LXXXIII. THE CATALYTIC DESTRUCTION OF CARNOSINE IN VITRO.

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IN a previous paper [Clifford, 1922] an account was given of the destruction of carnosine in beef and rat muscle kept in cold storage. Since the temperature at which the destruction occurred was at or below zero, it was improbable that it was due either to enzyme or bacterial action. The time taken however was prolonged—6–10 months—and therefore a slow change due to an enzyme or to bacteria might have been the cause. In order to exclude these possibilities experiments were carried out at 100°. A similar though far more rapid disappearance of carnosine took place at this temperature pointing to a simpler catalyst than an enzyme, while bacterial life is also inconsistent with a temperature range of 0°–100°.

EXPERIMENTAL RESULTS.

(1) *Destruction of carnosine in beef at 100°.*

From 3–5 g. of minced lean beef were weighed into test-tubes and 10 cc. water added. The tubes were then plugged with cotton wool and placed in a water-bath kept at 100°. When necessary more water was put in the tubes to prevent drying of the meat.

At intervals of 1–4 days a tube was removed, the contents made up to 95 cc. with distilled water and 5 cc. of 20 % metaphosphoric acid added to precipitate proteins. The carnosine present in the filtrate was estimated colorimetrically by the method described in a previous paper [Clifford, 1921, 1].

The numerical results of four experiments were as follows:

Days at 100°	S	15	29	29 A
Start	0.96 %	0.99 %	1.05 %	1.05 %
1	—	0.80	—	—
2	0.70	—	0.90	0.89
4	—	—	0.80	0.74
5	0.60	0.66	—	—
7	0.54	0.60	0.68	0.65
10	—	—	0.60	0.64
12	0.53	—	—	—
14	—	0.60	0.55	0.57
16	0.30	—	—	—
17	—	0.40	0.43	0.40
21	—	0.33	—	—

These results, Fig. 1, indicate the presence of a catalyst which removes carnosine from muscle. This removal is considerably hastened¹ by a rise of temperature and is therefore probably a chemical and not a physical change. On comparing the results with those previously published [Clifford, 1922] it is found that for the percentage of carnosine to fall to the same level 9–10 months is necessary at 0° as against 21 days with a temperature of 100°.

Fig. 1. 3–5 grams meat in 10 cc. water.

(2) *Catalyst not removed by boiling.*

Experiments were performed to find whether the catalyst were present in an extract made by boiling beef with water. Minced lean beef was put into a flask with water and boiled in a water-bath for 30–60 minutes. It was then filtered, and the residue again boiled with water for a similar period. The filtration was repeated and the two filtrates united. An estimation of the carnosine percentage of the extract was made, and portions of 10 cc. put in test-tubes plugged with cotton wool. The tubes were then placed in a water-bath at 100°.

In five experiments lasting 13–26 days no change took place in the carnosine content of the extract. Similar results were obtained in two other 13 day experiments, one kept at 60°, and the other at 37° after sterilisation by boiling.

¹ The coefficient is 1.5 for 10° rise whilst for physical actions it is generally 1.03–1.05.

Since the carnosine content of the extract in these experiments remained unchanged, the catalyst cannot be removed from beef by extraction with water for 1-2 hours at 100°.

(3) *Catalyst remains in beef after boiling.*

A series of test-tubes was taken containing 10 cc. of beef extract prepared as above together with 3 g. of boiled minced beef which had been washed in cold running water for 15 minutes to remove traces of extractives. The result as expected was identical with that obtained with fresh beef in water.

(4) *Catalyst removed by prolonged washing of boiled meat.*

Further experiments were carried out to find the result of prolonged washing of the boiled meat.

Five experiments with series of tubes containing 10 cc. of extract and 3 g. of boiled meat washed 3, 18, 24, 48, 48 hours respectively were kept at 100° with the following results:

	21 3 hours' washing	20 18 hours' washing	19 24 hours' washing	30 48 hours' washing	9 48 hours' washing
Days	%	%	%	%	%
Start	1.0	1.0	1.05	0.99	0.98
3	0.9	1.0	1.0	0.99	0.98
6	0.9	0.9	1.0	0.99	0.98
8	0.45	0.6	0.77	0.99	0.98
10	0.45	0.6	0.70	0.99	0.98
13	0.40	0.55	0.65	0.99	0.98
23	0.35	0.35	0.50	0.99	0.98
27	0.30	0.33	0.48	0.99	0.98

From these results it appears that the catalyst present in boiled beef which destroys carnosine can be removed by prolonged washing in running water, probably by separation of an adsorption compound from the muscle protein.

In one experiment the preliminary boiling of the meat was omitted and fresh unboiled minced beef was washed in running water for 24 hours. Three g. of this washed meat was then added to each of a series of tubes containing 10 cc. extract at 100°. The result was a disappearance of carnosine similar to that given by boiled beef washed for three hours.

Start	1.0 %	10 days	0.40 %
3 days	0.95 %	13 "	0.40 %
6 "	0.80 %	23 "	0.25 %
8 "	0.45 %	27 "	0.25 %

These results, Fig. 2, indicate that the catalytic destroyer of carnosine is present in some physical combination with the protein of beef muscle and may be removed by prolonged washing in cold running water. This removal is facilitated by boiling the meat before washing, though mere boiling for 1-2 hours does not extract the catalyst from the muscle.

The curve of disappearance of the base is peculiar and it is difficult to account for the delay in the start of the reaction with washed meat and extract as compared with fresh meat and water. In all the curves there is a sharp fall between the 6th and 8th days followed by a stationary period and then a slower steady fall.

This three-step curve is indicated in experiments on fresh meat and water (Fig. 1) and is strongly marked in experiments with cod muscle and with liver. A paper previously published [Clifford, 1922] shows a similar curve from beef kept in cold storage.

Fig. 2. 10 cc. meat extract + 3 grams washed meat.
 30, 9. Meat boiled 1-2 hours then washed 48 hours.
 19. " " " 24 "
 20. " " " 18 "
 21. " " " 3 "
 22. Meat unboiled but washed 24 hours.

(5) Presence of catalyst in muscles of white fish.

Carnosine has been shown [Clifford, 1921, 2] to be absent from the muscles of white fishes. A series of experiments was performed using 3 g. of fresh minced cod muscle in beef extract in place of 3 g. of boiled and washed beef. Unexpectedly it was found that the carnosine disappeared giving a similar curve to that with washed beef in its peculiar three-stepped nature, though the actual position of the stationary period varies (Fig. 3).

Three experiments were carried out at 60° after preliminary sterilisation by boiling and three were kept constantly at 100°.

	<i>P</i>	<i>H</i>	<i>7</i>	<i>E</i>	<i>14</i>	<i>24</i>
	60°	60°	60°	100°	100°	100°
Days	%	%	%	%	%	%
Start	0.96	0.99	0.99	0.99	1.04	1.0
1	—	—	—	—	0.80	—
2	0.90	0.90	—	0.90	—	—
3	—	—	—	—	—	1.1
4	—	—	0.95	—	—	—
5	0.85	—	—	—	0.80	—
6	—	—	—	—	—	0.75
7	0.58	0.80	0.72	0.90	0.55	—
8	—	—	—	—	—	0.60
9	—	—	—	—	—	—
10	—	0.80	—	0.88	—	0.46
11	—	—	0.60	—	—	—
13	0.58	—	—	—	—	0.46
14	—	0.66	0.55	0.50	0.50	—
16	—	0.66	—	0.44	—	—
17	0.52	—	—	—	—	—
18	—	0.44	0.55	—	0.45	—
20	—	—	—	—	—	—
21	—	0.40	0.50	0.40	0.35	—
23	—	0.35	—	—	—	—
25	—	0.30	—	—	—	0.30
28	—	—	0.40	—	—	—

Fig. 3. 10 cc. meat extract + 5 grams cod muscle.
Unbroken lines 100° C. constantly.
Broken lines 60° C. constantly after sterilisation by boiling.

(6) *Presence of catalyst in liver.*

An experiment was performed using tubes with 10 cc. of beef extract and 3 g. of calf liver kept constantly at 100°. Carnosine estimations again gave a stepped curve of disappearance of the base.

A second experiment in which 1 g. of freshly killed rat's liver was used in place of the 3 g. of calf liver, and in which the temperature was 60° in place

of 100°, also showed a loss. As was expected from the lower temperature and smaller amount of tissue taken, the rate of disappearance was slower than in the first experiment (Fig. 4).

Days of experiment	16 100° and 3 g. liver. %	Q 60° and 1 g. liver. %
Start	1.0	0.96
1	0.80	—
2	—	0.90
5	0.55	0.77
7	0.55	0.77
13	—	0.64
14	0.50	—
17	0.42	0.60
21	0.25	—

Fig. 4. 10 cc. meat extract + liver.
Q 1 g. rat liver kept at 60° C.
16 3 g. calf liver kept at 100° C.

A third experiment lasting 18 days, in which 3 g. of frozen calf liver was used as a source of the catalyst, showed no loss of the base. These findings point to the presence of the carnosine-destroying catalyst in liver as well as in muscle, whilst prolonged keeping in cold storage destroys the catalyst itself.

(7) *Absence of catalyst from kidney.*

Since skeletal muscle and liver had both been shown to cause the disappearance of carnosine from muscle extract, it was possible that the catalyst was present in all cells. Therefore an experiment was carried out using 1 g. of freshly killed rat's kidney, in place of liver or muscle, in 10 cc. of beef extract. The tubes were kept at 60°. No change in carnosine content took place till the 21st day, when a very slight fall took place. This fall however lay well within the limits of experimental error.

Similar results were obtained using 3 g. of sheep's kidney, 3 g. of bullock's kidney and 3 g. of frozen bullock's kidney, all kept at 100° for at least 20 days.

There is, therefore, no carnosine-destroying catalyst in the kidney, and consequently the possibility of existence of this catalyst in all cells cannot be maintained.

(8) *Absence of catalyst from invertebrate muscle.*

A series of test-tubes containing 10 cc. of beef extract together with 3 g. of fresh lobster muscle was left in a bath at 100°. No change in the carnosine content of the tubes was observed up to the end of 46 days, and therefore the catalyst was not present in the lobster muscle.

Oysters were next used as a further type of the invertebrata. Here from the first day onwards no pink colour could be obtained on diazotising the protein-free filtrate. However a deep yellow colour resulted and if estimated as carnosine showed no loss of base up to the 23rd day.

In all other experiments, *e.g.* with liver and muscle, or in simple keeping of meat in cold storage, disappearance of the base coincided with a less depth of colour, and not an alteration of hue. Therefore the lack of red colour on diazotising the oyster-beef extract was probably due to the presence of an inhibitory substance similar to that shown to exist in salmon muscle [Clifford, 1921, 2].

Unfortunately there was not enough of the beef extract with oyster muscle to use the precipitation method of Dietrich [1914] to show whether this were the case or not, but experiments are in progress to determine this point.

The definitely negative result with lobster muscle and the probably negative findings with oyster point to a lack of carnosine-destroying catalyst in the invertebrata.

DISCUSSION OF RESULTS.

The experiments described in this paper indicate the presence of a substance which is capable of destroying carnosine in muscle extracts, and owing to its temperature range it must be a relatively simple catalyst.

It is not known whether the absence or lessening of red colour on diazotising filtrates from meat kept several days in water at 100° is due to actual destruction of the iminazole ring, or to a synthesis which prevents coupling of this ring with the diazo-reagent. Experiments are in progress with the

object of elucidating this problem. In either case, whether the loss of carnosine is due to a synthesis or to a breaking up of the base, it will be of interest to know the fate of the β -alanine portion of the molecule, as this is the only known β -amino acid in the animal organism.

The stepped nature of the curves of loss of carnosine is peculiar and totally unlike any enzyme curve. It may indicate that not one but two agents are active in the change, and that they act in an analogous way to enzymes and co-enzymes. On the other hand the catalyst may be a single substance and the reaction take place in two stages separated by an inactive period. Neither of these explanations can account for the initial delay shown when using washed meat as a source of catalyst, since after this delay the three-stepped curve appears.

The catalyst is present in ox, rat, and cod muscle and therefore is probably found in vertebrate skeletal muscle generally, but experiments with lobster and oyster muscle indicate its absence from invertebrates. The livers of the rat and ox also contain this catalyst, which however is absent from frozen ox-liver. This indicates another connection between hepatic and muscular activity already seen in glycogen metabolism and also with urea, creatine and creatinine.

The catalyst is not present in all cells since experiments have proved it to be absent from the kidney of the rat, ox, and sheep. The existence of this catalyst may account for the non-appearance of ingested carnosine in the urine.

Earlier experiments in which carnosine was shown to be absent from the striped muscles of the white fishes and the finches appeared to suggest that carnosine is a merely accidental substance, somewhat arbitrary in its presence or absence. But the experiments detailed above, particularly those that show the presence of a carnosine-removing catalyst in the muscle of white fish, suggest that it is an intermediary product of metabolism and that its appearance and its percentage in muscle are determined by the rate at which it is formed and the rate at which it is removed in the different types of muscle.

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