Chromatographic Determination of Imidazole Compounds in the Whale Meat

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The two imidazole compounds, carnosine (β -alanylhistidine) and anserine (β -alanyl-1-methylhistidine), have been reported to occur widely in the muscle of various kinds of animal^{1,2)}. Besides these β -alanyldipeptides, the presence of a new dipeptide balenine (β -alanyl-3-methylhistidine) has been recently demonstrated in the extractives of the muscle of whale³⁻⁵⁾. The chromatographic analyses on carnosine and anserine, together with the basic amino acids, were made by several workers⁶⁻¹⁰⁾. The separation of balenine and 3-methylhistidine from these constituents, however, has not yet been carried out except by POCCHIARI *et al.*³⁾

The present investigation was undertaken to find out a separation method using ion exchange column chromatography for these dipeptides and related imidazole compounds, and to determine their concentrations in the extractives of whale meat. The details are given below.

Experimental

Materials. L-Histidine and L-carnosine purchased from Nutritional Biochemicals Corporation, L-anserine nitrate from Cyclo Chemical Corporation, L-1-methylhistidine from California Corporation for Biochemical Research, L-3-methylhistidine from Mann Research Laboratories, were used. Balenine was obtained from the extractives of fin whale meat by the procedures of HORISAKA and MUSASHI¹¹⁾ and of NAKAI and TSUJIGADO¹²⁾. The paper chromatographic separation using a solvent system¹³⁾ of phenol and hydrochloric acid was also used. The purity of these compounds was examined by the ion exchange column chromatographic method of SPACKMAN *et al.*⁷⁾ and by nitrogen determination, respectively.

Preparation of the extractives of whale meat. Fresh whale meat, obtained from the dorsal portions of duplicate specimens and stored in the frozen state, was used. The meat was extracted with 1% picric acid in a blender as described by HAMILTON and TARR¹⁴⁾. The extractives were filtered and freed from picric acid by means of a column of Dowex 2- $\times$ 8 in chloride form according to the procedure of STEIN and MOORE¹⁵⁾. The effluent was evaporated to small volume in a rotary evaporator, diluted quantitatively, and kept in the frozen state until used.

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Analytical methods. Chromatographic analyses were, for the most part, performed with an amino acid analyzer (Hitachi KLA-2) according to the technique of SPACKMAN *et al.*⁷⁾ using a 0.9×50-cm column of Amberlite IR-120. In order to see the availability of the procedure of POCCHIARI *et al.*³⁾, a 0.9×150-cm column of Amberlite IR-120 was used at 50°C. For DAVEY's method⁶⁾, a 0.9×55-cm column of Dowex 50-×4 and 0.2 M borate buffer solution of pH 8.00 were prepared, and amino acid concentrations in 1-ml fractions obtained at the rate of 5 ml per hour by the collector were determined by the photometric ninhydrin method¹⁶⁾.

Results and Discussion

Separation of carnosine, anserine and balenine by the procedure of DAVEY. An aliquot of each solution of these β -alanyldipeptides was chromatographed by the procedure of DAVEY⁶⁾. The results are summarized in Fig. 1 and indicate that the elution peaks for carnosine, anserine and balenine are at 59-, 66- and 68-ml effluents, respectively. When a mixture of these peptides was chromatographed, two peaks are obtained as shown in Fig. 2. It is evident from these figures that anserine emerges together with balenine just behind carnosine from a 55-cm column, and the method

Fig. 1. Elution peaks of carnosine, anserine and balenine from a 55-cm column of Dowex 50-×4.
Conditions: 0.2 M borate buffer, pH 8.00 and 0.181 N Na ions, at 25°C.

Fig. 2. Chromatographic analysis of a mixture of carnosine, anserine and balenine from a 55-cm column of Dowex 50-×4.
Conditions: Same as those in Fig. 1.

seems not applicable for the determination of these dipeptides.

DAVEY^{6,17)} reported the presence of relatively large amounts of carnosine and anserine in the muscle tissues of blue and fin whales using his procedure, and LUKTON and OLCOTT¹⁸⁾ recognized a similar trend in a humpback whale by DAVEY's method with the exception that the column length was extended from 50 cm to 65 cm. The amount of anserine in the muscle of these whales, however, should be re-examined, since a large amount of balenine is found in *Mystacoceti* as shown in Table 1.

Examination on the procedure of POCCHIARI *et al.* The position of the elution peaks of basic amino acids, carnosine and related compounds was determined applying each specimen alone or in combination to the column according to the technique of POCCHIARI *et al.*³⁾ When the mixture was developed, the peaks for balenine and carnosine were at about 990 and 1035 ml effluents corresponding to 33 and 34.5 hours after the start, respectively. The resolution time thus extended 1.3 folds as compared to that of POCCHIARI *et al.* though the reason was not obvious.

From a 150-cm column, anserine and 1-methylhistidine were eluted in overlapping 3-methylhistidine and ammonia, respectively. It has already been shown that anserine is eluted as a zone following but partially overlapping 3-methylhistidine from a 50-cm column of SPACKMAN *et al.*⁷⁾ These results, therefore, suggest that the accurate determination of these imidazole compounds may be not attained by these two methods.

Effect of pH on resolution of imidazole compounds using a 50-cm column of SPACKMAN *et al.* To improve the resolution, the imidazole compounds were eluted by using 0.38 N sodium citrate buffer solutions differing in pH values, from 3.96 to 4.26, at the rate of 30 ml per hour at 30°C, as described previously for a 50-cm column by SPACKMAN *et al.*⁷⁾ The results are shown in Fig. 3.

Fig. 3. Effect of pH on resolution of basic amino acids from a 50-cm column of Amberlite IR-120.

Conditions: 0.38 N sodium citrate buffer, at 30°C.

Table 1. Concentration of imidazole compounds in the extractives of the whale muscle.

	Total N	Carnosine	Anserine	Balenine	Histidine	1-Methyl- histidine	3-Methyl- histidine	Imidazole-N per total N (%)
(mg%)								
<i>Mystacoceti</i>								
Fin whale	637	140	5.0	1500	2.2	0	2.8	60.8
	624	103	4.4	1510	1.0	0	3.0	60.8
Sei whale	715	131	6.4	1840	0.5	0	2.6	64.9
	709	138	7.5	1830	1.8	0	7.9	65.5
Little piked whale	780	154	22.7	1930	4.4	0	6.6	63.6
(*)	718	117	49.3	1700	3.1	0	9.5	61.1
<i>Odontoceti</i>								
Sperm whale	440	196	126	3.2	2.7	0	0.9	18.1
	466	206	83.6	1.8	2.8	0	0.5	16.3
Pilot whale	533	227	39.0	515	2.0	0	9.2	35.3
	549	275	37.5	590	3.3	0	4.6	39.3

(*) An infant specimen.

Concerning the amino acids emerging after lysine, it is apparent that the eluting position is changed as the pH is lowered. At pH 3.96, three dipeptides separate each other satisfactorily but histidine emerges on lysine. In addition, the peaks are broad and the time consuming for separation is inconvenient for daily run. At pH 4.06, although histidine is eluted as a zone following but partially overlapping lysine, the resolution is adequate and the time necessary for the exit of carnosine is 22 hours after the start.

Figure 4 shows the effect of concentrations on resolution of balenine and carnosine, namely, as the concentration increases, the peaks become wider and give the steep acclivity at the beginning of the emergence. When the mixture of a small amount of balenine and a large amount of carnosine is chromatographed, the peak of the latter hanging over that of the former is obtained.

Fig. 4. Effect of concentration on resolution of carnosine and balenine.
a: synthetic mixture; b and c: extractives of whale meat.
Conditions: 0.38 N sodium citrate buffer, pH 4.06, at 30°C.

Determination of imidazole compounds in the extractives of whale meat. The imidazole compounds in the extractives of whale meat were chromatographed with 0.38 N sodium citrate buffer solution of pH 4.06 using a 50-cm column of Amberlite IR-120 at 30°C. For the determination of histidine, a buffer solution of pH 4.26 was used. The identification of β -alanyldipeptides was made by chromatographic analysis of the fractions which were hydrolyzed with 6 N HCl solution. The results are given in Table 1.

The significant feature of the results is the variation in the concentration of carnosine, anserine and balenine. Among the whales examined so far, the fin, sei and little piked whales, which belong to *Mystacoceti*, contain a large amount of balenine, an intermediate amount of carnosine, and a relatively small amount of anserine in their muscle. The sperm and pilot whales, which belong to *Odontoceti*, on the other hand, contain an intermediate amount of carnosine and anserine, differing from the members of *Mystacoceti*. The sperm whale holds only a small amount of balenine, but pilot whale a relatively large amount of it. In general, the contents of carnosine and anserine are not so high as they are reputed to be so by DAVEY^{6,17)} and LUKTON and OLCOTT¹⁸⁾.

Among histidine and its methyl compounds, histidine and 3-methylhistidine are detected, but their levels are less than 10 mg%. 1-Methylhistidine is not found. In samples of fin, sei and little piked whales, the imidazole compounds account for over 60% of the extractive nitrogen, and more than 99% of imidazole nitrogen are occupied by the dipeptides. In sperm and pilot whales, however, imidazole compounds account for only 16-18 and 35-39% of the extractive nitrogen, respectively.

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