DISTRIBUTION OF AMINO ACID IN PROTEINS FROM VARIOUS PARTS OF WHALE BODY

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INTRODUCTION

Numerous studies have been already made on amino acid distribution in tissue and organ proteins of terrestrial mammals, and most of their results have been taken in the Block and Weiss' handbook (1956). As regards whales, however, there have been only a few works. It is not only a contribution to our biochemical knowledge of whales to clarify the amino acid distribution in tissue and organ proteins of whales but also necessary in order to find out better utilization of whales. The present study was undertaken with the author's such intention. Proteins obtained from muscle meats of fin, sei, sperm, and baird beaked whales, muscular tissues of diaphragm and heart, mucous membranes of first stomach and small intestine, pancreas, liver, cerebrum, lung, spleen, testis, crystalline lens, aorta and epidermis (black skin) of baird beaked whale, and also gum and baleen plate of whalebone whales were quantitatively analyzed for their constituent amino acids, and results obtained were discussed.

AMINO ACID DETERMINATION

In the present study, the author intended to carry out the determination of amino acids chiefly by means of the direct photometry on ascending one-dimensional paper chromatograms. Many solvent systems which had been recommended in literature for quantitative separation of each amino acid on one-dimensional paper chromatograms were traced in the present work, but unsatisfactory results were obtained in these many cases. Probably it was due to circumstances that the experimental conditions in the present work, especially the kind of filter paper, the method of development and temperature during developing were unable to be brought into line with those in the literature. On the basis of results of the author's many experiments, a method for direct estimation of 18 amino acids on paper chromatograms was devised, which was summarized in Table 1. Estimation of amino acids on the chromatograms that were revealed with color reagents was carried out by the author's modification of the procedure of measuring maximum color

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densities of individual spots (Block, 1950; McFarren and Mills, 1952) except the case of methionine where the method of measuring area of the spot (Block et al. 1958) was employed.

Histidine estimation was not mainly made by this method, but by the elution method, i.e., aqueous eluate of the histidine area of the finished one-dimensional chromatogram was subjected to the Pauly's diazo-reaction and estimated colorimetrically.

By means of these methods, 18 kinds of common, naturally occurring amino acids except tryptophan and hydroxyproline were easily and economically determined with satisfactory results.

EXPERIMENTAL

I. *Direct Estimation on Paper Chromatograms*

1. *Filter Paper.* Toyo No. 51 filter paper sheets 30 *cm.* wide and 30 *cm.*, 40 *cm.* and 43 *cm.* long respectively are used for 24 *cm.*, 30 to 35 *cm.* and 36 to 38 *cm.* of solvent run (cf. Table 1).

TABLE 1. OUTLINE OF THE PRESENT METHOD FOR DIRECT ESTIMATION OF AMINO ACIDS ON PAPER CHROMATOGRAMS

	Solvents	Lengths of solvent run beyond original spots	Color reagents*	Amino acids estimated
1.	n-Butanol · acetic acid · water (4:1:1 v/v)	33–35 *cm.* Develop 3 times	I	Arginine, cystine, hydroxylysine, phenylalanine
2.	n-Butanol · acetic acid · water (25:6:25 v/v) (Block and Weiss, 1956)	35 *cm.* Develop 3 times	I	Lysine, histidine, arginine, alanine, tyrosine
		24 *cm.*	II	Proline
			III	Methionine
3.	n-Butanol · acetic acid · water (40:5:7 v/v) (Rao and Wadhwani, 1955)	35 *cm.*	I	Phenylalanine, tyrosine
4.	n-Butanol · ethanol · water (20:1:4 v/v)	36–38 *cm.* Develop 3 times	I	Leucine
			II	Proline
5.	Phenol saturated with pH 12 phosphate buffer of 0.067 *M.* (Treat filter paper with the same buffer before sample application) (McFarren, 1951, 1952)	30–32 *cm.*	IV	Threonine, glycine, serine, glutamic acid, aspartic acid
6.	Pyridine·isoamyl alcohol·water (1:2:1 v/v)	35 *cm.* Develop 3 times	I	Isoleucine
7.	Pyridine·isoamyl alcohol·water (10:10:7 v/v) (Giri, 1953) (Treat filter paper with 0.05 *M* sodium acetate solution before sample application)	34–35 *cm.*	I	Valine

* Color reagents:
I = 0.4% (w/v) ninhydrin in acetone.
II = 0.2% (w/v) isatin in acetone.
III = 4 *cc.* of 0.002 *M* PtK_2Cl_6, 0.25 *cc.* of 1*M* KI, 0.4 *cc.* of 2*N* HCl, 76 *cc.* of acetone (Toennies and Kolb, 1951; Block and Weiss, 1956).
IV = 0.4% (w/v) ninhydrin, 4% (v/v) acetic acid in acetone.

The buffered paper sheets (No. 5 and No. 7 in Table 1) are prepared by dipping filter paper sheets, 30×60 *cm.*, into the buffer solutions, air-drying by suspending by one end and cutting into the desired size. pH 12 phosphate buffer solution is prepared according to McFarren (1951).

2. *Preparation of Protein Hydrolyzate.* 200 *mg.* of protein is hydrolyzed with 2 *cc.* of 6 *N* HCl in sealed glass tube in an oven at 110°C for 24 hrs. The contents of the tube are filtered through a glass filter, and after thoroughly washing the precipitate thus obtained with water, the combined filtrate and washings are concentrated to dryness on a steam bath. The residue is then dissolved in 10 *cc.* of water, again evaporated to dryness, and placed in a vacuum desiccator over KOH for 24 hrs. The residue is finally taken up in 2.0 *cc.* of water (Original solution).

For estimations of leucine and hydroxylysine, this original solution is used as it is, and for estimations of the others, four or two times dilution of the original is used in most cases.

3. *Preparation of Standard Solution.* 50 *mg.* glycine, 50 *mg.* DL-alanine, 70 *mg.* DL-valine, 80 *mg.* L-leucine, 60 *mg.* DL-isoleucine, 50 *mg.* DL-serine, 50 *mg.* DL-threonine, 30 *mg.* L-methionine, 10 *mg.* L-cystine, 50 *mg.* L-phenylalanine, 50 *mg.* L-tyrosine, 65 *mg.* L-proline, 70 *mg.* L-aspartic acid, 120 *mg.* L-glutamic acid, 40 *mg.* L-histidine·HCl·H_2O, 70 *mg.* L-arginine·HCl and 100 *mg.* L-lisine·HCl are dissolved in water under addition of an adequate quantity of 6 *N* HCl, and the solution is made up to a final volume of 10.0 *cc.* 2.0 *cc.* of the solution is evaporated to dryness on a steam bath. The residue is dissolved in 10 *cc.* of water, again evaporated to dryness, and placed in a vacuum desiccator over KOH for 24 hrs. The residue is finally dissolved in 2.0 *cc.* of water (Original standard solution).

For estimation of leucine, this original solution is employed as it is, and for estimation of the others, four or often two times dilution of the original is employed.

As the standard of hydroxylysine, a gelatin hydrolyzate in which hydroxylysine content was already determined by periodate oxidation (Block and Bolling, 1951) is employed.

4. *Application of Solutions.* On a line 5 *cm.* from the lower edge of the paper, seven points are marked at 2.5 *cm.* or 3.0 *cm.* intervals. The standard and the test sample are applied at alternate points with a self-filling micropipette. The desired concentration on the paper is built up by repeated sequence of spotting and drying. Every spotting must be

Amino acid specimens employed as the standards were manufactures of Ajinomoto Co., Ltd. and Tokyo-Kasei-Kogyo Co., Ltd. These were paper-chromatographically pure.

made in the same quantity of the solutions. In the author's experiment, one spotting was 77×10^{-5} *cc.* in quantity. The standard solution is applied 1, 2, 3, and 4 times at the range of four respective points, and the test solution, in most cases, 2 to 3 times at the range of three respective points.

5. *Developing Solvents.* The solvent mixtures in Table 1 are prepared in the usual fashion.

6. *Development of Chromatograms.* Being spotted, the paper sheet is suspended in air at room temperature for more than one hour. Then, the sheet is formed into cylinder, and subjected to the ascending development.

In the case that one of the solvents No. 1, 2, 4, and 6 is used, the multiple development technique (Jeanes et al., 1951) is adopted, i. e., the sequence of developing and air-drying is repeated for a total of 3 times. In the case of 35 *cm.* solvent run using one of the solvents No. 2, 3, and 6, it is recommended that the paper is allowed to remain in the developing chamber for several hours more after the solvent front has reached the upper edge of the paper.

The development on the pH 12 buffered paper should be commenced within 20 hrs. from the time when the air-drying of the paper moistened with the buffer solution was commenced. If not so, the resolution of amino acids will grow worse owing to decline of the buffer action of the sheet.

7. *Color Formation of Amino Acids.* At the completion of the development, the paper is dried in a gentle current of air at room temperature. The sheet is then dipped in a color reagent and again dried at room temperature. When one of the ninhydrin solutions in Table 1 is employed as the color reagent, the color is developed by heating in an oven at 60° to 63° C. for 15 minutes. When the isatin solution is used to reveal proline, the color is developed by heating at 70° to 73°C. for 10 minutes. Proline gives a blue spot on a yellow background. The oven should be saturated with water vapor in every case.

Platinum reagent for methionine must be prepared with acetone purified with use of $KMnO_4$ and K_2CO_3 (Block and Weiss, 1956). On treating the sheet with this reagent, methionine gives a white spot on a red-purple background without heating.

8. *Measurement of Maximum Color Densities of Amino Acids except Methionine.* After the revealed chromatogram has been allowed to keep at room temperature for more than one hour, the color densities of the spots are determined with a photoelectric colorimeter as in the following.

Each spot is cut out of the chromatogram and fastened with the aid of clips to a sample holder which contains an aperture of suitable size.

In order to reduce the experimental error which may be derived from unevenness of thickness of paper in the same sheet, it is advisable that the size of the aperture is made as large as possible within the size of the spot to be estimated, provided that the separation of the spot allows. Shape and size of the aperture should be properly changed according to shape, size, and separation of the spot to be estimated. However, when a series of spots of the same acid is estimated on the same chromatogram, the same aperture must be used for every spot. In the author's experiment, circular apertures 7 *mm.*, 9 *mm.*, 11.5 *mm.*, and 14 *mm.* in diameter and rectangular apertures 3×14 *mm.*, 5×14 *mm.*, and 10×14 *mm.*, have been employed.

9. *Measurement of Areas of Methionine Spots.* Circumferences of white spots revealed with the platinum reagent are marked in pencil, and the areas are determined with a planimeter.

10. *Calculation.* The standard curve for each amino acid is prepared from the values at individual concentrations of the standard. The amino acid concentration in each spot of the test solution is estimated by interpolation from the curve, and then the content of each amino acid in the sample of protein is calculated. The result obtained at every spot of each amino acid is averaged. An example of calculation formula is as follows:

If A=quantity (*mg.*) of protein before hydrolysis contained in 2.0 *cc.* of the hydrolyzate (original),

B=the number of times of spotting the hydrolyzate (4 times dilution of the original),

C=quantity (*mg.*) of each amino acid in 10.0 *cc.* of the standard solution (original), and

D=the number of times of spotting the standard solution (4 times dilution of the original) being equivalent to B ('D' is estimated from the standard curve), then

each amino acid content in protein (*g.* per 100 *g.* of protein)$=20\cdot C\cdot D/A\cdot B$

In order to employ this formula, concentrations of the amino acid and test sample in every spot should be expressed in numbers of times of spotting the standard solution and hydrolyzate respectively, as mentioned above.

II. *Determination of Histidine by the Elution Method*

0.025 to 0.1 *cc.* of a solution containing 25 to 200 γ histidine is applied as a streak, 18 *cm.* long and about 3.0 *mm.* wide, on a line 5 *cm.* from the bottom of a sheet 30 *cm.* square of Toyo No. 131 filter paper. One drop of the same solution is also placed at each point 1 *cm.* from each end of the same line, with the object of preparing guide strips. After

the solvent, n-butanol·acetic acid·water (4 : 1 : 1 v/v), has upwards run about 17 *cm.* past the line, the guide strips are cut off and sprayed with a diazo-reagent (Block et al., 1958, p. 133) to reveal histidine spots. The location of histidine area in the remaining part of the sheet is presumed from the results of the guide strips. A strip about 2.4 *cm.* wide which contains the whole histidine area is cut out of the remaining sheet and irrigated with water by the same technique as descending paper chromatography. The lower end of the strip should be cut in a wedge form. First 2 *cc.* of the eluate is subjected to the Macpherson's modification (1946) of the Pauly-reaction to estimate histidine, excepting the electrodialysis in his method.

III. *Determination of Tryptophan and Hydroxyproline*

Tryptophan was determined by Spies and Chambers' 'procedure H' (1949). Test solution for the determination was prepared as in the following: About 0.5 *g.* of each protein was weighed accurately, boiled with 10 *cc.* of 5 *N* NaOH in an oil bath at 125° C. under reflux for 5 hours, and then the solution was made up to a whole volume of 50.0 *cc.* with adding water.

Hydroxyproline was determined by Neuman and Logan's method (1950). The preparation of the test solution was as follows: 200 *mg.* of protein was hydrolyzed with 2 *cc.* of 6 *N* HCl in a sealed glass tube in an oven at 110° C. for 24 hours. The contents of the tube were filtered through a glass filter, and after washing the precipitate with 10 *cc.* of water, the combined filtrate and washings were concentrated to dryness on a steam bath. The residue was then taken up in 10 *cc.* of water, again concentrated to dryness, dissolved in 30 *cc.* of water, and after neutralized with *N* NaOH, evaporated to dryness. The residue was finally taken up in 10.0 *cc.* of water. At the time of the determination the solution was diluted so as to contain 10–40 γ of hydroxyproline per *cc.*

DISCUSSION

The previously published methods of amino acid estimation using one-dimensional paper chromatography have adopted mostly the descending developing technique. With the descending technique, the desirable prolongation of the length of solvent run is easily achieved by properly extending the length of development time, and consequently, there is the possibility of promoting the resolution of amino acids in this way (Roland and Gross, 1954). With the ascending development technique, there is a limit to the length of solvent run as is generally known, and

consequently the resolution of amino acids are restricted in some measure by this fact. However, the ascending technique has been exclusively adopted in the present work because of its merits of simplicity of apparatus and the ease with which a large number of analyses may be made (Williams and Kirby, 1948).

I. *The Direct Estimation on Paper Chromatograms*

The resolution patterns (Fig. 1) is scarcely distorted by considerable variations of experimental conditions, especially temperature during the development. In choosing the solvents, the author paid attention to this point also, because all the developments in the present work had to be made at room temperature. Conditioning the filter paper in the developing chamber before development is unnecessary.

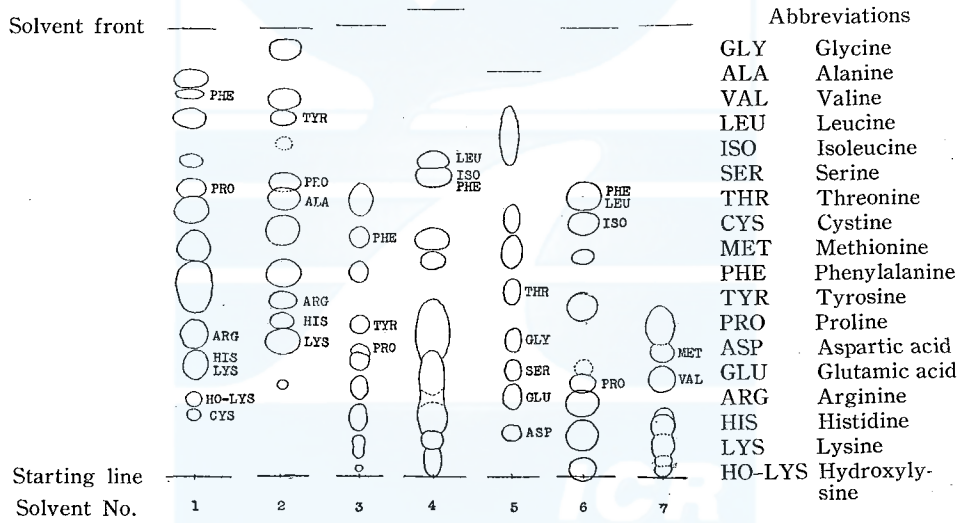

Fig. 1. Resolution Patterns

In most cases of paper chromatography with the ordinary solvents, leucine and isoleucine as well as methionine and valine overlap each other. It is these amino acids that the author took pains in resolving.

The solvent No. 4 used for separation of leucine is the author's modification of one of the Rao and Wadhwani's solvents (1955) used in their quantitative circular paper chromatography. Leucine and isoleucine as well as phenylalanine still overlap slightly, but the estimation of leucine is completely achieved with use of a narrow aperture at the time of measuring color densities of the spots. In the present experiment, apertures 3 *mm.* and 5 *mm.* wide were employed for estimation of leucine.

Isoleucine is sharply separated from other amino acids with the aid of the solvent No. 6.

With the use of the solvent No. 7, valine is separated even on unbuffered paper, but in such a case, the color of the spot is uneven and consequently the estimation is unable to be made. The unevenness of the color is presumably caused by hydrochloric acid contained in the protein hydrolyzate and the standard solution. The author could remove this hindrance by means of buffering the paper sheet with sodium acetate solution before the sample application.

A portion of methionine is autoxidized into methionine sulfoxide on the paper during development and air-drying, and so, applying the multiple development technique to the estimation of methionine (Block and Weiss, 1956, p. 78) is unadvisable. Thus, some amino acid and methionine sulfoxide often overlap each other on the chromatograms made by the multiple development technique. The author, therefore, paid attention to this point when this technique was adopted for resolution of amino acids.

In descending (McFarren, 1951, 1952; Reidel and Bienenfeld, 1956; Lange, 1956; Schnuchel, 1956; Roberts and Kolor, 1957) and circular (Krishnamurthy and Swaminathan, 1955) paper chromatography, the pH 12 buffered phenol has been favorably used for separation of aspartic acid, glutamic acid, serine, glycine, threonine and alanine, but in ascending chromatography, according to the author's experience, alanine is incompletely separated. In the author's method, therefore, this solvent is used only for separation of the five amino acids other than alanine.

The location of hydroxysine on the chromatogram was experimentally determined basing on the author's investigation of chromatogram of a hydrolyzate of gelatin which contains a comparatively large amount of this amino acid, the fact that this amino acid is precipitated with phosphowolframic acid, and Inskip's study (1951).

Hitherto, determination of hydroxylysine has been made exclusively by periodate oxidation (Block and Bolling, 1951; Akabori and Mizushima, 1954), but its procedure is complicated and a relatively large amount of the test sample is required for one estimation. By use of the above-mentioned technique, the determination of this amino acid is simplified and enabled to be made in a micro- or submicro-scale.

The present method was successfully applied to determination of 17 amino acids in casein.

Casein (E. Merck) desiccated in vacuo over H_2SO_4 was hydrolyzed with 6 *N* HCl, as above described, for 24 hrs. for estimation of methionine, phenylalanine and tyrosine, and for 48 hrs. for all the other amino acids.

The values obtained in the present experiment and also the values

reported in literature are listed in Table 2. There is a good agreement between the values in both cases. The values for serine and threonine in the literature include corrections made for decomposition of these acids during acid hydrolysis of protein, but the values obtained in the present experiment include no correction. In the literature, tyrosine was estimated on alkaline hydrolyzates, and in the present experiment, on the acid hydrolyzate. This is presumably the reason of the fact that the present value for tyrosine is somewhat lower than the values in the literature.

TABLE 2. RESULTS OF AMINO ACID DETERMINATION ON CASEIN

Constituent	Values expressed in *g.* per 100 *g.* protein			Values expressed in *g.* amino acid N per 100 *g.* protein N	
	Present Work	Gordon et al. (1949, 1950)	Sundararajan et al. (1957)	Present Work	Gordon et al. (1949, 1950)
Total N	15.02	15.63	15.56		
Glycine	2.0	2.00	1.90	2.5	2.39*
Alanine	3.4	3.20	3.38	3.6	3.22*
Valine	7.2	7.2	6.94	5.7	5.5
Leucine	9.7	9.2	9.54	6.9	6.3
Isoleucine	6.1	6.1	6.13	4.3	4.2
Proline	10.9	10.6	11.72	8.8	8.3*
Phenylalanine	4.9	5.0	4.98	2.8	2.7
Tyrosine	5.2	6.3	6.32	2.7	3.1
Serine	5.0	6.3	6.27	4.4	5.4
Threonine	4.3	4.9	5.28	3.4	3.7
Cystine	0.31	0.34	0.35	0.24	0.3
Methionine	2.9	2.8	2.93	1.8	1.7
Arginine	3.8	4.1	3.92	8.1	8.4
Histidine	2.9	3.1	3.03	5.2	5.4
Lysine	7.6	8.2	8.18	9.7	10.1
Aspartic acid	6.5	7.1	7.66	4.6	4.8
Glutamic acid	21.4	22.4	21.62	13.6	13.6

* The author's calculation from figures in the literature expressed in *g.* per 100 *g.* protein (Gordon et al., 1950).

Because accuracy of the quantitative paper chromatography using the maximum color density technique was exhaustively discussed already (Salander et al., 1953; Roland and Gross, 1954; Block and Weiss, 1956; Roberts et al., 1957, 1958), no attempt on it has been made in the present study.

Block (1958, p. 117) pointed out that cystine was decomposed when a hydrolyzate was allowed to stand. In the present experiment the same tendency was observed with methionine.

II. *Determination of Histidine by the Elution Method.*

In the Macpherson's method (1946) for histidine estimation, histidine is separated from tyrosine, which also presents the Pauly-reaction, by means of electrodialysis. In the author's procedure, the separation is easily achieved by means of the one-dimensional paper chromatography.

There is such satisfactory reproducibility as the results of two times determination on 3 samples of protein are shown in Table 3.

TABLE 3. HISTIDINE CONTENT IN THREE SAMPLES OF PROTEIN DETERMINED BY ELUTION METHOD

Values are expressed in *g.* per 100 *g.* of dry protein.

	Total N	Histidine
Meat, fin whale	15.29	2.69 2.63
Liver, baird beaked whale	14.59	2.81 2.88
Pancreas, baird beaked whale	15.23	2.79 2.42

After the present experiment of determining histidine contained in tissue and organ proteins of whales was finished, Frank and Petersen (1956) published a similar technique in their study on estimation of histidine concentration in blood-plasma.

MATERIALS AND PREPARATION OF SAMPLES OF PROTEIN

All the muscle meats used in the present work were dorsal muscles.

Muscle Meat of Fin Whale (*Balaenoptera physalus*). The material was taken from a male fin whale 61 feet in body length which was caught at the position of 54° 14′ N, 166° 40′ W on Sept. 19th 1954. About 2.5 hours elapsed from the time of killing to the commencement of the treatment. A lump of meat was taken out of the carcass by Mr. K. Fujino of the Whales Research Institute and refrigerated until the commencement of the experiment (Oct. 10th 1954).

4 *kg.* of minced meat was boiled with water twice as much as the meat for 5 minutes and then filtered under pressure. The residue was boiled again with water and filtered as above. The final residue was air-dried. 50 *g.* of the air-dried matter was treated with tepid water, boiling water, alcohol, and ether successively as muscle meat of baird beaked whale mentioned below.

Muscle Meat of Sei Whale (*Balaenoptera borealis*). The material was taken from a male whale 44 feet in body length which was killed at the position of 51° 44′ N, 173° 00′ E on Aug. 4th 1952. About 4 hours elapsed from the time of killing to the commencement of the treatment. The material was taken by Mr. K. Fujino and refrigerated until the

commencement of the experiment (Oct. 1st 1952). 16.4 *kg.* of minced meat was treated similarly as fin whale meat.

Muscle Meat of Sperm Whale (Physeter catodon). A piece of muscle meat was taken from a male whale 41 feet in body length which was killed off the coast of Kinkazan Island on Dec. 6th 1948. Presumably about 18 hours elapsed from the time of killing to the arrival at the landstation. The grade of freshness of the carcass (Nakai, 1948) at the commencement of treating was about 70. Without delay, the material was minced, treated with acetone and ether successively and then air-dried. Thus treated material was further treated with tepid water, boiling water, alcohol, and ether as muscle meat of baird beaked whale in the following.

Muscle Meat of Baird Beaked Whale (Berardius bairdii). A lump of meat was taken from a male whale which was killed off the coast of Kinkazan Island in the summer of 1953. Presumably about 10 hours elapsed from the time of killing to the arrival at the landstation. The carcass at the commencement of treating was in good fresh condition. 60 *g.* of minced and then thoroughly mixed material was mixed with 60 *cc.* of acetone to prevent subsequent decay and brought to the laboratory. Acetone was distilled off on a steam bath. The residue was extracted with 120 *cc.* of tepid water for 30 minutes under occational stirring and then filtered. The filtrate yielded no proteinous coagulation when it was boiled for a short time, and so it was abandoned. The extraction was repeated twice. The meat residue was treated 3 times with succesive 120 *cc.* portions of boiling water for 5 minutes each on a boiling water bath under occational stirring and filtered with suction. 120 *cc.* of alcohol was added to the residue, boiled in a water bath for ten minutes and sucked off. The extraction was repeated twice with renewal of alcohol at each time. Alcohol which was included in the final meat residue was evaporated on a water bath. Then, the meat residue was submitted to the ether extraction using the Soxhlet's apparatus and then air-dried. Protein thus obtained was desiccated over sulfuric acid under a reduced pressure.

Muscular Tissues of Diaphragm and Heart of Baird Beaked Whale. The whales from which the materials were taken, were killed off the coast of Kinkazan Island in the summer of 1953 as in the foregoing. The muscular tissue of ventricle of the heart was taken from a male whale and the phrenic muscular tissue from a female one. These materials were similarly treated as muscle meat of baird beaked whale.

Various Tissues and Organs. Cerebrum, aorta, epidermis (black skin) and mucous membrane of small intestine were sampled from male baird beaked whales. Pancreas, lung and mucous membrane of first stomach

were sampled from female ones. Lenses were taken from male and female baird beaked whales, and mixed. Sex of baird beaked whales from which spleen and liver were taken and also sex of the sei whale from which gum* was taken, are unknown. These whales were killed off the coast of Kinkazan Island in the summer of 1953 and were in good fresh condition until the commencement of the treatment at the landstation. A baleen plate which had been taken from a whalebone whale (probably, *Balaenoptera edeni*) caught in the adjacent waters of Ogasawara Islands and then air-dried was used as a material for the present experiment.

From mucous membranes loose connective tissue under the membranes (tela submucosa) and others were removed as perfectly as possible by macro-observation, and then the membranes were washed with water to remove mucos.

From pancreas, liver, cerebrum, lung, spleen, and testis, were removed capsules, connective tissues, and blood vessels as much as possible.

From epidermis the outermost thin layer, namely, horny layer (stratum corneum) being apt to peel off was removed. Thus treated material, therefore, consists mainly of germinal layer (stratum germinativum).

The aorta was free from surrounding connective tissues.

The lenses taken from the whales were slightly washed with water to remove the remainder of vitreous body and others.

The minced materials, except the baleen plate, were mixed with the same amount of acetone to prevent subsequent decay and brought to the laboratory. Acetone was distilled off on a steam bath. The residue was treated with tepid water, boiling water, alcohol, and ether succesively in the same manner as the baird beaked whale meat. The baleen plate was cut in thin slices and then treated with tepid water and others as above. Samples of protein thus prepared were desiccated over sulfuric acid under a reduced pressure.

RESULTS

Results obtained are listed in Table 4. The blank means that the corresponding amino acid was not determined because its existence in the test sample was not confirmed by means of qualitative paper chromatography. For comparing with whale meat, values of beef reported in literature are also listed in Table 4.

* The 'gum', in the present paper, means a elastic tissue which occupies the gaps between roots of adjacent baleen plates and is grayish white in color.

DISCUSSION

The whale meat is an important proteinous food-stuff in Japan. The result of the feeding experiment with albino rats carried out by Suzuki et al. (1919) revealed that its protein seems to be not inferior to proteins of other food-stuffs in nutritive value. However, very few studies (Okuda and Okimoto, 1919; Yamakawa and Shibuya, 1926; Schulze, 1949; Carpenter et al., 1955; Ogawa et al., 1958) have been made of its amino acid composition by which the nutritive value should be substantiated, and Ogawa and co-worker's study (1958) on fin whale meat is the only one which achieved neary complete amino acid analysis on whale meat. In the present work, amino acid composition of muscle proteins of four important species of whales in the adjacent waters of Japan was revealed.

As shown in Table 4, there is a close resemblance of the amino acid composition of the first five voluntary muscles. Especially, muscle meats of the two species of toothed whales (sperm and baird beaked whales) resemble very closely each other. It seems that the resemblance between muscle meats of the two species of whalebone whales (fin and sei whales) is somewhat inferior to that between the muscle meats of the toothed whales. It is interesting that in the amino acid composition of protein of muscle meat, sperm and baird beaked whales which are classified into different families (*Physeteridae* and *Ziphiidae* respectively) resemble each other rather more closely than the two species of whalebone whales which are classified into the same family (*Balaenopterydae*).

In comparing the whalebone whales with the toothed whales on each amino acid content in protein of muscle meat, it is noticed that the former is richer in arginine, methionine, cystine, aspartic acid and valine, and the latter in histidine, phenylalanine, tryptophan and glycine. It can readily be imagined that histidine content in muscle protein is pretty affected by myoglobin content in muscle because of the large content of histidine in myoglobin (Schmid, 1949). The relatively large content of histidine in muscle meats of the two species of toothed whales and in the phrenic muscular tissue of baird beaked whale is presumably due to the large content of myoglobin in these tissues. It is already known that the myoglobin content in muscles of sperm whale is much larger than that in muscles of sei whale (Tawara, 1950). Muscle meat and phrenic muscular tissue of the beaked whale are almost black in color as sperm whale meat, and so myoglobin content in these tissues must be also very large. From the results in Tawara's work (1950), it is probable that the amount of myoglobin contained in muscles of

TABLE 4. AMINO ACID COMPOSITION OF

Values expressed in

Protein of	Species of whale	Total N	GLY	ALA	VAL	LEU	ISO
Muscle meat	Fin	15.29	3.8	6.4	5.3	9.9	5.5
Muscle meat	Sei	15.01	4.0	6.1	5.6	10.4	6.5
Muscle meat	Sperm	15.78	4.4	6.2	5.1	10.1	5.4
Muscle meat	Baird beaked	15.52	4.3	5.8	4.4	11.4	5.1
[Beef (Bigwood, 1953)			4.5	6.4	5.0	8.2	5.2
Phrenic muscular tissue	Baird beaked	14.87	4.5	6.0	4.2	11.1	5.3
Cardiac muscular tissue	〃	15.26	5.1	6.0	5.0	10.2	3.9
Mucous membrane of first stomach	〃	15.90	11.8	6.1	4.4	7.3	3.5
Mucous membrance of small intestine	〃	16.11	12.6	6.8	4.3	6.5	2.9
Pancreas	〃	15.23	7.8	6.1	5.3	8.7	4.5
Liver	〃	14.59	5.6	6.8	5.7	9.5	4.4
Cerebrum	〃	13.42	4.4	5.7	5.3	9.2	4.0
Lung	〃	15.32	7.2	7.4	6.4	11.2	2.8
Spleen	〃	15.34	6.5	7.5	6.3	10.7	2.8
Testis	〃	14.34	8.5	6.3	4.9	7.5	3.4
Lens	〃	15.44	4.4	3.3	5.1	9.2	4.6
Aorta	〃	15.48	17.8	11.7	9.3	6.5	2.9
Epidermis	〃	15.45	5.8	4.5	4.5	9.0	4.1
Gum	Sei	15.38	5.2	4.6	4.9	10.2	4.5
Baleen	*Balaenoptera edeni*?	14.69	6.2	4.1	4.3	8.3	3.5

Abbreviations:

GLY	Glycine	LEU	Leucine	TRY	Tryptophan
ALA	Alanine	ISO	Isoleucine	PHE	Phenylalanine
VAL	Valine	PRO	Proline	TYR	Tyrosine

TABLE 5. AMINO ACID COMPOSI

Amino acid content is expressed in

Investigator	Sample	Total N	GLY	ALA	VAL	LEU	ISO	PRO
Ogawa et al.*	Fresh meat	3.85	4.8	5.1	3.5	5.1	3.2	2.6
Nakai	Protein	15.29	4.6	6.6	4.1	6.9	3.8	3.0

* Values of amino acids were calculated from the figures in the original paper ex-

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g./100 *g.* protein

PRO	TRY	PHE	TYR	SER	THR	CYS	MET	ARG	HIS	LYS	ASP	GLU	HO-PRO	HO-LYS
3.8	1.06	4.2	3.8	4.4	5.0	0.97	2.5	7.2	2.66	9.4	9.1	16.0		
3.6	1.08	3.8	3.5	4.2	5.4	1.0	2.4	6.6	3.6	9.5	9.9	13.6		
3.7	1.23	4.8	3.4	4.5	5.2	0.77	2.2	5.9	4.3	9.8	8.7	16.2		
3.4	1.22	4.6	3.4	4.5	5.2	0.83	2.1	5.9	5.6	9.8	8.5	15.1	0.24	
3.6	1.1	4.5	4.1	4.5	4.7	1.3	2.9	5.4	3.8	9.3	9.8	15.9]		
4.4	1.05	4.7	3.3	3.8	5.2	0.75	2.1	6.0	4.5	9.4	8.2	15.1		
4.7	0.83	4.0	2.9	4.9	5.1	0.99	2.4	6.0	2.7	7.8	9.1	12.9	0.86	±
8.7	0.57	3.3	2.7	4.7	4.1	1.0	1.4	7.4	1.7	5.8	7.9	10.9	6.3	0.37
8.6	0.57	3.3	3.2	4.7	4.0	1.0	1.3	7.6	1.7	5.1	7.3	10.5	7.2	0.43
5.8	0.94	4.2	3.6	4.2	4.7	1.4	1.8	7.1	2.6	7.1	7.5	10.4	2.7	+
6.1	0.87	4.3	3.1	4.2	5.5	0.86	1.6	6.3	2.84	6.7	8.3	10.2	1.2	±
4.0	0.89	4.4	4.0	4.9	5.0	1.4	2.0	6.0	2.5	6.9	7.5	10.0		
5.7	0.82	4.5	2.7	4.1	4.7	1.0	1.3	5.0	4.2	7.3	9.4	9.8	2.0	+
4.9	0.92	5.0	3.1	4.2	6.3	0.97	1.5	5.3	3.6	8.6	8.3	8.4	0.95	±
6.5	0.81	3.6	2.9	4.2	4.6	1.1	1.3	6.1	1.9	6.0	7.3	11.2	2.94	+
4.6	2.58	6.6	8.0	6.9	3.4	2.0	2.2	11.7	4.1	4.8	9.2	13.0		
12.4		3.7	3.0	2.5	2.8	0.58	0.34	4.6	0.62	2.3	3.6	5.7	4.84	0.23
6.5	0.46	3.4	3.5	6.2	4.6	3.0	1.8	6.2	2.0	7.8	7.8	14.3		
4.1	0.47	3.5	3.9	6.8	5.2	3.2	1.3	7.3	1.9	5.9	8.9	16.0		
6.1	0.47	2.7	6.3	7.4	5.0	6.5	0.99	7.7	1.8	4.5	7.6	12.3		

SER Serine MET Methionine LYS Lysine HO-PRO Hydroxyproline
THR Threonine ARG Arginine ASP Aspartic acid HO-LYS Hydroxylysine
CYS Cystine HIS Histidine GLU Glutamic acid

TION OF FIN WHALE MEAT

g. amino acid N/100 *g.* total N

TRY	PHE	TYR	SER	THR	CYS	MET	ARG	HIS	LYS	ASP	GLU
0.89	2.0	1.5	3.7	3.2	0.31	1.5	9.7	6.9	8.5	6.1	7.6
0.95	2.3	1.9	3.8	3.8	0.74	1.5	15.2	4.72	11.8	6.3	10.0

pressed in *g.* amino acid/100 *g.* total matters.

whales is closely associated with their habitual lengths of time of diving, which are relatively constant depending on whale species. As is generally known, both sperm and baird beaked whales are long and deep divers, and their chief foods are both alike and mostly consist of squids. Thus, the author believes that the similarity of the amino acid composition of muscle proteins of these animals is closely connected with the similarity of their ecology.

As compared with beef, whale meat seems to be inferior to it in contents of the sulfur-containing amino acids, namely, methionine and cystine. However, in contents of the so-called essential amino acids other than methionine, whale meat is not always inferior to beef.

The heart muscle protein contains larger amounts of hydroxyproline, proline, and glycine, and smaller amounts of isoleucine, tryptophan, tyrosine, histidine, lysine, and glutamic acid than the voluntary muscle proteins. The existence of hydroxylysine in the heart muscle protein was also proved. As described below, hydroxyproline and hydroxylysine are nearly peculiar to collagen, and so the collagen content in heart muscle must be much larger than that in voluntary muscles. Presumably, this fact suggests that heart muscle contains the intercellular substance more abundantly than voluntary muscles. Assuming that whole amount of hydroxyproline in proteins of muscle meat and heart muscle exists as collagen and that collagen contains hydroxyproline at the ratio of 13.5% (Buddecke, 1958), the collagen content in proteins of muscle meat and heart muscle of baird beaked whale comes to 1.8% and 6.4% respectively.

In Table 5, the present results of protein of muscle meat of fin whale are compared with the Ogawa and co-workers' values of fin whale meat (1958). There is considerable disparity of each amino acid content between both cases. It is probably due to the facts that in Ogawa and co-workers' study, fresh meat was itself hydrolyzed, but in the author's experiment, the protein free from nitrogenous extractive, fat, etc. was hydrolyzed and also both cases differed from each other in the technique of protein hydrolysis.

Striking similarity of the amino acid composition of mucous membranes of the first stomach and small intestine should be noticed.

It is worth noticing that all four samples of protein of digestive system have relatively large contents of hydroxyproline and hydroxylysine. (The author found also that protein of parotid gland of a rabbit contained hydroxyproline at the ratio of 3.5%.) Especially, the mucous membranes contain much greater quantities of these amino acids than all the other samples. The distribution of both amino acids in nature seems to be nearly confined to collagen. The hydroxyproline content in collagen is about 14% (Akabori and Mizushima, 1954-5;

Buddecke, 1958; Block and Weiss, 1956). According to results of some investigators' experiments, hydroxyproline seems to be contained also in elastin, but the content in this protein is only about 1.5% (Block and Weiss, 1956). As shown in Table 4, the hydroxyproline content in proteins of mucous membranes and pancreas is much greater than the content in elastin. Accordingly, it is evident that the greater part of hydroxyproline in proteins of mucous membranes and pancreas exists as collagen or collagen-like substance. The existence of hydroxylysine in proteins other than collogen and gelatin hasn't been confirmed. From these facts, it is inferred that each of the analyzed tissues and organs of digestive system contains a large quantity of collagen or collagen-like substance. The much larger content of glycine in proteins of mucous membranes as compared with the alanine content is also an evidence for this inference, because collagen contains a very large quantity of glycine and a slightly large quantity of alanine while elastin contains very great quantities of both glycine and alanine (cf. Table 6.). Assuming that

TABLE 6. APPROXIMATE AMINO ACID COMPOSITION OF COLLAGENS AND ELASTINS

Calculated to 16.0 *g.* of Nitrogen (Block & Weiss, 1956, p. 343)

	Collagens	Elastins		Collagens	Elastins
Glycine	21.9	24.2	Threonine	2.6	1.1
Alanine	9.1	21.3	Cystine	0.2	0.2
Valine	2.8	15.6	Methionine	0.9	0.2
Leucine	3.2	7.6	Arginine	7.7	1.2
Isoleucine	1.6	3.4	Histidine	0.8	0.1
Proline	12.8	13.0	Lysine	4.0	0.5
Tryptophan	0.0	0.1	Aspartic acid	5.7	0.7
Phenylalanine	2.2	4.3	Glutamic acid	10.1	2.8
Tyrosine	0.9	1.9	Hydroxyproline	12.4	1.5
Serine	4.0	0.9			

whole amount of hydroxyproline in proteins of mucous membranes exists as collagen and that collagen contains hydroxyproline at the ratio of 13.5% (Buddecke, 1958), the collagen content in proteins of mucous membranes of first stomach and small intestine comes to 46.6% and 53.3% respectively, namely about half of the whole quantity of each sample. It is supposed that such a large content of collagen in mucous membranes has some important meaning concerning their physiological functions. Collagen is a kind of scleroprotein and not easily affected by proteolytic enzymes. It is, therefore, presumable that the large content of collagen (or collagen-like substance) in mucous membranes of stomack and small intestine may be one of the reasons why these mucous membranes can withstand the action of their own proteolytic enzymes.

The nutritive value of mammalian liver has been often discussed from old times. When liver is regarded as a kind of protein food-stuff, the liver of baird beaked whale is probably inferior to muscle meat in nutritive value because its contents of a number of essential amino acids, namely, methionine, lysine, tryptophan and isoleucine are considerably smaller than the contents of these amino acids in muscle meat.

The cerebrum protein, as compared with other tissue and organ proteins, contains a remarkably small amount of total nitrogen. The similar fact is also observed with other kinds of animals (Block and Weiss, 1956, p. 284). In the amino acid composition, this protein shows no noteworthy special feature.

The amino acid composition of crystalline lens protein is considerably characteristic. In contents of arginine, tryptophan, phenylalanine and tyrosine, the lens protein is the largest among all the samples of protein treated in the present work, and the smallest in the alanine content.

The amino acid composition of aorta protein is specific. The value of nearly every amino acid contained in this protein is either always larger or always smaller than values of the same amino acid in all other kinds of protein analyzed in the present work. As is generally known, elastin is principal constituent protein of arteries (Akabori and Mizushima, 1954, Vol. II, p. 2 and 7). As shown in Table 4, the aorta protein contains a pretty large quantity of hydroxyproline and also an appreciable quantity of hydroxylysine. It is, therefore, undoubted that collagen is also an important constituent of the aorta. The amino acid composition of aorta protein shows that the protein consists mainly of elastin and collagen, which is easily understood when the aorta protein is compared with elastin and collagen in amino acid composition (Table 6).

Epidermis and gum resemble closely each other in amino acid composition. From their good similarity in amino acid composition, elasticity, appearance and others, the author believes that both tissue proteins are substantially the same. From their relatively high content of cystine, it is probable that these proteins contain some amounts of keratin.

The baleen is regarded as a kind of crude keratin. Its high content of cystin is an evidence for that.

From the results obtained in the present work, generally, it is found that internal organ proteins, as compared with protein of muscle meat, contain larger quantities of glycine, proline, hydroxyproline, and hydroxylysine and smaller quantities of isoleucine, tryptophan, methionine, lysine, and glutamic acid. This fact shows that internal organs are much richer in quantity of collagen than muscle meat. That is easily understood

from the amino acid composition of collagen and from the fact that hydroxyproline and hydroxylysine are almost peculiar to collagen as stated before. In other words, the aforesaid fact shows that internal organs, as compared with muscle meat, are much richer in quantity of connective tissue, because collagen is the principal constituent protein of connective tissue.

Internal organ proteins are probably inferior to protein of muscle meat in nutritive value on account of their smaller contents of the above-mentioned essential amino acids, namely, isoleucine, tryptophan, methionine and lysine.

SUMMARY

1. In order to carry out the present study, a simple and economical method for direct estimation of 18 common amino acids except tryptophan and hydroxyproline on ascending one-dimensional paper chromatograms was devised and Macpherson's method for dermination of histidine was modified. By use of the former, determination of hydroxylysine was also enabled to be made in a micro- or submicro-scale.

2. Amino acid distribution in proteins obtained from muscle meats of fin, sei, sperm, and baird beaked whales, muscular tissues of diaphragm and heart, mucous membranes of first stomach and small intestine, pancreas, liver, cerebrum, lung, spleen, testis, lens, aorta, and epidermis of baird beaked whale, and also gum and baleen plate of whalebone whales was investigated.

3. Proteins of voluntary muscles, especially muscle proteins of sperm and baird beaked whales resemble very closely each other in amino acid composition. It seems that the similarity of the amino acid composition of muscle proteins of these animals is closely associated with the similarity of their ecology.

4. The whale meat seems to be inferior to beef in contents of sulfur-containing amino acids, but not always inferior to beef in contents of essential amino acids other than methionine.

5. Heart muscle differs considerably from voluntary muscles in amino acid composition. Collagen content in heart muscle is much larger than that in voluntary muscles. From this fact it is presumed that heart muscle contains the intercellular substance more abundantly than voluntary muscles.

6. Large content of collagen in each digestive organ protein was inferred from its amino acid composition. The collagen content in proteins of mucous membranes of first stomach and small intestine reaches about half of the whole quantity of each sample. The large content

of collagen (or collagen-like substance) in the mucous membranes may be one of the reasons why these membranes can withstand the action of their own proteolytic enzymes.

7. Lens protein is characterized by its large contents of tryptophan, phenylalanine, tyrosine, and arginine.

8. Aorta protein consists mainly of elastin and collagen. The value of nearly every amino acid contained in this protein is either always larger or always smaller than values of the same amino acid in all other samples of protein analyzed in the present work.

9. Gum and epidermis without horny layer are substantially the same.

10. Generally, internal organ proteins, as compared with protein of muscle meat, contain larger quantities of glycine, proline, hydroxyproline, and hydroxylysine and smaller quantities of isoleucine, tryptophan, methionine, lysine, and glutamic acid. This fact shows that internal organs are much richer in quantity of connective tissue than muscle meat, and that internal organ proteins are probably inferior to protein of muscle meat in nutritive value.

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