XC. THE DISTRIBUTION OF CARNOSINE IN THE ANIMAL KINGDOM[1].

By WINIFRED MARY CLIFFORD.

Household and Social Science Department, King's College for Women, Kensington.

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CARNOSINE, which was first isolated by Gulewitsch [1900], has now been proved to be β-alanylhistidine in two independent investigations [Baumann and Ingvaldsen, 1918; and Barger and Tutin, 1918].

This dipeptide is to be found in all mammalian skeletal muscle and also to a less extent in mammalian cardiac muscle. E. Mellanby [1908] found that creatine is present in vertebrate muscle whilst absent from that of invertebrates, and he suggested that the distribution of carnosine might follow along similar lines. Accordingly a research was made into the distribution of the base in the muscles of various animals. It has been possible to extend the enquiry amongst some of the rarer species, owing to the kindness of Dr Sonntag of the Zoological Gardens.

METHODS USED IN THE RESEARCH.

1. *Colorimetric method.* The quantitative estimation of carnosine in the muscles of the animals investigated was carried out according to the method described by Clifford [1921].

Essentially this consists in making a watery extract of muscle, removing soluble proteins with metaphosphoric acid, and diazotising the resultant protein-free solution. The depth of colour is then matched against a standard in a Duboscq colorimeter.

This method has advantages over the older precipitation methods in that it is rapid and obviates loss of the base by repeated precipitations. Consequently results far higher than those previously given for the carnosine content of muscles have been obtained. This is to be expected since, at every stage of preparation of the pure base, either according to the method of Dietrich [1914] or Gulewitsch [1900], the diazo-reaction shows incomplete precipitation of the base by the reagents used in the final stage. Also part of it is left behind in every one of the huge and numerous precipitates formed in the removal of proteins and inorganic salts.

Therefore, for the estimation of carnosine in a given muscle, the colorimetric method appears preferable to the isolation method. A further advantage

[1] Part of this paper was incorporated in a Thesis for the M.Sc., London.

lies in the fact that small amounts of tissue are sufficient for an estimation. For quantitative isolation of the base, it is useless to work with less than 1000 g. of muscle, whilst colorimetrically 1 g. or even less has been used with success.

2. *Precipitation methods.* Whenever necessary to confirm or supplement the colorimetric readings, a precipitation method has been used.

The necessity for such a procedure was realised on diazotising salmon extract. Since this fish belongs to a carnosine containing family, the Physostomi, it was expected that a positive reaction would be given by the protein-free filtrate.

However, the extract from 12·7 g. of salmon muscle when diazotised gave only a fleeting pink tinge and rapidly turned yellow. On increasing the amount of the extract a red-orange colour resulted which again in a few seconds changed to a deep yellow. When a stronger solution of diazo reagent was added to this yellow liquid fleeting red streaks appeared, which faded almost as soon as they were formed. The presence of carnosine was thus indicated in the solution, but at the same time accompanied by some other substance masking the diazo reaction.

Accordingly, an endeavour was made to obtain pure carnosine from 4 lbs. muscle treated according to the method of Dietrich [1914]. No red colour was obtained on diazotisation until after removal of excess of lead by means of sulphuric acid, when the carnosine reaction became strongly positive in the filtrate. The final re-crystallisation gave a small amount of 95 % pure carnosine.

Pike, salmon-trout, and whitebait gave a similar result to that given by salmon and, therefore, in these four fishes it is impossible to state the amount of carnosine in the muscles, though the base is present, as seen on diazotisation of the lead-free filtrate.

This suggested that other negative results were also due to the presence of an inhibitory substance and therefore 2–4 lbs. of every apparently carnosine-free fish muscle and as much as possible of all other negative muscles was treated according to the precipitation method. The colorimetric findings with the Anacanthini were thus confirmed. Cod, haddock, hake, whiting, brill, halibut, plaice, sole and turbot gave as an end product a sticky mass which on diazotisation gave an intense yellow colour with no trace of red. A similar lack of the base was found with extracts from 100 g. each of oyster and mussel, 30 g. of house-fly and 60 g. of common sparrow.

The actual precipitation method used was as follows.

The tissue was extracted with distilled water at 80–90° for three periods of 1½ hours each. The three extracts were united and the tissue pulp squeezed dry through muslin. A slight excess of lead acetate was added to the hot solution, and the resultant precipitate filtered off. The filtrate was neutralised with 20 % caustic soda at about 45° when another precipitate formed. This was removed, and the filtrate heated to about 60°. It was then freed from excess lead by the addition of sulphuric acid. (At this stage, if carnosine were

present, the diazo reaction of the filtrate became positive even if negative in the original muscle extract.) After filtration the acid solution was neutralised with baryta, re-filtered, and the filtrate evaporated to 50–100 cc. over a water-bath. Absolute alcohol was added till a faint permanent cloudiness appeared. Approximately twice as much alcohol as filtrate was used. A small amount of ethyl ether was added followed by 10 % mercuric sulphate in 5 % sulphuric acid as long as a mercury precipitate formed. After 24 hours the solution was filtered and the precipitate well washed with 5 % sulphuric acid. It was then suspended in water and decomposed by hydrogen sulphide. The mercuric sulphide was filtered off, and the solution neutralised with baryta, excess of this being removed by carbon dioxide. The new filtrate was concentrated on a water-bath to a thick syrup which became crystalline on cooling. This was ground in a mortar with a mixture of four parts of ethyl alcohol and one part of water. The filtrate from this process was evaporated and gave a yield of impure carnosine. The grinding with alcohol and evaporation were repeated twice and in this manner a small amount of 97 % pure carnosine was obtained.

Possibility of Putrefactive Change.

A further suggestion to account for the absence of carnosine from the white fishes was that post-mortem changes had set in and had led to the disappearance of the base. In order to test this, ten sticklebacks were obtained and killed. Five were ground up at once (after removal of the head and intestines) and were extracted with water. The resultant solution when diazotised and examined colorimetrically gave 0·14 % of carnosine for stickleback muscle and bone.

The other five fishes were left for four days exposed to the air at room temperature in the summer. They were then extracted and treated in the same way as the freshly killed ones. Again the yield of carnosine was 0·14 %, showing that keeping fish for four days after death caused no alteration of carnosine content.

One sample of plaice analysed and found carnosine-free had not been dead more than 24 hours when extracted. This estimation was carried out in the winter and extensive bacterial action would hardly have set in in such a short time.

The crayfish, sea anemones, oysters, mussels, scallops, snails, flies and fly maggots were killed immediately before extraction and, therefore, putrefactive changes can be excluded as the cause of the negative diazo reaction in these experiments. With rabbit muscle from (1) a freshly killed animal; (2) a sample sent by post from Cambridge; and (3) a rabbit bought from a poulterer's shop, the yields of carnosine varied from 0·13–0·15 % only. Therefore, it may be said that in fresh muscle with no definite signs of putrefaction the carnosine content remains unchanged at any rate up to four days after death. On the other hand meat kept till definitely putrid gave less carnosine when extracted than the fresh sample.

From these results it was decided to use any sample of muscle from the Zoological Gardens as representing the normal animal unless a smell of putrefaction could be detected. The animals had never been dead more than two days before extraction, but if definite putrefaction had set in the sample was rejected as useless, and no carnosine estimations were made on it.

Constancy of Carnosine Content in the Species.

The amount of carnosine in the muscles of any animal species is constant in every case where it has been possible to make several different extractions. In this it resembles creatine [Myers and Fine 1913]. Owing to this fact, it has seemed legitimate to take a single estimation in the case of rare animals as truly representing the carnosine content of that species.

Actual experiments performed to test this carnosine constancy are as follows.

(1) *Rabbit.*

3	rabbits,	2 days old,	0·135, 0·14, 0·14 %
3	„	4 „	0·13, 0·14, 0·15 %
2	„	28 „	0·15, 0·15 %
2	„	49 „	0·14, 0·16 %
4	„	adults	0·15, 0·15, 0·14, 0·15 %

(2) *Rats.*

2	rats,	30 minutes old,	0·10 %
4	„	2 weeks old	0·11, 0·12, 0·10, 0·10 %
3	„	3 „	0·11, 0·12, 0·11 %
1	„	8 „	0·12 %
22	„	adults	0·10, 0·125 %

(3) *Beef.*

English beef, 6 different samples: 1·1, 1·0, 1·1, 0·98, 1·0, 1·0 %
English veal, 2 different samples: 1·12, 1·05 %

(4) *Sheep.*

English mutton, 3 samples: 0·38, 0·37, 0·38 %
English lamb, 2 samples: 0·40, 0·42 %

(5) *Fish.*

In every case of non-carnosine-containing white fish at least two separate samples were taken and each proved negative on applying the diazo reaction. With cod seven different samples have been tested by two separate methods, always with a negative finding.

Carnosine in Skeletal and Cardiac Muscle.

When possible the carnosine content of cardiac, as well as of skeletal muscle, was investigated. The unexpected result obtained was that in every case the cardiac content was very much lower than the skeletal.

Actual findings were:

	Carnosine in skeletal muscle %	Carnosine in cardiac muscle %
Cockatoo	0·07	0·009
Rhesus monkey	0·34	0·03
Rat	0·11	0·02
Rattlesnake	0·045	Faint trace
Testaceous snake	0·06	„
Sheep	0·38	0·017

This is in contradiction to the findings of Bubanović [1918] who stated that human cardiac and skeletal muscle gave the same carnosine content. However, although he carried out the estimations on human cardiac muscle himself, those on skeletal muscle with which he compares them are those of v. Fürth and Hryntschak [1914] on horse flesh, and the carnosine content of muscle varies enormously in different species.

Carnosine in Red and White Muscle.

With the rat, rabbit and domestic fowl, separate estimations were carried out on samples of red and white skeletal muscle. In no case was any difference of carnosine content obtained.

Distribution of Carnosine in the Animal Kingdom.

(1) *Invertebrates.*

All invertebrate muscle, so far examined, has been free from carnosine. The distribution thus resembles that of creatine which has been shown by various observers to be absent from invertebrates.

A crayfish was killed and 10 g. of its muscle extracted with water, and diazotised after precipitation with metaphosphoric acid as previously described [Clifford, 1921]. No pink coloration resulted, but a yellow was produced. Since a minute trace of carnosine (0·00001 g.) gives rise to a definite pink colour when treated with diazobenzenesulphanilic acid and sodium carbonate, the negative result showed absence of the base from crayfish muscle. This experiment was repeated on two other occasions with similar results.

The following invertebrates have been examined:

	Carnosine in muscle
(1) *Actinozoa.*	
Sea anemone (*Taelia*, 2 varieties)	Absent
(2) *Arthropoda.*	
i. Crustacea.	
Crayfish (*Astacus fluviatilis*)	Absent (2 methods)
ii. Insecta.	
Honey bee (*Apis mellifica*)	Absent
Blue bottle fly (*Calliphora*)	„ (2 methods)
Blue bottle larvae	„ „
iii. Arachnida.	
House spider (*Tegenaria domestica*)	Absent
(3) *Mollusca.*	
i. Gastropoda.	
Land snail (*Helix*)	Absent
Sea „ „	„
ii. Pelecypoda.	
Oyster (*Ostrea*)	Absent (2 methods)
Mussel (*Mytilus edulis*)	„ „
Scallop (*Pecten*)	„ „

It may, therefore, be concluded that carnosine is absent from the muscles of invertebrates, since neither by direct diazotisation of muscle extracts, nor by an isolation method could a trace of the base be found in any invertebrate examined[1].

(2) *Vertebrates.*

A. TELEOSTEI.

(i) *Non-fatty fishes. Anacanthini.*

Cod muscle was extracted as typifying the teleostean fishes. There was every expectation of a good colour reaction since Suzuki and his co-workers [1909] claimed to have isolated 0·2 % of the base from the bonito, a Japanese fish belonging to the Acanthopteri. However, when the cod extract was diazotised there was no trace of pink in the solution but merely a clear pure yellow. The Japanese observers in their paper state that their "carnosine" prepared from the bonito by precipitation with silver nitrate and baryta gave a negative Pauly reaction. Since pure carnosine when diazotised gives an intense red colour, their final product must have consisted of some other substance.

This negative finding with cod led to the investigation of other Anacanthini with the following results:

Anacanthini	Carnosine in muscle
Cod (*Gadus morrhua*)	Absent (2 methods)
Brill (*Psetta laevis*)	„ „
Haddock (*Gadus aeglefinus*)	„ „
Hake (*Merluccius vulgaris*)	„ „
Halibut (*Hippoglossus vulgaris*)	„ „
Plaice (*Pleuronectes platessa*)	„ „
Sole (*Solea vulgaris*)	„ „

(ii) *Fatty fishes. Acanthopteri and Physostomi.*

The results previously described point to the absence of carnosine from fishes, but when the dark muscled members of two other sub-orders of the Teleostei, viz. the Acanthopteri and Physostomi were examined, they were found to contain considerable amounts of carnosine in their muscles.

With *Acanthopteri* the results were:

Mackerel (*Scomber scomber*)	0·50 % carnosine
Mullet (grey) (*Mugil capito*)	0·18 „
„ (red) (*Mullus barbatus*)	0·16 „
Sea bream (*Abramis brama*)	0·14 „
Stickleback (muscle and bone) (*Gastrosteus pungitius*)	0·14 „

[1] This suggested a possible means of identifying the source of commercial fish and meat pastes. On testing it was found that there was a difference between fish and meat paste, for "crab," "anchovy" and "bloater" pastes gave negative reactions, whilst "wild duck" and "ham and tongue" gave 0·50 %. Hence, contrary to general opinion there probably is a true distinction between the two classes of paste, though since "crab" and "bloater" give similar negative results and "wild duck" and "ham and tongue" both gave 0·5 %, there are probably but two basal pastes and no more, for bloaters would be expected to give a positive reaction; and wild duck less than ham and tongue.

With *Physostomi*:

Barbel (*Barbus vulgaris*)	0·18 % carnosine
Eel (*Anguilla vulgaris*)	0·37 „
Gold fish (*Carassius auratus*)	0·11 „
Grayling (*Thymallus vulgaris*)	0·11 „
Herring (*Clupea harengus*)	0·16 „
Minnow (muscle and bone) (*Leuciscus*)	0·09 „
Pike (*Esox lucius*)	Present (precipitation method)
Roach (*Leuciscus rutilus*)	0·18 % carnosine
Salmon (*Salmo salar*)	Present (precipitation method)
Salmon trout (*Salmo trutto*)	„ „
Smelt (*Osmerus eperlanus*)	0·18 % carnosine
Sprat (*Clupea sprattus*)	0·47 „
River trout (*Salmo fario*)	0·11 „
Tench (*Tinca vulgaris*)	0·11 „

Hence, it seems that the distribution of carnosine in teleostean muscle follows the morphological classification, being absent from the Anacanthini and present in the Acanthopteri and Physostomi. It was also present in the one member examined of the

(iii) *Chondrostei*:

Sturgeon (*Acipenser sturio*)	0·23 % carnosine

The classification of these fishes is that of the older zoologists led by Gunther, who put the flat fishes with the other white fishes, *e.g.* the cod and haddock, but in the modern classification they are placed with the Acanthopteri for morphological reasons, the whole system being based on the anatomy of the skull. On chemical grounds, however, the older classification is supported since the flat fishes (Pleuronectidae) lack carnosine in their muscles like the other Anacanthini.

B. Reptiles and Amphibians.

Muscle from several of these animals gave the following results:

(i) *Amphibia.*	
Axolotl (*Amblystoma tigrinum*)	0·19 % carnosine
Frog (*Rana temporaria*)	0·25 „
Frog, young tadpole stage	Present (too little for quantitative estimation)
Newt (*Triton vulgaris*)	0·19 % carnosine
(ii) *Reptilia.*	
Lacertilia.	
Green lizard (*Lacerta viridis*)	0·10 % carnosine
Ophidia.	
American black snake (*Zamenis constrictor*)	Present (too little for quantitative estimation)
Cape Bucephalus (*Dipsas bucephala*)	0·03 % carnosine
Corais snake (*Coluber corais*)	0·32 „
English grass snake (*Tropidonotus natrix*)	0·20 „
Indian python (*Python molurus*)	0·13 „
Rattlesnake (*Crotalus horridus*)	0·045 „
Testaceous snake (*Zamenis flagelliformis*)	0·06 „
Chelonia.	
Speckled terrapin (*Malacoclemmys terrapin*)	Carnosine absent

From these results it seems that carnosine is present in the reptiles and amphibians, but the reptiles, like the fishes, may contain a carnosine-free family. Only one member of the Chelonia, the speckled terrapin, has been examined, and it is carnosine free. However, there was insufficient muscle for the precipitation method to be carried out, and therefore it may be that a substance masking the diazo-reaction, similar to that found in the salmon, was present and prevented the development of the red colour of diazotised carnosine.

C. Birds.

Extracts of bird muscle gave the following results:

(*a*) *Sphenisci.*	
Black-footed penguin (*Spheniscus demereus*)	0·055 % carnosine
(*b*) *Falconidae.*	
Sparrow hawk (*Accipiter nisus*)	0·18 „
(*c*) *Columbae.*	
Common pigeon (*Columba livia*)	Trace of carnosine
Southern triangular spotted pigeon	0·033 % carnosine
(*d*) *Paradiseidae.*	
King Bird of Paradise (*Cicinnurus regius*)	0·059 „
(*e*) *Corvidae*	
Blue jay (*Garralus cyanocitta*)	0·043 „
Common jay (*Garralus glandarius*)	0·04 „
(*f*) *Phasianidae.*	
Domestic fowl (*Gallus domesticus*)	0·17 „
Hybrid pheasant fowl	0·02 „
Golden pheasant (*Chrysolophus pictus*)	0·04 „
Partridge, English (*Perdix*)	0·11 „
„ French (*Caccabis rufa*)	0·11 „
Quail (*Coturnix communis*)	0·08 „
(*g*) *Psittacidae.*	
Blue-fronted Amazon (*Chrysotis aestiva*)	0·03 „
Budgerigar (*Melopsittacus undulatus*)	Absent by diazo-reaction, insufficient for precipitation method
Greater sulphur crested cockatoo (*Cacatua galerita*) ...	0·07 % carnosine
Lesser sulphur crested cockatoo (*Cacatua sulphurea*)	0·10 „
Illiger's macaw (*Ara maracana*)	0·04 „
(*h*) *Fringillidae.*	
Bicheno's finch (*Stictoptera bichenovia*)	Carnosine absent
Indigo bunting (*Passerina cyanoa*)	„
Long-tailed finch (*Poëphila acuticauda*)	„
Siskin (*Chrysomitris spinus*)	„
Red-crested cardinal (*Carduelis*)	„
Sparrow (*Passer domesticus*)	„
Tanager (*Rhamphocelus brasilius*)	(Two methods) Carnosine absent
(*i*) *Strigidae.*	
Eagle owl (*Bubo ignavus*)	Carnosine absent

The birds here, as the fishes, fall into two classes, one carnosine-free and the other with carnosine in the muscles. The finches and possibly the owls

resemble the Anacanthini in being carnosine-free, whilst the penguins, birds of prey, pigeons, birds of paradise, jays, game birds and parrots are like the Physostomi, Acanthopteri and Chondrostei in having the base in their muscles.

D. Mammals.

The mammals examined differ from the other big classes in the animal kingdom in that no member of the group has yet been found to be carnosine-free. Estimations were carried out on the following of the mammalia:

(1) *Rodentia.*	
Capybara (*Hydrochaerus*)	0·20 % carnosine
Common marmot (*Arctomys*)	0·06 „
Crested porcupine (*Hystrix cristata*)	0·05 „
Jerboa (*Dipus*)	Present (not enough for quantitative estimation)
Mouse (*Mus domestica*)	0·09 % carnosine
Oak doormouse (*Muscardinus*)	0·09 „
14 specimens rabbit (*Lepus cuniculus*)	0·15 „
32 specimens rat (*Muridae*)	0·11 „
(2) *Ungulata.*	
Antelope (*Anoa*)	0·16 „
6 specimens bull (*Bos*)	1·11 „
2 „ „ (calf)	1·12 „
Horse (*Equus caballus*)	0·90 „
Kashmir deer (*Cervidae*)	0·05 „
Pig (*Sus*)	0·64 „
3 specimens sheep (*Ovis*)	0·38 „
2 „ lamb	0·41 „
(3) *Carnivora.*	
Common fox (*Canis vulpes*)	0·09 „
Leopard (*Felis pardus*)	0·12 „
(4) *Primates.*	
Rhesus monkey (*Macacus rhesus*)	0·34 „

Discussion of Results.

It is difficult to believe that carnosine has little or no significance though it cannot be an absolute necessity since large families of animals exist without it. It is a dipeptide and until recently was the only one known in the body. Another, glutathione, consisting of cystein and glutamic acid was recently isolated by Hopkins [1921] and shown to be a factor in cell respiration.

There are several indications of the importance of carnosine to the organism. It is constant in amount in any given species and is often found in a high percentage. Thus in beef there is 1 % carnosine in the fresh muscle, and since beef is about 66 % water, carnosine is present in the solids to the extent of 3 % whilst even in the pheasant with the low carnosine content of 0·04 % in fresh muscle, the percentage of carnosine in the solids is as high as 0·17 %. It is difficult to believe that a muscle substance of this high concentration should be useless to the organism.

Again carnosine is not found in the urine and therefore the ingested substance must be metabolised as also must that of the muscles. Carnosine is said to be stable to pepsin and trypsin but to be split up by the action of erepsin. This splitting up and subsequent conversion of the β-alanine and histidine to their end products will liberate a definite amount of energy which can be utilised by the organism. In this case, contrary to general opinion, meat extracts which contain from 7–11 % of carnosine are not without any food value to the organism.

Carnosine is the only known substance containing a β-amino-acid in the body and this may be of great importance, but so far the significance of this fact is unknown.

It was hoped that an investigation into the distribution of the base in the animal kingdom would give a clue as to its physiological function, but this has not been the case.

It seemed possible that carnosine might be correlated with muscular activity, but the active cod and the sluggish plaice are alike in lacking it, and the mackerel with similar activities and environment to the cod has a plentiful supply. With birds also the active finches are carnosine-free and the equally active game birds possess the base.

There is again no difference in the carnosine content of red and white muscle though their rate of contraction differs greatly and the heart contains very little of the extractive in comparison with either red or white skeletal muscle. This suggests that carnosine has no functions concerned with oxidations. If it were of importance in this connection it would be expected that the heart, as the most constantly active organ of the body, would contain at least as much, and probably more, than the rest of the musculature.

No connection between diet and carnosine has been found, though it seemed probable that there should be a definite relationship. The carnivorous leopard and the herbivorous antelope have almost the same percentage of carnosine in their muscles, whilst the bull and the sheep, with their practically identical dietaries, show a great difference, the bull containing almost three times more in its muscles than the sheep.

The only relation brought out by this investigation is a morphological one. If the base is absent from one member of a zoological family, it appears to be absent from all. This is well seen in birds where no examined finch has been found to have carnosine.

A similar thing is seen in the fishes under the old classification (with the flat fish in the Anacanthini) where no member of the Anacanthini is found with carnosine, though all the others have a definite amount in their muscles.

It is possible that other extractives such as creatine may also show a selective distribution in the animal kingdom, and if so, generalisations as to the presence of a substance in all muscles, based on estimations of one kind of animal, are unsound.

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