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DETERMINATION OF OPHIDINE IN HUMAN URINE

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SUMMARY

Ophidine (β-alanyl-3-methylhistidine) was first detected in the urine of two patients and later in two members of the laboratory staff loaded with whale meat, by column chromatography, high-voltage paper electrophoresis and two-dimensional paper chromatography.

The ophidine peak was detected between homocarnosine and dimethylarginine using a lithium buffer gradient in column chromatography. In paper chromatography the ophidine spot was detected at a position close to anserine and homocarnosine. The ophidine in the urine from the patients was of dietary origin since it was absent in the urine a few weeks later.

INTRODUCTION

One or two of the dipeptides carnosine (β-alanyl-histidine), anserine (β-alanyl-1-methylhistidine) or ophidine (β-alanyl-3-methylhistidine) (Fig. 1) occur in the muscle tissue of most vertebrates so far examined. In muscles of whales and snakes the main imidazole dipeptide is ophidine [1] (Table I).

The physiology of these dipeptides is unknown. Their possible role in glycolysis, muscle contraction, or as buffers associated with muscles that maintain their energy-rich phosphate ester supplies by anaerobic means has been suggested [1].

Urine samples from two brothers with mental retardation were examined for inborn errors of metabolism. By column chromatography using a lithium buffer a large unknown peak was found about 30 min after 3-methylhistidine. The urine was then examined using high-voltage paper electrophoresis and two-dimensional paper chromatography. The urine was concentrated and the unknown ninhydrin-positive peak was collected, hydrolyzed and identified as ophidine.

Two normal adults were then loaded with whale meat, and when their urines were examined they both contained large amounts of ophidine.

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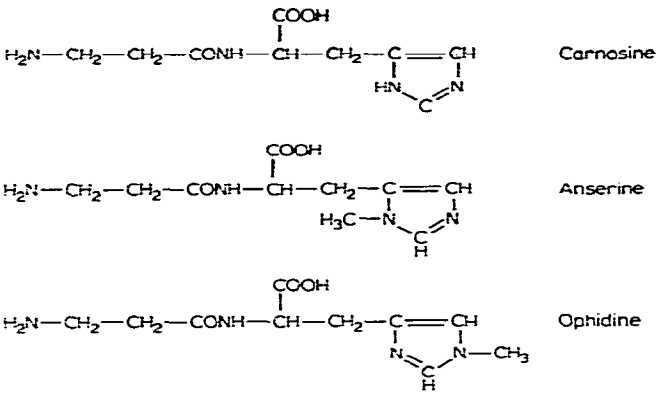


Fig. 1. Structural formulae of three imidazole dipeptides of β-alanine.

TABLE I

AMOUNTS OF CARNOSINE, ANSERINE AND OPHIDINE FOUND IN VERTEBRATE MUSCLE BY VARIOUS WORKERS*

Class	Common name	Muscle	Dipeptide** conc. (μmol/g tissue)		
			Car	Ans	Oph
Mammalia	Ox	Thigh	6.6	1.0	
	Horse	Longissimus dorsi	19.3		
	Cat	Gastrocnemius	6.6	8.3	
	Rabbit	Gastrocnemius	3.5	20.9	
	Fine whale, *Balaenoptera physalus*	L. dorsi	4.3		45.0
	Blue whale, *B. musculus*	L. dorsi	0.4		44.9
	Sei whale, *B. borealis*	Dorsal			37.5
	Bottle-nosed dolphin (newborn)		2.8		3.0
	Dolphin (adult)		5.8		20.2
	Sperm whale	L. dorsi	2.9	7.9	
Aves	Chicken	Pectoral	12.3	40.9	
	Pigeon	Pectoral	0.9	4.6	
Reptilia	Boa constrictor		1.8		0.8
	Cotton-mouthed moccasin		2.2		2.9
	King cobra				5.0
	Sea snake				23.3
	Black-banded sea snake				33.1
	Crocodile		1.4		
Osteichthyes	Atlantic salmon		17.7		

*Modified from Crush [1].
**Car = carnosine, Ans = anserine, Oph = ophidine.

MATERIALS AND METHODS

Urines were obtained from two brothers with mental retardation and from two normal persons. The normal persons were loaded with meat from the whale *Balaenoptera acutorostrata*, which lives along the west coast of Norway. The urines were collected over 4 h after the meal.

Sample preparation

For the determination of the urinary amino acid, the urine was deproteinized by adding 5 mg of sulfosalicylic acid per ml of urine. Before collecting the "ophidine fraction" the urine was first extracted with three volumes of ethyl acetate (pH 1.5—2) and the aqueous layer was concentrated by a rotavapor at 40°C. The concentrated urine was then filtered on an ultrafilter (molecular weight cut-off, > 10,000) and the filtrate was evaporated to dryness on a rotavapor at 40°C. Before analyzing, the dried residue was suspended in water corresponding to ten times the concentration of the urine.

Hydrolysis

The concentrated fraction (100 µl) was hydrolyzed in 6 *N* HCl for 20 h at 100°C. After evaporation to dryness, the hydrolyzates were dissolved in 100 µl of distilled water.

Two-dimensional paper chromatography

Whatman paper No. 1, 20 × 20 cm, was used in Shandon equipment for ascending chromatography at 23°C. In the first dimension the solvent was pyridine—water—acetone—ammonia (45 : 30 : 20 : 5, v/v). In the second dimension the solvent was isopropanol—formic acid—water (75 : 12.5 : 12.5, v/v).

Spray reagents

Ninhydrin (3 g) was dissolved in collidine (50 ml) and made up to 1000 ml with isopropanol.

High-voltage paper electrophoresis

A Pherograph original (Nach Wieland Pfleiderer DBCM. No. 1713858, Frankfurt, G.F.R.) was employed with Macherey Nagel paper (No. 214, 35 × 40 cm). The buffer (0.05 *M* $NaB_4O_7 \cdot 10H_2O$) was adjusted to pH 9.25 with 0.1 *N* NaOH and the electrophoresis was run for 4 h. The paper was then dried at 50°C and a second run carried out with isopropanol—acetic acid—water (8 : 1 : 1, v/v) for ascending paper chromatography.

Liquid column chromatography

The amino acids were analyzed using a Technicon amino acid analyzer. The analyzer was equipped with a glass column (140 × 0.6 cm I.D.) packed with Chromobeads Type B resin. The buffer solution for the gradient elution contained lithium according to the method of Perry et al. [2]. The column was operated at 35°C until the glutamine was eluted, whereupon the temperature was raised to 70°C. The Technicon integrator—calculator was used to

quantitate all of the amino acids. Norleucine was used as the internal standard. The concentration of the ninhydrin-positive substances is expressed as μmol/mmol of creatinine in the urine.

RESULTS

An unknown ninhydrin-positive peak was detected in the urine from the two brothers about 30 min after the 3MeHis peak on the amino acid analyzer (Fig. 2). The retention time was 1134 min (see Table II). When two members of the laboratory staff were loaded with whale meat, a peak with the same retention time was obtained (Fig. 3).

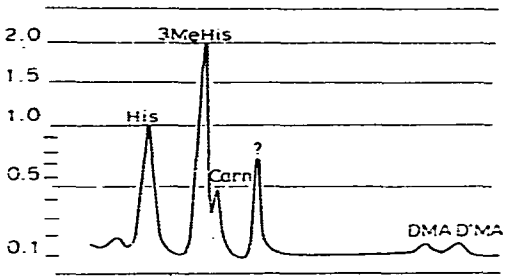


Fig. 2. The chromatogram of ninhydrin-positive histidine compounds from one of the patients using the Technicon amino acid analyzer. The unknown peak was eluted about 30 min after 3MeHis. DMA = Guanidino-N,N-dimethylarginine; D'MA = guanidino-N,N'-dimethylarginine.

TABLE II

THE RETENTION TIMES OF HISTIDINE AND ITS DERIVATIVES, USING THE TECHNICON AMINO ANALYZER WITH LITHIUM GRADIENT

Histidine compounds	Retention time (min)
Histidine	1064
1-Methyl-histidine	1085
Anserine	1098
3-Methyl-histidine	1102
Carnosine	1111
Homocarnosine	1114
Ophidine	1134

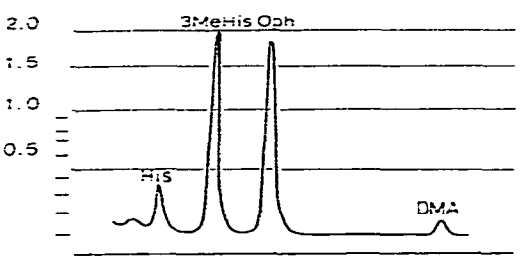
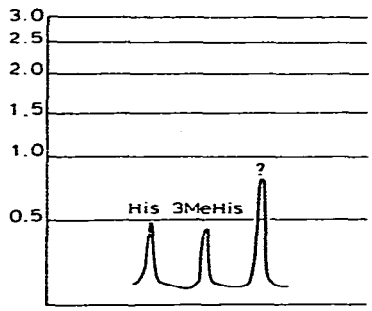


Fig. 3. The chromatogram shows the histidine and histidine derivatives from one of the two normal persons loaded with whale meat. The ophidine peak was eluted about 30 min after 3MeHis.

After extraction with ethyl acetate and ten-fold concentration of the urine samples from the brothers and the normal persons, the unknown ninhydrin-positive peak was collected on a sample collector. Fig. 4 shows the concentrated fraction from one of the brothers with added His and 3MeHis. In Fig. 5 the hydrolyzed products from the same fraction are shown together with added His. The hydrolyzed fraction contained only βAla and 3MeHis, which proves that the unknown peak is ophidine.

The positions of the histidine and the histidine derivatives occurring in human urine upon two-dimensional paper chromatography are shown in Fig. 6.



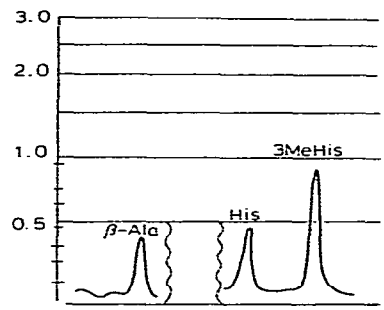


Fig. 4. Concentrated fraction of the unknown substance from the urine of one of the patients. 3MeHis and His are added to the fraction as reference substances.

Fig. 5. Hydrolysis products of the fraction shown in Fig. 3. His was added as a reference.

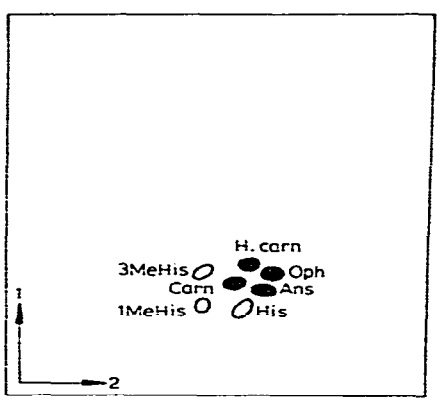
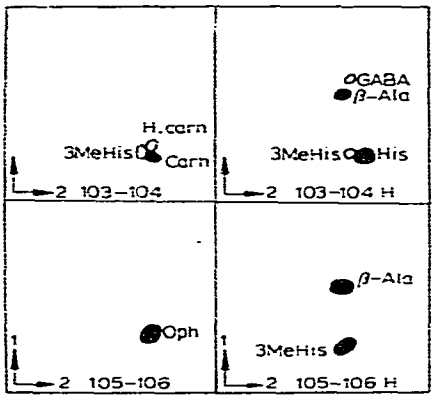


Fig. 6. Separation of standard solutions of histidine and histidine derivatives by two-dimensional paper chromatography.

When the concentrated fractions of the urines from the two brothers and two normal persons were collected, both the fractions and their hydrolysis products were separated by two-dimensional paper chromatography. Fig. 7 shows the unknown ninhydrin-positive fraction (105—106) from one of the brothers' urine, and the fraction (103—104) before it. The same fractions (103—104 H and 105—106 H) after hydrolysis prove that fraction 103—104 contains both homocarnosine (γ-aminobutyryl-histidine) and carnosine, while fraction 105—106 contains only ophidine.

In Fig. 8 the paper chromatogram of urine from one of the two persons loaded with whale meat is shown. No homocarnosine was present, but otherwise the hydrolyzed carnosine and ophidine fractions (103—104 H and 105—106 H) show the same picture as the hydrolyzed fractions from the patient (Fig. 7).



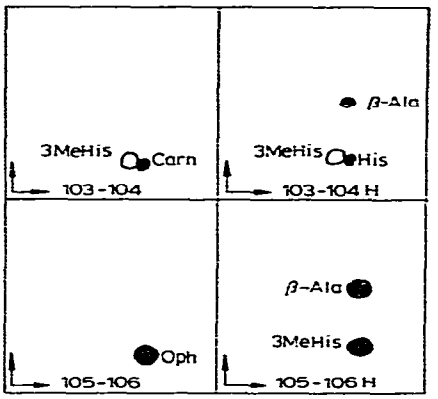


Fig. 7. Paper chromatography of two concentrated fractions collected after column chromatography of the urine from one of the two patients. The two chromatograms to the right are of the hydrolysis products. H = hydrolyzed.

Fig. 8. Paper chromatography of the two concentrated fractions collected from a normal person loaded with whale meat. H = hydrolyzed.

To verify further that the patients really excreted ophidine (containing 3MeHis) and not anserine (containing 1MeHis), the hydrolyzed fraction of the unknown peak was separated by high-voltage paper electrophoresis. In this run the histidine and methylhistidines are clearly separated. In fraction 105—106 H from the patient only 3MeHis was detected (as shown in Fig. 9), which proves the presence of ophidine.

Urine samples received from the two brothers six weeks later did not contain ophidine.

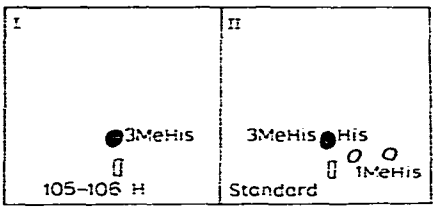


Fig. 9. Separation of histidine and histidine derivatives using high-voltage electrophoresis as the first run and paper chromatography as the second run. (I) hydrolyzed fraction from the concentrated urine from one of the patients. (II) standard solution of His, 1MeHis and 3MeHis. ▯ represents the spot of application. H = hydrolyzed.

DISCUSSION

Quantitative determination of amino acids in physiological fluids using

an automatic amino analyzer is one of the routine procedures for detecting inborn errors of metabolism. This analyzer was used to quantitate the amino acids in the urine from two brothers with mental retardation. They were excreting an excessive amount of an unknown compound and when their urines were hydrolyzed a large amount of βAla and 3MeHis appeared. The unknown peak was collected from the amino acid analyzer and examined before and after hydrolysis by paper chromatography and high-voltage electrophoresis and identified as the dipeptide ophidine.

There is evidence that the diet can affect the dipeptide content of tissues. It has been found that the carnosine content of white muscle of the cat could be decreased by fasting the animal or by preventing use of the muscles. On the other hand, carnosine could be increased by liberal feeding of meat [3]. In salmon on a histidine-deficient diet, the histidine and carnosine in the skeletal muscle were depleted, but the anserine concentration was maintained [4].

Anserine is normally excreted by man after eating rabbit or chicken meat which are rich in anserine (see Table I).

Ophidine has been described in earlier papers [1] as a peptide in the muscle of whales and snakes, together with carnosine (see Table I). When two normal persons were loaded with whale meat they both excreted large amounts of ophidine, just as the two brothers did.

Urine samples from the two brothers collected six weeks after the first sample did not contain any ophidine. This fact indicates that the dipeptide ophidine in the urine from these two brothers was of dietary origin.

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