Confirmation of Carnosine and Its Methylated Compounds in the Muscles of Some Animals

Michizo SUYAMA and Michie MARUYAMA*

(Received March 18, 1969)

Carnosine (β -alanylhistidine) and its methylated compounds occur widely in the muscles of many species of animals. Anserine (β -alanyl-1-methylhistidine**) was first detected in the goose¹⁾ and later in many species^{2,3)}. Ophidine (β -alanyl-2-methylhistidine**) has been reported to be present in the cobra and some other snakes^{4~9)}. Finally, balenine (β -alanyl-3-methylhistidine**) was recognized in the meat extract of whales^{10~12)}, and proved to be identical with cetanine^{11,13)}.

TSUNOO *et al.*¹⁴⁾ recently reported the presence of ophidine in the three species of dolphin, *Tursiops truncatus*, *Grampidelphis griseus*, and *Feresa attenuata*, on the basis that the infrared absorption spectra and melting point agreed with those of ophidine from the snakes. The authors¹⁵⁾ have determined the amounts of carnosine, anserine and balenine, together with the constitutional imidazole compounds, in the extractives of whale meat by the chromatographic technique using an Amberlite IR-120 column (0.9 $\times$ 50 cm). However, the indication showing the occurrence of ophidine and 2-methylhistidine in the Odonotoceti has not been observed so far on the chromatograms.

The present investigation was undertaken to identify the main imidazole dipeptide in the muscles of several kinds of animal. The peptide preparations obtained from the extractives of snake and dolphin by the procedure of NAKAI *et al.*¹⁶⁾ and from those of dolphin by the method of TSUNOO *et al.*¹⁴⁾ showed the same infrared absorption spectrum as of ophidine described by TSUNOO *et al.*¹⁴⁾ It has become clear from nuclear magnetic resonance (NMR) study that the peptide is β -alanyl-3-methylhistidine and not β -alanyl-2-methylhistidine. The details are given below.

Experimental

Preparation of carnosine and its methylated compounds from the animal meat. By the procedure of NAKAI *et al.*¹⁶⁾, carnosine and anserine were prepared from the histidine plus arginine fraction of the meat extracts of common dolphin, *Delphinus delphis*, and from the

* Tokyo University of Fisheries, Konan, Minato-ku, Tokyo (須山三千三・丸山美智恵: 東京水産大学).

** The trivial names "1-methylhistidine, 2-methylhistidine, and 3-methylhistidine" are inconsistent with the accepted numbering method for imidazole derivatives, the systematic names being α -amino- β -(1-methyl-5-imidazolyl)propionic acid, α -amino- β -(2-methyl-4(or 5)-imidazolyl)propionic acid, and α -amino- β -(1-methyl-4-imidazolyl)propionic acid, respectively. We have, however, used the trivial names for brevity.

lysine fraction of those of bigeye tuna, *Parathunnus mebachi*, respectively. Two specimens of balenine were obtained from the sei whale, *Balaenoptera borealis*, and the common dolphin, and two specimens of ophidine from the snakes, *Natrix tigrina* and *Elaphe quadrivirgata*, respectively. The snake, *N. tigrina*, has been reported to contain ophidine by TAKEDA⁹⁾. Beside these, the meat of common dolphin was treated by the method of TSUNOO *et al.*¹⁴⁾ in order to obtain ophidine.

All these specimens were prepared as free base, and recrystallized from ethanol in clumps of fine needles. They were hygroscopic to some extent, and stored in a dehydrated state.

Measurement of infrared absorption spectra. An infrared spectrometer (Nihon Bunko Kogyo, Model IR-S) was used throughout this experiment.

Mesurement of NMR spectra. The proton resonance spectra were measured by a Varian HA-100 NMR spectrometer operated at 100 Mcps, using deuterium oxide solution and sodium 2,2-dimethyl-2-silapentane sulfate (DSS) as solvent and internal reference, respectively.

Results

Properties of the specimens. Carnosine gave a red color with Pauly's reagent, but the other specimens gave no color. Elemental analyses showed the approximate formulae, $C_9H_{14}N_4O_3$ for carnosine, and $C_{10}H_{16}N_4O_3 \cdot \frac{1}{2}H_2O$ for the others. The melting points of the specimens determined by the use of prismoscopic apparatus (Mitamura Riken Kogyo Co. Ltd.) were 229–231°C for carnosine, 224°C for anserine, and 222–224°C for the others.

Grouping of specimens by infrared absorption spectra. The infrared absorption spectra observed are shown in Fig. 1, and three types of spectrum are apparent, respectively represented by carnosine, anserine and the others. Among the five samples from No. 3 to 7, including balenine and ophidine, no marked difference is recognized. It may be noteworthy that TSUNOO *et al.*¹⁴⁾ obtained a quite identical infrared absorption spectrum on their ophidine preparations with that of balenine reported by NAKAI and TSUNIGADO¹¹⁾ but they did not notice the identity.

NMR signals of carnosine. The NMR spectrum of carnosine together with peak assignments is shown in Fig. 2.

The spectrum is analyzed from chemical shift and line shape as follows: The resonance lines at 7.50–6.70 τ are due to the four protons (H_a , $H_{a'}$, H_b , and $H_{b'}$) of two CH_2 groups of the β -alanyl residue and to the two protons (H_d and H_e) of β - CH_2 of the histidine moiety. Owing to the asymmetric α -carbon of histidine, H_d should not be equivalent to H_e , and the pattern is considered as the AB portion of an ABX system. The quartet at 5.55 τ is due to H_c of the α -carbon of histidine. As illustrated in spin-decoupling (445 cps, denoted by (1) in Fig. 2), H_c is coupled to H_d and H_e , and gives a quartet assignable to the X portion

Fig. 1. Infrared absorption spectra of carnosine and its methylated compounds in KBr.

No. 1: Carnosine from common dolphin. No. 2: Anserine from bigeye tuna. No. 3: Balenine from sei whale. No. 4: Balenine from common dolphin. No. 5: Ophidine from common dolphin. No. 6: Ophidine from *Natrix tigrina*. No. 7: Ophidine from *Elaphe quadrivirgata*.

Specimens No. 1–4, 6 and 7 were prepared by the method of NAKAI *et al.* and specimen No. 5 by the method of TSUNOO *et al.*

of an ABX system. The strong peak at 5.20τ is attributed to the presence of HDO; the exchangeable protons of NH , NH_2 , and COOH are displaced by deuterium and their resonances are included in the peak.

The two protons of the imidazole moiety, H_f and H_g , show absorptions at 3.07τ and 2.30τ , and the respective positions are decided by spin-decoupling as follows: The peaks at 2.30τ and 3.07τ are converted to sharp peaks by irradiations of 693 cps (denoted by (2) in Fig. 2) and of 770 cps (denoted by (3) in Fig. 2), respectively; the two peaks are mutually coupled. Irradiation of 693 cps (2) shows a distinct influence on the resonance lines at $7.03\text{--}6.90\tau$ corresponding to H_d and H_e , whereas irradiation of 770 cps (3) has no effect on the signals of H_d and H_e .

From the formula of carnosine, it may be assumed that the coupling constants $J_{d,f}$

Fig. 2. NMR spectra of carnosine prepared from the muscle of common dolphin. Irradiations of H_e , H_f and H_g are denoted by (1), (2) and (3), and, the heights of resonances before and after irradiations are by N and I, respectively. Spectrometer, Varian HA-100; internal standard, DSS; solvent, D_2O ; sweep time, 500 sec; sweep width, 500 cps.

Fig. 3. NMR spectra of anserine prepared from the muscle of bigeye tuna.

and $J_{e,f}$ in $\text{H}_f\text{-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}=\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}$ would be larger than $J_{d,g}$ and $J_{e,g}$ in $\text{H}_g\text{-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}\text{-NH-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}\text{-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}$ or $\text{H}_g\text{-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}=\text{N-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}\text{-}\overset{\overset{\text{H}_d}{|}}{\underset{\underset{\text{H}_e}{|}}{\text{C}}}$. The signals of H_d and H_e are thus affected by the irradiation of 693 cps (H_f) but little affected by that of 770 cps (H_g). These findings indicate that the signal at 3.70τ is assigned to H_f and that at 2.30τ to H_g .

NMR signals of anserine. The NMR spectrum of anserine is shown in Fig. 3. The signals at $7.50\text{--}6.70\tau$, 5.55τ (quartet), and 5.20τ seem to be in good accordance with the above elucidation on carnosine. The two signals at 3.19τ and 2.40τ are assigned to H_f and H_g of imidazole by the technique of spin-decoupling, respectively. A sharp peak at 6.36τ is assigned to the N-methyl group from the evaluation of chemical shift.

The position of the methyl group of imidazole is decided as follows: Irradiation of H_g (760 cps, denoted by (3) in Fig. 3) heightens the N-methyl peak, but shows almost no

Fig. 4. NMR spectra of balenine and ophidine.

No. 3: Balenine from sei whale. No. 4: Balenine from common dolphin. No. 5: Ophidine from common dolphin. No. 6: Ophidine from *Natrrix tigrina*. No. 7: Ophidine from *Elaphe quadrivirgata*.

Fig. 5. NMR spectra of balenine prepared from the muscle of sei whale.

effect on the peaks of H_d and H_e . Irradiation of H_f (681 cps, denoted by (2) in Fig. 3) has no effect on the peak of N-methyl, but heightens the peaks of H_d and H_e . These findings mean that H_g is far from H_d and H_e , but not far from N-methyl; and that H_f is not far from H_d and H_e , but far from N-methyl. Therefore, the structure of anserine is unequivocally expressed as 1-methyl compound.

NMR signals of balenine and ophidine. The NMR spectra of balenine and ophidine are summarized in Fig. 4, in which five specimens show identical NMR spectra. The data led to a problem as to which compound shows such a spectrum, the 3-methyl compound or 2-methyl compound.

To solve a question, the signals were analyzed by spin-decoupling as follows (Fig. 5): The resonance lines at 7.50–6.70 τ , 5.58 τ (quartet), and 5.20 τ are interpreted in the same way as described above. The signals at 6.35 τ , 3.10 τ , and 2.46 τ are assigned to be N-methyl, H_f , and H_g , respectively. Irradiation of H_f ((2) in Fig. 5) makes the resonance line of N-methyl higher, and also irradiation of H_g ((3) in Fig. 5) makes it higher in the same degree. It may therefore be concluded that the N-methyl is located between H_f and H_g , and this allows one to identify the moiety $H_f-C-N-C-H_g$, a 3-methyl compound (balenine).

Discussion

Ophidine was reported to be present in the muscle of cobra by IMAMURA⁴), and to be

β -alanyl-2-methylhistidine by KENDO⁵⁾, since it exhibited the positive Pauly's reaction beside some other features. Since Pauly's reaction is, in the case of imidazole compound, known to develop only in the compounds in which the NH group remains in the free state¹⁷⁾, the methyl group had thus been assumed to be located at 2-position of the imidazole ring. ONO¹⁸⁾ and ONO and HIROHATA¹⁹⁾ have recognized that chemically synthesized 2-methylhistidine was strongly positive in the reaction.

However, there have been conflicting data on this subject. KENDO⁵⁾ and TAKEDA⁹⁾ have reported that both specimens of ophidine prepared from the cobra and *Natrix tigrina* gave the weak but positive Pauly's reaction, and TSUNOO *et al.*^{7,8)} have shown that the specimens obtained from the snake, *Trimeresurus flavoviridis flavoviridis*, and from the sea snakes, *Laticauda semifasciata* and *L. laticaudata*, were negative in the reaction.

Putting these mentions together, the authors have some doubts about the purity of the specimens, and presume that the positive reaction to Pauly's reagent may be due to the presence of impurities such as carnosine (e.g.: The muscle of *Natrix tigrina* contains 209 mg% carnosine in addition to 111 mg% balenine²⁰⁾). In fact, the purified specimen of balenine gave a negative reaction in the present study.

The misconception of ophidine is, also, attributable to ONO's report in which the same Rf value was given to the synthesized β -alanyl-2-methylhistidine and the preparations from the snake. To state, the identification of carnosine and its methylated compounds by the technique of paper chromatography seems to be difficult, because these compounds give very close Rf values and are not distinguishable by the Rf values and the color developed with ninhydrin reagent. The same difficulty was also recognized in thin layer chromatography²⁰⁾.

Summing it up, the authors are positive in denying the existence of β -alanyl-2-methylhistidine in the muscles of snakes and common dolphin. It is apparent that the compound so far designated as ophidine is nothing but identical with balenine (β -alanyl-3-methylhistidine). If the previous assumption on β -alanyl-2-methylhistidine is true, the NMR spectrum due to the 2-methylimidazole moiety should be evidently different from 1- or 3-methyl compound in the number of CH protons; 2-methylimidazole should have one CH proton as opposed to 1- or 3-methylimidazole having two CH protons. Such a spectrum was not obtained on the specimens analyzed in the present investigation.

WOLFF *et al.*²¹⁾ recently reported that the structure of ophidine, obtained from the whale meat (species is not clarified), reveals the presence of 3-methylhistidine moiety on the basis of the comparison of NMR spectrum of sample with those of 1-, 2-, and 3-methylhistidine as well as anserine.

The authors wish to express their sincere thanks to Central Research Laboratories, Ajinomoto Co., Inc. for technical assistance on NMR spectral study.

References

- 1) D. ACKERMANN, O. TIMPE, and K. POLLER: *Z. physiol. Chem.*, **183**, 1–10 (1929).
- 2) W. A. WOLFF and D. W. WILSON: *J. Biol. Chem.*, **95**, 495–504 (1932); **109**, 565–571 (1935).
- 3) J. A. ZAPP and D. W. WILSON: *ibid.*, **126**, 9–18, 19–27 (1938).
- 4) H. IMAMURA: *J. Biochem.*, **30**, 479–490 (1939).
- 5) K. KENDO: *ibid.*, **36**, 265–276 (1944).
- 6) T. TOMITA: *Z. physiol. Chem.*, **304**, 72–76 (1956).
- 7) S. TSUNOO, K. HORISAKA, Y. MIYASHITA, J. TAKEDA, and N. TAKATAMA: *Jap. J. Pharmacol.*, **14**, 401–411 (1964).
- 8) S. TSUNOO, K. HORISAKA, K. MOTONISHI, and J. TAKEDA: *J. Biochem.*, **56**, 604–606 (1964).
- 9) J. TAKEDA: *J. Biochem.*, **57**, 1–6 (1965).
- 10) F. POCCHIARI, L. TENTORI, and G. VIVALDI: *Sci. Repts. Ist. Super. Sanità*, **2**, 188–194 (1962).
- 11) T. NAKAI and N. TSUJIGADO: *J. Biochem.*, **57**, 812–814 (1965).
- 12) P. O. DENNIS and P. A. LORKIN: *J. Chem. Soc.*, **1965** (Sept.), 4968–4972.
- 13) E. MÜLLER: *Z. physiol. Chem.*, **287**, 40–42 (1951); **317**, 190–192 (1959).
- 14) S. TSUNOO, K. HORISAKA, H. TANABE, M. MURATA, S. TAKAHASHI, A. MUSASHI, and J. TANABE: *Jap. J. Pharmacol.*, **16**, 98–109 (1966).
- 15) M. SUYAMA, T. SUZUKI, and J. NONAKA: *This Bull.*, **33**, 141–146 (1967).
- 16) T. NAKAI, N. TSUJIGADO, and S. AKIYA: *J. Biochem.*, **54**, 541–549 (1963); **56**, 482–483 (1964).
- 17) K. HOFMANN: *Imidazole and Its Derivatives*, Part 1, 136, Interscience Publishers, Inc., New York (1953).
- 18) T. ONO: *J. Jap. Biochem. Soc. (Seikagaku)*, **27**, 278–283 (1955).
- 19) T. ONO and R. HIROHATA: *Z. physiol. Chem.*, **304**, 77–81 (1956).
- 20) M. SUYAMA: Unpublished data.
- 21) J. WOLFF, K. HORISAKA, and H. M. FALES: *Biochem.*, **7**, 2455–2457 (1968).