copepod *Calanus helgolandicus* (Cowey & Corner, 1962). The results indicated that amino acids accounted for a relatively small proportion (33–45%) of the total nitrogen. However, this work was done at a time when considerable numbers of *Calanus* were needed for other experiments, so that only minimal quantities of material were used in the amino acid analyses, and no replication was possible. Because of this it was considered desirable to obtain further information with fresh material collected in large quantities, and we therefore determined the total and individual concentrations of amino acids present in the copepod *C. finmarchicus*, (Gunner.) which we obtained in plentiful amounts at Millport in May 1962.

The larger amounts of material available also enabled us to estimate some of the non-protein nitrogenous substances in *Calanus*, the presence of which could have accounted for the disparity we noted earlier between contents of total nitrogen and amino acid nitrogen. Analyses of the non-protein nitrogen fraction seemed particularly necessary. Apart from the work of Norris & Benoit (1945), who reported a concentration of 0·63 mg trimethylamine oxide nitrogen/g wet weight in a mixed sample of copepods, there is no information about the trimethylamine oxide and betaine contents of zooplankton or about the composition of their exoskeleton. This may contain non-protein nitrogenous substances (e.g. chitin) and the nitrogen present in them might account for a considerable amount of the total nitrogen present in the animal.

The problem also has an important nutritional aspect. Thus, it is generally assumed that phytoplanktonic protein is quantitatively assimilated by zooplankton and laid down as animal protein. There is the further possibility, however, that zooplankton convert some of this plant protein into nitrogenous compounds which are relatively unimportant in the marine economy, and in order to appreciate the extent of such conversion it is necessary to obtain quantitative information about the non-amino acid nitrogen of zooplankton.

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Finally, by using the large amounts of *Calanus* available at Millport, we have also been able to estimate amino acids in both the free amino acid fraction and the protein fraction, thereby providing information about the size and nature of the free amino acid pool in *Calanus*. This may have future importance from the viewpoint of amino acid turnover and nitrogen excretion in zooplankton, Harris (1959) having shown that the latter is extremely high.

MATERIAL AND METHODS

Calanus were taken from the surface of the sea in the Firth of Clyde with a coarse tow net (25 m.p.i.) and brought alive to the Millport laboratory in breffits. Unwanted planktonic animals and other visible contaminating matter were removed from the water, but no attempt was made to sort the animals according to stage or sex. Dr S. M. Marshall of the Millport laboratory kindly examined samples of the *Calanus* we used and found that 59 % were adult females, 28 % Stage V, and 13 % males. The sea water from the breffits was filtered through fine bolting silk and the retained *Calanus* were rapidly washed with distilled water, removed from the bolting silk and gently blotted with filter paper to remove excess water. Portions of the moist *Calanus* were then analysed as follows.

Total nitrogen was determined by a micro-Kjeldahl procedure; digestion was carried out as described by Chibnall, Rees & Williams (1943) and the ammonia was distilled into 4 % boric acid as described by Barnes (1959). Dry matter was determined by drying to constant weight at 105° C. Acid hydrolysis was carried out as described by Cowey & Corner (1962).

An aqueous extract of *Calanus* was prepared as described by Wood (1958). Essentially this method consists in making five successive extractions of the tissue (or animals) with 80 % ethanol. The ethanolic extracts are then combined and shaken with three volumes of chloroform, when an upper aqueous layer containing the non-protein nitrogenous substances separates out. To ensure complete maceration of the *Calanus* we used a Potter-type homogenizer, but because of its limited capacity (about 20 ml.) five 80 % ethanol extractions were made on three separate batches of *Calanus* (collected on successive days). The resulting 80 % ethanol extracts were then combined and treated with chloroform.

In this aqueous extract total nitrogen was determined by the micro-Kjeldahl procedure and amide nitrogen, volatile base nitrogen, trimethylamine oxide nitrogen and betaine nitrogen were determined as described by Kermack, Lees & Wood (1955). An acid hydrolysate of the extract was prepared as described by Cowey (1961).

The residues of the *Calanus* remaining from the aqueous extract preparation were presumed to be largely precipitated protein, fat and exoskeleton. They were collected, air-dried and then continuously extracted in a Soxhlet for 6 h with diethyl ether. The residue of protein and exoskeleton was air-dried and ground to a fine white powder, samples of which were used for ash, moisture and nitrogen determinations, and for acid hydrolysis as described by Cowey & Corner (1962). Tryptophan in this *Calanus* protein was determined by the method of Spies & Chambers (1949), cystine by the method of Schram, Moore & Bigwood (1954) and amide nitrogen by the method of Chibnall, Mangan & Rees (1958), except that ammonia was not determined separately.

The concentrations of other (acid-stable) amino acids in hydrolysates of whole *Calanus* and *Calanus* protein, and in the aqueous extract of *Calanus* both before and after hydrolysis was determined by the method of Moore, Spackman & Stein (1958).

A large sample (92 l.) of sea water was collected from the same area as was the

Calanus and transported to the Plymouth laboratory. The particulate material (phytoplankton, nanoplankton and detritus) was separated from the water as previously described (Cowey & Corner, 1962), dried to constant weight, and its nitrogen and amino acid contents were determined by the methods used for *Calanus*.

RESULTS

The nitrogenous substances found in *Calanus* are listed in Table 1. Protein nitrogen (i.e. that portion of the total nitrogen not present in the non-protein fraction) accounted for 76% of the total nitrogen. The free amino acids in the non-protein fraction made up a further 14% of the total nitrogen. Thus, amino acid nitrogen (free and combined as proteins) represented about 90% of the total nitrogen. Trimethylamine oxide nitrogen and betaine nitrogen made up 6 and about 1·5% of the total nitrogen respectively.

TABLE 1. SOME NITROGENOUS CONSTITUENTS OF *CALANUS FINMARCHICUS*

All values are mg N/g wet weight

Total N	17·69	Non-protein N:	
Total non-protein N	4·22	Free amino acids*	2·45
Total protein N (by difference)	13·47	Trimethylamine oxide	1·06
		Betaine	0·28
		Amide	0·09
		Volatile base	0·08

* By summation of N present in free amino acids (Table 3) but excluding amide N, which was independently estimated.

The total amount of amino acids found in the hydrolysate of whole animals (Table 2) agrees well with the nitrogen figures in Table 1. Thus, 15·63 mg N/g wet weight were found in the total amino acids of whole *Calanus*; the sum of free amino acid nitrogen and protein nitrogen was 15·92 mg N/g wet weight (Table 1). This finding confirms the earlier assumption that nearly all the nitrogen precipitated during the preparation of the aqueous extract was contained in proteins. The total of all the amino acids present in *Calanus* was 112·5 mg/g wet weight, and, as the animals contained 77·6% water, amino acids made up 50% of the dry weight.

These figures indicate that little nitrogen is contained in the exoskeleton other than as protein. It has been asserted (Curl, 1962) that chitin is not estimated by the Kjeldahl procedure. If this were so and chitin were present to a large extent in the exoskeleton our estimates of total nitrogen in *Calanus* would obviously be low. However, when we used the Kjeldahl procedure to determine the nitrogen in a sample of chitin supplied by B.D.H., Ltd. (Poole, Dorset), we obtained theoretical recoveries.

Under the conditions used by us to hydrolyse *Calanus* (6 N-HCl under reflux for 24 h) any chitin present would have been initially broken down to glucosamine residues. No glucosamine peak was present in the effluent

curve of the Moore & Stein column when the *Calanus* hydrolysate was analysed, but recent experiments have shown that under the conditions of our hydrolysis glucosamine is almost completely deaminated.

The protein extracted from *Calanus* (Table 2) contained 16·9% nitrogen on an ash-free, moisture-free basis. It is evident that good recoveries of amino acids have been obtained, because 97·7% of the protein nitrogen was found in the constituent amino acids.

TABLE 2. THE AMINO ACID COMPOSITION OF *CALANUS FINMARCHICUS* AND OF PROTEIN EXTRACTED FROM *C. FINMARCHICUS*

	Calanus finmarchicus			Protein extracted from *C. finmarchicus*
	mg/g wet weight	mg N/g wet weight	g amino acid N/100 g total amino acid N	g amino acid N/100 g protein N
Aspartic acid	10·09	1·06	6·78	6·54
Threonine	4·78	0·56	3·58	3·41
Serine	4·51	0·60	3·84	3·70
Glutamic acid	13·85	1·32	8·45	8·02
Proline	6·39	0·78	4·99	3·17
Glycine	7·72	1·44	9·21	7·27
Alanine	6·80	1·07	6·85	6·97
Valine	6·44	0·77	4·93	4·93
Methionine	2·25	0·21	1·34	1·86
Isoleucine	5·76	0·61	3·90	3·94
Leucine	9·73	1·04	6·65	6·21
Tyrosine	6·36	0·49	3·13	2·19
Phenylalanine	5·59	0·47	3·01	2·93
Lysine	9·32	1·79	11·45	11·01
Histidine	2·36	0·64	4·09	4·03
Arginine	7·62	2·45	15·67	14·33
Cystine/2*	0·37	0·04	0·26	1·03
Taurine	2·60	0·29	1·86	0·00
Tryptophan	—	—	—	0·94
Amide N	—	—	—	5·22
Total	112·54	15·63	—	97·70

* Both cysteine (C-S-H-) and cystine (C-S-S-C) are here included; they are not differentiated by the method used.

The composition of the free amino acid fraction of *Calanus* tissues is shown in Table 3. The concentration of some of these amino acids increased by more than the margin of experimental error (about ±5%) after acid hydrolysis. This is presumably because the amino acids of this fraction have been released during the hydrolysis from small peptides present in the original extract. Some, but not all, of the increase in aspartic and glutamic acid concentrations must have resulted from the hydrolysis of their respective amides. There were several unidentified peaks in the effluent fractions of the Moore & Stein column when the unhydrolysed extract was analysed; they occurred between (i) taurine and aspartic acid, (ii) glycine and valine, (iii) leucine and tyrosine, and are considered to be additional evidence of the presence of peptides in the extract. Similarly the higher concentrations of serine and histidine in the

unhydrolysed extract are probably due to these amino acids being eluted from the Moore & Stein column simultaneously with a peptide.

The amino acid content of the particulate matter from Millport sea water is shown in Table 4, and for comparative purposes similar data relating to

TABLE 3. THE COMPOSITION OF THE FREE AMINO ACID POOL OF *CALANUS FINMARCHICUS*

	Before hydrolysis, μmoles/g wet weight	After hydrolysis	
		μmoles/g wet weight	μg N/g wet weight
Aspartic acid	0·89	3·95	55·4
Threonine	2·13	2·88	40·3
Serine	3·10	2·40	33·6
Glutamic acid	3·20	10·40	145·5
Proline	9·29	9·95	139·0
Glycine	26·81	31·48	441·0
Alanine	9·29	11·50	161·0
Valine	3·04	4·37	61·2
Methionine	1·42	1·30	18·2
Isoleucine	2·60	2·64	37·0
Leucine	3·72	4·43	62·2
Tyrosine	2·38	2·39	33·5
Phenylalanine	1·69	1·72	24·1
Lysine	3·90	5·78	162·0
Histidine	1·05	0·53	22·3
Arginine	15·40	15·00	840·0
Taurine	19·20	18·72	262·0
Glutamine + asparagine	6·31	0	—
Total	—	—	2538·3

TABLE 4. THE AMINO ACID CONTENT AND COMPOSITION OF PARTICULATE MATTER FROM MILLPORT SEA WATER

	μg amino acids/l. of sea water	g amino acid N/100 g total amino acid N in particulate matter	
		Millport	Plymouth (May 1961)
Aspartic acid	63·8	7·1	8·0
Threonine	35·9	4·5	4·6
Serine	54·9	7·7	7·7
Glutamic acid	111·5	11·3	7·6
Proline	15·5	1·9	2·1
Glycine	59·6	11·7	12·4
Alanine	49·3	8·1	7·6
Valine	36·5	4·6	6·5
Methionine	1·7	0·2	L
Isoleucine	18·5	2·1	4·3
Leucine	54·3	5·3	6·7
Tyrosine	13·3	1·1	2·1
Phenylalanine	25·3	2·2	3·2
Lysine	44·8	9·3	10·3
Histidine	12·9	2·6	1·1
Arginine	48·5	16·6	15·8
Total	646·3	—	—

L = Lost.

Plymouth sea water collected at the same time of year is also shown. The particulate matter in Millport sea water amounted to 5 mg dry weight/l., and amino acids in it totalled 646·3 μg/l. Similarly the nitrogen content of the particulate matter was 241 μg/l., whereas nitrogen present in the amino acids amounted to 94 μg/l. Thus, in the particulate matter, amino acid nitrogen accounted for only about 40% of the total nitrogen.

DISCUSSION

In an earlier study of the distribution of nitrogenous substances in copepods Krey (1958) determined protein by a biuret reaction using albumin as a standard. He found that in copepods a variable amount of the total nitrogen (65–74%) was accounted for as protein (albumin) nitrogen and that in a mixture of *Calanus* species only 47·7% of the total nitrogen was present as protein nitrogen. Our preliminary analyses of *C. helgolandicus* (Cowey & Corner, 1962) agreed with Krey's finding and indicated that nitrogenous substances other than amino acids and proteins might occur in relatively large quantities in *Calanus*. Subsequently we have been unable to confirm this and the results now reported show that amino acid nitrogen accounts for 90% of the total nitrogen present in *C. finmarchicus*. Similarly in many other samples of *C. helgolandicus* which we have examined more recently (Cowey & Corner, 1963) amino acid nitrogen again accounted for most of the total nitrogen. We have no explanation for the discrepancy between total nitrogen and amino acid nitrogen reported earlier.

It follows from our results that nitrogenous compounds other than amino acids are quantitatively unimportant in the zooplankton, unless trimethylamine oxide is rapidly formed and excreted. However, there is no evidence that trimethylamine oxide is an excretory product in Crustacea. Similarly, it has not been shown to function in biological methylations in marine animals (Groninger, 1959) and it is probably metabolically inert.

The protein extracted from *Calanus* is unremarkable in its amino acid composition. It is likely to have a high biological value in mammalian nutrition because of its balanced content of essential amino acids. In a bacterial assay of protein quality (Ford, 1960) a sample of whole *C. finmarchicus* had per gram of nitrogen 85% of the value of casein (Dr K. W. Daisley, private communication).

The free amino acid fraction of *Calanus* is large (16–20% of the protein content of the animal). It represents the sum of separate free amino acid fractions in different tissues, of which muscle is probably quantitatively the most important. Because the exoskeleton of *Calanus* almost certainly contains protein but no free amino acids, the free amino acid fraction of the internal organs is probably even higher than 16–20% of their protein content. Other marine Crustacea have also been found to contain high concentrations of free

amino acids, at least in their muscle tissue. Thus, for example, Duchâteau, Florkin & Sarlet (1954) reported 30·44 mg free amino acids/g fresh muscle in *Homarus* (*ca.* 5·0 mg free amino acid nitrogen/g wet muscle). Further, the free amino acid fraction of *Homarus* muscle (and that of other decapods) is characterized by very large quantities of glycine, arginine and proline and large amounts of alanine and glutamic acid; large quantities of these same amino acids typify the free amino acid fraction of *Calanus*. Taurine was also present in large amounts, a finding consistent with that reported by Kermack *et al.* (1955) for *Homarus* muscle. Duchâteau *et al.* (1954) did not measure taurine.

The similarity in size and composition between the free amino acid fraction of *Calanus* and that of higher marine Crustacea is therefore very marked. The large amounts of free amino acids in the muscles of some of these higher marine Crustacea appears to be involved in their adaptation to, and survival in, sea waters of differing salinity. Thus, when *Eriocheir sinensis* and *Carcinus maenas* are adapted to dilute sea water, the concentrations of free amino acids in the muscles are greatly reduced, the muscle fibres probably being always in osmotic equilibrium with the blood (Shaw, 1958). It is not unlikely that the large free amino acid fraction of *Calanus* fulfills a similar function. Marshall & Orr (1955) report that *Calanus* can readily adapt to sea water of a salinity of 27‰ and can survive in water having a salinity as low as 12–17‰. The ability of *Calanus* so to adapt may be dependent on a decrease in concentrations of free amino acids, and it would be interesting to know the concentrations of free amino acids after such an adaptation.

The amino acid composition of the particulate material from Clyde sea water is essentially similar to that of another coastal water (Plymouth) which we have examined. The disparity between Kjeldahl nitrogen and amino acid nitrogen may be due to the presence of nitrogenous compounds in detritus and other non-plant matter, which were included in the particulate material we analysed. From a study of the ratios, carotenoid:Chlorophyll 'a', and Chlorophyll 'a':N, in pure cultures of *Phaeodactylum tricornutum* and in natural populations of phytoplankton Yentsch & Vaccaro (1958) calculated that phytoplanktonic nitrogen accounted for 13–25% of the total particulate nitrogen in Vineyard Sound and for 43–58% in inshore stations of the continental shelf. Thus, it is clear that large quantities of non-phytoplanktonic particulate nitrogen are characteristic of inshore waters. If it be assumed that this non-phytoplanktonic particulate nitrogen is not protein (or amino acid) in character then there is fair agreement between our results and those of Yentsch & Vaccaro.

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SUMMARY

Amino acids, both free and combined as protein, and some other nitrogenous constituents of *Calanus finmarchicus* have been examined. Seventy-six per cent of the total nitrogen in *C. finmarchicus* was present in protein amino acids, 14% in the free amino acid fraction, 6% in trimethylamine oxide, and 1·5% in betaine. These findings are discussed in relation to previous work on nitrogenous constituents of *Calanus*.

The free amino acid fraction of *Calanus* is compared with that of higher Crustacea and it is suggested that this fraction may be important in the adaptation of the animal to dilute sea water.

Amino acid nitrogen made up 40% of the total nitrogen of particulate material from Clyde sea water. This finding is compared with other recent analyses of the phytoplanktonic nitrogen and total particulate nitrogen of inshore waters.

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