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Some Non-protein Constituents of the Tissues of the Lobster

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The object of this work has been to obtain information about some non-protein constituents, more especially the amino acids, of muscle, hepatopancreas, and blood of the lobster (*Homarus vulgaris*). Some preliminary results were included in an earlier communication (Fraser, Kermack, Lees & Wood, 1952).

MATERIALS AND METHODS

Removal of tissue. The lobster was killed by a spike inserted below and just behind its head; the blood that dripped from the wound was collected in a beaker. When the flow of blood had ceased, the shell was cut open and the tail muscle and hepatopancreas removed.

Extraction with ethanol. The tissue was macerated in an Atomix homogenizer in 4 vol. 96% (v/v) ethanol for 5 min. at 12000 rev./min. The suspension was kept at room temperature overnight and then filtered on a Büchner funnel. The ethanol was removed as far as possible from the filtrate by distillation under reduced pressure at 60°. Carotenoid material was extracted by light petroleum, b.p. 60–80°.

Extraction with water. The tissue was macerated with an equal volume of water for 5 min. in an Atomix homogenizer. After standing for a further 5 min. the suspension was treated with an equal volume of 10% (w/v) trichloroacetic acid and the precipitated proteins were filtered off.

Water and ethanol both extracted non-protein nitrogenous constituents of lobster muscle and hepatopancreas. Successive extractions with both water and ethanol were carried out on muscle samples from the same lobster, and the total N of the individual extracts was measured (Table 1). If a sufficient number of extractions are carried out, the same total amount of nitrogenous material is brought into solution by both ethanol and water, but extraction with water is more rapid. Analyses, unless otherwise indicated, were made on solutions obtained by combining the filtrates from two successive aqueous extracts.

Autolysis. In order to study the effects of autolysis, lobster muscle or hepatopancreas (25–50 g.) was cut into small pieces and incubated in presence of 1 ml. toluene in a stoppered flask.

Blood. Lobster blood very rapidly congeals on shedding. The clot was extracted with ethanol in the same way as muscle. The extract from the blood clot was desalted by passing it through a column of sulphonated polystyrene resin and eluting the amino acids from the column with $N-NH_4OH$. The displaced material was concentrated under reduced pressure and the solution chromatographed.

Table 1. *The nitrogenous material removed from lobster muscle and hepatopancreas by successive aqueous and ethanol extractions*

All figures are given in mg./100 g. wet muscle.

Extraction no.	Total N	
	Ethanol	Water
1	500	644
2	176	105
3	54	28
4	20	8
5	18	3
6	7	—
Total	775	788

Chromatographic solvents. Phenol (400 g.) was treated with 100 ml. water. A solution of 0.5N ammonia was placed in the tank, the atmosphere of which was saturated with coal gas. Collidine-water (3:1, by vol.) and *n*-butanol-water-glacial acetic acid (5:4:1, by vol.) were used.

Two-dimensional paper chromatograms of muscle and hepatopancreas aqueous extracts were run in phenol and collidine, and the relative amounts of the individual amino acids were estimated approximately by visual examination of the size and intensity of the spots.

The procedure for ion exchange chromatography was that described by Partridge & Brimley (1952), using a sulphonated polystyrene resin; displacement was carried out by 0.075N-NaOH. Fractions from the columns were examined for amino acids by one-dimensional chromatograms run in phenol or butanol-water-acetic acid.

Analytical methods

Total N. The total N of the extracts was measured by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943), distillation being carried out in the apparatus described by Markham (1942). The distillate was collected in 1% (w/v) boric acid containing the indicator of Conway & O'Malley (1942).

α -Amino N. This was estimated by the method of Pope & Stevens (1939).

Trimethylamine oxide. This was estimated by reducing it to trimethylamine, and measuring the amount of amine produced. A measured volume of extract (1–3 ml.) was mixed with 3 ml. of the reducing agent (1 g. $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 3 ml. conc. HCl) and placed in a boiling-water bath for 15 min. The cooled solution and the unreduced extract were both suitably diluted and analysed for volatile bases by the Conway (1950) technique. Because of the high concentration of acid in the reduced sample, 40% (w/v) NaOH was a more suitable alkali than saturated K_2CO_3 which produced undesirable effervescence. The 40% NaOH was also used for the unreduced samples in these experiments. This use of 40% NaOH had a further advantage. Any glutamine present was hydrolysed by the acid used in the reduction process with the formation of NH_3 , and therefore contributed to volatile base. However, 40% NaOH under conditions obtaining in the Conway units likewise hydrolysed glutamine and so a similar contribution from glutamine to volatile base was ensured in the non-reduced sample. Thus the difference in the volatile base produced by reduction was attributable entirely to trimethylamine oxide.

Glutamine amide N. The observation that glutamine was hydrolysed in the Conway unit by 40% NaOH suggested a method of glutamine estimation. Simultaneous determinations of the volatile base N of the sample were carried out with (a) saturated K_2CO_3 and (b) 40% NaOH as the alkali in the Conway units. The difference in the values obtained was attributable to NH_3 released from glutamine.

Betaine N. This was determined by precipitation with ammonium reineckate followed by colorimetric estimation of the reineckate in acetone solution, appropriate allowance being made for the co-precipitated trimethylamine oxide. Preliminary experiments showed that when precipitation was carried out at pH 2.2, betaines and trimethylamine oxide were precipitated along with the basic amino acids. The reineckates of the amino acids could be removed from the precipitate by washing with *n*-propanol. The insoluble reineckates obtained from lobster muscle by this process were examined qualitatively by paper chromatography of the regenerated bases. Only trimethylamine oxide and glycine betaine could be detected.

The method of betaine estimation consisted in saturating a buffer solution (0.4 ml. 0.2M- Na_2HPO_4 + 19.6 ml. 0.1M citric acid) with ammonium reineckate and adding 3 ml. of this solution to 1 ml. of sample. The mixture was allowed to stand at room temp. for 2–3 hr. and filtered through a no.4 sintered-glass crucible. The precipitate was washed thoroughly with *n*-propanol, dissolved in acetone, the solution made up to 10 ml. and estimated colorimetrically in a photoelectric colorimeter (Evans Electroselenium Ltd.) fitted with an EEL green 404 filter (maximum transmission at 530 m μ). Both trimethylamine oxide and betaine contribute to the colour, so it is necessary to determine trimethylamine oxide independently. Analyses carried out on mixtures of glycine betaine, trimethylamine oxide, and glutamine, showed that glutamine did not interfere with the estimation of trimethylamine oxide or betaine and that the method was satisfactory for estimating betaine in the presence of trimethylamine oxide.

Arginine. This was determined by the method of Dubnoff (1941).

Creatine and creatinine. These were estimated by the method of Folin & Wu (1919).

Amino acids. An approximate estimate of the amounts of the individual amino acids was obtained by the method of Fowden (1951) which involves the elution of the separate spots from an untreated chromatogram and estimation of the amino acid in each eluate by ninhydrin. These investigations were carried out on ethanol extracts of muscle.

RESULTS

The amounts of various nitrogenous constituents in aqueous extracts of muscle and hepatopancreas are shown in Table 2. The relative abundance of the various amino acids present in aqueous extracts of muscle, hepatopancreas and blood, is shown in Table 3. The scoring in this table is admittedly very rough, but quantitative figures for ethanolic extracts of muscle are given in Table 4. These figures are based on single extracts from a number of different lobsters.

The non-protein nitrogenous constituents of muscle and hepatopancreas increased in quantity during incubation of the tissues at 30° in the presence of toluene (Table 5). For muscle this increase occurred almost entirely during the first 1–2 hr. incubation, little further change occurring during the next 16 hr. Observations on the hepatopancreas were limited to periods of incubation up to

Table 2. *The nitrogenous compounds present in aqueous extracts of lobster muscle and hepatopancreas*

All figures are given in mg./100 g. wet tissue.

Tissue	Total N	α -Amino N	Trimethyl- amine oxide N	Betaine N	Glutamine amide N	Volatile base N
Muscle	820	358	100	92	29	10
Muscle	762	306	104	96	30	8
Muscle	805	280	106	—	—	—
Muscle	749	369	110	—	26	14
Hepatopancreas	628	248	17	91	12	30
Hepatopancreas	625	233	21	85	19	16

2 hr. by which time relatively large increases had already taken place in all fractions except the trimethylamine oxide N.

Reducing substances were present in blood to the extent of 20 mg./100 ml. (calculated as glucose) when determined by the method of Nelson (1944).

The reducing compound was shown to be glucose by preparing ethanol extracts of lobster blood and running chromatograms in phenol and butanol-acetic acid when spots with R_f values corresponding to glucose were detected by *p*-anisidine-HCl spray. No other spot was observed.

Table 3. *The amino acids present in aqueous extracts of muscle and hepatopancreas*

The relative abundance was estimated from visual inspection of the spots on a two-dimensional chromatogram. - Indicates not detected.

Amino acid	Relative abundance		
	Muscle	Hepatopancreas	Blood
Proline	+++++	++	-
Glycine	+++++	+++++	++
Taurine	+++++	+++++	-
Alanine	+++	++	+
Arginine	+++	-	-
Glutamine	+++	-	-
Leucine	+++	+	+
Valine	+++	+	+
Glutamic acid	++	-	+
Lysine	++	-	-
Serine	++	+	-
Tyrosine	++	-	-
Histidine	+	-	-
Threonine	+	-	-
Aspartic acid	Trace	-	+

Table 4. *The proportions of the individual amino acids in ethanolic extracts of lobster muscle as determined by the method of Fowden (1951)*

Each figure is based on values obtained from the analysis of extracts of two or more lobsters.

Amino acid	Amount (as % of total α -amino N)
Alanine	5.1
Arginine	5.7
Aspartic acid	Trace
Glutamic acid	0.8
Glutamine	6.2
Glycine	21.9
Histidine	0.5
Leucine	4.1
Lysine	2.3
Proline	37.3
Serine	2.2
Taurine	(10.1)
Threonine	0.6
Tyrosine	0.9
Valine	6.0
Total (excluding taurine)	93.6

Table 5. *Changes in the non-protein nitrogenous constituents of lobster muscle and hepatopancreas during incubation at 30° in the presence of toluene*

All figures are given in mg./100 g. wet tissue.

Time (hr.)	...	...	...	...	...	0	1	2	6	18
Total N	Muscle 1					820	836	854	860	—
	Muscle 2					762	—	916	926	—
	Muscle 3					805	—	962	—	—
	Muscle 4					749	965	990	987	979
	Hepatopancreas					625	—	1438	—	—
α -Amino N	Muscle 1					358	381	391	396	—
	Muscle 2					306	—	363	371	—
	Muscle 3					280	—	360	—	—
	Muscle 4					369	404	404	403	411
	Hepatopancreas					233	—	698	—	—
Trimethylamine oxide N	Muscle 1					100	99	98	102	—
	Muscle 2					104	—	123	118	—
	Muscle 3					106	—	110	—	—
	Muscle 4					110	123	122	121	120
	Hepatopancreas					21	—	23	—	—
Volatile base N	Muscle 1					10	13	15	21	—
	Muscle 2					8	—	19	23	—
	Muscle 3					—	—	—	—	—
	Muscle 4					14	19	26	28	36
	Hepatopancreas					16	—	28	—	—
Betaine N	Muscle 1					92	92	96	93	—
	Muscle 2					96	—	87	92	—
	Hepatopancreas					85	—	114	—	—
Glutamine amide N	Muscle 1					29	33	30	33	—
	Muscle 2					30	—	37	41	—
	Muscle 4					26	32	28	30	28
	Hepatopancreas					19	—	75	—	—

DISCUSSION

Camien, Sarlet, Duchateau & Florkin (1951) have investigated the free amino acids of the muscles of various marine crustacea, including lobsters. They used microbiological methods of assay, as opposed to the chemical methods we have employed. Moreover, their extraction procedure, which consisted in boiling the tissue with water for 5 min. followed by tungstic acid precipitation of the proteins, was more vigorous than the cold water or ethanol extractions used in this investigation. Despite these differences in techniques, there is general agreement between their results and ours.

Both investigations show large amounts of free proline and glycine to be present in fresh lobster muscle, together with smaller quantities of glutamine and alanine and traces of aspartic acid, glutamic acid, histidine, lysine, threonine and tyrosine. However, Camien *et al.* (1951) found somewhat larger quantities of arginine, and smaller quantities of valine and leucine than we did. The methods used by the Belgian workers did not estimate taurine which we have found to be present to the extent of about 300 mg./100 g. wet muscle. As taurine does not contribute to the α -amino N estimated by the method of Pope & Stevens (1939), the total α -amino N obtained by the microbiological methods should be comparable with the values found in the present work. Camien *et al.* found 349 mg. α -amino N/100 g. wet muscle, and this figure is well within the range of α -amino N values we have observed. The relatively small amount of histidine present was in striking contrast to the large amount of this amino acid found in certain fishes, particularly those of the herring and mackerel family (Shewan, private communication).

Lobsters show considerable individual variation in the concentration of non-protein nitrogenous constituents of their muscles. The α -amino N had the greatest variation, the range being 280–370 mg./100 g. wet muscle. By contrast, the trimethylamine oxide and glycine betaine were more constant, varying from 100–110 and 92–96 mg./100 g., respectively. The percentage variation of volatile base N was considerable, but as volatile base N accounted for less than 5 % of the total N, the effect of this variation on the total non-protein N was small.

Of the total water-soluble non-protein nitrogen of lobster muscle, about 84 % has been accounted for. This 84 % consists of 42 % α -amino N, 13 % trimethylamine oxide N, 12 % glycine betaine N, 2 % volatile base N, 11 % nitrogen atoms in the α -amino acids other than α -amino N, and 4 % taurine-N. It may be mentioned that, according to Shewan (1951), only 30 % had previously been accounted for.

When these figures are compared with the corresponding ones for the fresh hepatopancreas, the only significant difference is in the value of the trimethylamine oxide N which is much smaller in the hepatopancreas (3 %).

Although the α -amino N of the muscle extract varied considerably among different lobsters, chromatographic examination showed that the relative proportions of the individual amino acids remained fairly constant.

Every amino acid found in extracts of lobster hepatopancreas or blood was also present in the muscle, but the converse was not true. Of the fifteen amino acids present in muscle, seven were detected in the hepatopancreas and six in the blood. Glycine and taurine were relatively more abundant in the hepatopancreas than in the muscle, and appeared to account for most of the ninhydrin-reacting compounds in the hepatopancreas (Table 3).

The concentration of α -amino N in extracts of lobster blood (about 10 mg./100 ml.) was much lower than that found in muscle or hepatopancreas. On the other hand, the inorganic salt concentration was much higher. This contrasts with insect blood where the salt concentration is fairly low and the amino acid concentration high.

The soluble non-protein nitrogenous constituents of lobster muscle increased slightly during the first 2 hr. of incubation at 30° in the presence of toluene, most of the increase being in the form of α -amino N. There was little further change over the next 18 hr. except in the amount of volatile base N which increased slowly but steadily over the whole period.

Reed, Rice & Sinclair (1929) investigated the changes due to autolysis (in the presence of toluene) in the non-protein nitrogenous constituents of lobster muscle. They observed a steady increase in the total N of the muscle extractives throughout 48 hr. incubation. However, they used a muscle homogenate as compared with the whole muscle studied in the present work. This may account for the differences between their results and ours.

Changes in the non-protein nitrogenous constituents of lobster hepatopancreas during incubation at 30° were also studied. Here a striking increase in the total non-protein nitrogen was observed within 2 hr., most of it being attributable to α -amino N. This α -amino N was probably released from protein material by the action of proteolytic enzymes such as are known to be present in lobster hepatopancreas.

SUMMARY

1. The water-soluble non-protein nitrogenous constituents of lobster muscle, hepatopancreas and blood have been investigated.

2. Over 80 % of the total N of the muscle non-protein nitrogen has been accounted for as 42 %

α -amino N, 13 % trimethylamine oxide N, 12 % glycine betaine N, 2 % volatile base N, 11 % nitrogen atoms other than α -amino in the α -amino acids, and 4 % taurine-N.

3. The water-soluble non-protein nitrogen of the hepatopancreas was partially accounted for as 38 % α -amino N, 3 % trimethylamine oxide N, 14 % glycine betaine N, and 5 % volatile base N.

4. The non-protein α -amino N in muscle and hepatopancreas lies between 200 and 400 mg./100 g. wet tissue; in blood the value is less than 10 mg./100 ml. Fifteen free amino acids have been detected in the muscle, seven in the hepatopancreas and six in the blood. Free glycine is present in relatively large amounts in all three tissues as is proline in muscle and taurine in hepatopancreas.

5. The reducing power of lobster blood is equivalent to 20 mg. glucose/100 ml. and the reducing substance has been demonstrated chromatographically to be glucose.

6. The changes occurring in water-soluble non-protein N of lobster muscle and hepatopancreas during incubation at 30° have been studied.

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Studies in Immunochemistry

16. THE ISOLATION OF BLOOD-GROUP ACTIVE MUCOIDS FROM OVARIAN CYST FLUIDS*

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The isolation and characterization of human blood groups A, B, H and Le^a specific substances from ovarian cyst fluid have been described in considerable detail by Morgan & Waddell (1945), Aminoff, Morgan & Watkins (1950), Kekwick (1950), Annison & Morgan (1952*a, b*), Kekwick (1952*a, b*), Gibbons & Morgan (1954) and Caspary (1954). The group materials, which sedimented and migrated as single substances during ultracentrifuging and electrophoresis, were characterized as mucoids and were found to be chemically similar, though not identical. Before attaching definite genetical significance to the differences in the analytical figures found, one might consider whether these differences can be correlated with the serological specificity observed, or whether the nature of the final product depends on the methods of fractionation employed. The results of the examination of nine cyst materials derived from 'secretors'

belonging to different phenotypes within the ABO system are summarized in this paper. Cyst fluids obtained from the so-called 'non-secretors', which almost always contain Le^a substance only (Grubb, 1948; Grubb & Morgan, 1949; Morgan, 1950) are not included. The cyst contents were fractionated until they yielded at least one mucoid material which showed no evidence of inhomogeneity when examined in the ultracentrifuge or by electrophoresis.

MATERIALS AND METHODS

Cyst fluids. The fluids were kept at 0–4° and dried from the frozen state as soon as possible after removal from the cystadenoma. In general the cyst fluids were more than 1 l. in volume, but fluids of smaller volume were used when they showed some special serological character.

Fractionation procedures. The methods of purification employed are described by Aminoff *et al.* (1950), Annison & Morgan (1952*a, b*), and Gibbons & Morgan (1954). Alkaline conditions were not used in any stage of the isolation

* Part 15 of this series: Davies, Morgan & Record (1955).