CLV. THE DETERMINATION OF CARNOSINE.

BY WINIFRED MARY CLIFFORD AND VERNON HENRY MOTTRAM.

*From the Physiological Laboratory, King's College of Household
and Social Science, Kensington, London, W. 8.*

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EARLY in the year 1921 Clifford published a method of estimating carnosine, which is based on the colour produced when carnosine reacts with diazobenzene-sulphonic acid. The resultant coloured solution is matched in a colorimeter against a standardised mixture of Congo red and methyl orange [Koessler and Hanke, 1919].

The method was extended to the estimation of chromogenic substances in muscle (mainly, no doubt, carnosine). The muscle, in this process, is minced, weighed, and extracted with water, the proteins in the extract precipitated by metaphosphoric acid, and the filtrate treated as if it were a carnosine solution. Based on this method a series of papers has been published [Clifford, 1921, 1, 2, 1922, 1, 2, 1923] with results which are consistent with each other, and which form a starting-point for the investigation of the functions of carnosine.

Unfortunately the other two workers who have concerned themselves with carnosine estimations do not agree with Clifford in some, and those the simplest and most striking, of her findings. G. Hunter has sorely criticised many details of the method [Hunter, 1921, 1922, 1924] and even suggests that the carnosine nitrate upon which Clifford's estimations are based was only 43 % pure. As this was supplied to her by Dr H. H. Dale as pure, and as the melting-point was correct, it is a suggestion not easily accepted. Mitsuda [1923] agrees with Hunter in saying that the carnosine content of cats' muscle varies.

Now this disagreement is intolerable, and it was resolved to re-examine some of the salient points of difference. The experimental work has been shared, so that one of us (V. H. M.) was able to hand over to the other (W. M. C.) clear, colourless solutions for estimation, the source, previous history, and carnosine content of which were quite unknown to the estimator.

METHOD OF ESTIMATION.

(1) *On solutions of pure carnosine.*

The method follows that of Koessler and Hanke [1919]. To 10 cc. of 1.1 % sodium carbonate solution are added 2- x cc. of distilled water, 4 cc. of the diazo-reagent (kept cool by ice in hot weather) and x cc. of the solution

to be estimated. The volume of the last naturally varies inversely as the strength of the solution. The mixed solutions are left standing at room temperature for 3–5 minutes to develop the full colour. Then the colour is estimated in a Duboscq colorimeter with Koessler and Hanke's C.R.-M.O. standard mixture as a comparison. No deviations from the Koessler and Hanke technique have been adopted except that the minute directions as regards time of mixing the reagents have not been found necessary, and the strength of the indicator chosen is such that a match at 30 mm. with the carnosine solution set at 20 mm. corresponds to 0.0001 g. of carnosine.

(2) *On tissues containing iminazole compounds.*

The tissue is minced, and from 1 to 6 g. are weighed accurately into a small Monax beaker. From 80 to 90 cc. of distilled water are added, and the whole brought slowly to the boil. 5 cc. of a 20 % solution of metaphosphoric acid are then added, and the mixture is allowed to stand (best overnight). The mixture is filtered and the precipitate washed. The filtrate is made up to 100 cc. Of this crystal clear fluid about 1 cc. is used for the estimation and the technique is exactly that of the preceding paragraph.

REINVESTIGATION OF THE METHOD.

(1) *On pure carnosine.*

(a) *Accuracy of the method.* As the original work was carried out in the difficult post-war period when dyes for the C.R.-M.O. indicator were hard to obtain, and as the original sample of carnosine nitrate, guaranteed pure as it was by Dr Dale, was rather small, it appeared to us necessary to re-standardise the old indicator (the stock solutions of which are still in use) with a new pure sample of carnosine.

Attempts to make in this laboratory, or to purchase on the market, samples of carnosine having proved abortive, this crucial test could not have been made if it had not been for the kindness of Dr Dudley, to whom we are indebted for half a gram of the pure base carnosine.

Solutions of known strength were made in distilled water from this sample and estimated, with the results shown in Table I. Carefully calibrated pipettes, measuring flasks and burettes were used.

Table I.

Solution	Calculated %	Found %	Difference g.
1	0.1460	0.146 (5)	+0.0005
2	0.0730	0.073	Nil
3	0.0487	0.050	+0.0063
4	0.0365	0.036 (6)	+0.0001
5	0.3595	0.359	-0.0005
6	0.2200	0.219	-0.0010
7	0.6190	0.617	-0.0020
8	0.3650	0.365	Nil

The figures speak for themselves. With one exception (Solution 3), the agreement is remarkably close, and in this case there is a note in the protocol (V. H. M.) that the emptying of the automatic pipette did not appear satisfactory. Neglecting this one solution, the agreement is little short of perfect. There seemed to be no reason for going any further with this part of the work, which indicates (i) that the original carnosine was not, as has been suggested, 43 % pure only, but chemically pure; (ii) that the stock solutions of dyes keep perfectly satisfactorily over a number of years; and (iii) that the method of estimation has a high order of accuracy.

Fig. 1. Readings on the colorimeter plotted against carnosine content of solution.
The straight line represents the true linear relation.

(b) *Linear agreement between colour and amount of carnosine.* It has been stated [Clifford, 1921, 1] that the production of diazo-colour is not directly proportional to the amount of carnosine present in the solution to be estimated, whereas Hunter [1921] claims a complete proportionality. Mitsuda [1923], while agreeing with Clifford that the relation is not linear, states that the deviation from linearity is not so great as Clifford found.

In making the estimations given in Table I some 32 adjustments of the strengths of the solutions were made before the end-point was reached. Fortunately, accurate colorimeter readings were taken of the matches given before the correct dilutions were obtained, so that we have a large number of readings comparing colour produced with the known amounts of carnosine present. These are given in Fig. 1. It will be seen that there is a fair approximation to linearity. None the less, the best and most normally displaced from the mean are those when the reading of the C.R.-M.O. in the colorimeter is at 30 mm. and the unknown is set at 20 mm., the conditions under which estimations are usually made.

(2) *On muscle extracts.*

The chief interest of a method of estimating carnosine lies in its applicability to tissues. There are often large amounts in vertebrate muscle of substances which give the diazo-reaction. The iminazole compounds present are carnosine, purine compounds, histamine and possibly histidine; but no one doubts that it is carnosine which is responsible for the major part of the colour. Hunter [1922] estimates it at 95 % of the total, so that an accurate and consistent method of estimating the chromogenic substances in muscle is likely to throw some light upon the functions of carnosine.

Now one thing which appeared certain from Clifford's work [1921, 2; 1922, 2] on the distribution of carnosine in animal tissues, is that all the skeletal muscles of any animal of any one species have an almost constant amount of carnosine, but that each species has its own constant which may range from zero (*e.g.* cod, sole, finch) to 1 % (*e.g.* ox).

Both Hunter and Mitsuda maintain, on the other hand, that there is no constancy in the carnosine content of different animals of the same species. Estimates are given for the gastrocnemius of the cat which run from 0.007 % to 1.293 %, while Clifford's figures lie between 0.48 % and 0.51 %.

To eliminate the personal factor in the colour comparisons a large series of estimations of the chromogenic substances in ox and cat muscle has been made, using 13 different muscles or groups of muscle in the ox and 9 different muscles and 4 groups of muscle in the cat. In all these estimations except the last four, by which time it was considered proved that the personal factor did not count at all, the weighings and the extractions of the muscle were made by one of us (V. H. M.) and nothing but clear solutions handed over to the other (W. M. C.) for estimation.

Bullocks' muscle is a convenient tissue to investigate, but it must be freshly killed and not imported [Clifford, 1922, 1]. Various "cuts" were bought on various dates and the chromogenic substances found are expressed as carnosine in Table II. Each figure given is the mean of at least three parallel estimations agreeing within 1 %—indeed the greatest difference between highest and lowest estimate of any one sample is 8 parts in 970.

Table II. *Chromogenic substances in bullocks' muscle expressed as carnosine.*

"Cut"	Carnosine %
Rump steak	0.983
Shoulder steak	0.990
Fillet steak	0.981
Flank	0.981
Silver side	0.974
Shin	0.981
Skirt	0.975

It is obvious that there is great constancy in the amount. The figures show that the amount of carnosine is constant from animal to animal and for different groups of muscle. To get discrete muscles and not "cuts," five pairs and an odd one were dissected from a bullock's head. Their analyses are given in Table III.

Table III. *Chromogenic substances in muscles of bullock's head expressed as carnosine.*

Name of muscle	Carnosine %
Superior constrictor (?)	0.987, 0.991
Masseter	0.986
Buccinator	0.994, 0.991
Trapezius	0.991, 0.986
Pterygoideus externus	0.992, 0.990
„ internus	0.990, 0.991

It thus appears from the results in Tables II and III that the carnosine content of the different skeletal muscles of the ox is approximately constant both from muscle to muscle and from animal to animal. The greatest variation is from 0.975 % to 0.994 %—at the most a little under 2 %.

Table IV. *Chromogenic substances in cats' muscles expressed as carnosine.*

No. of Exp.	Name of muscle	Carnosine %	Notes
1	Gastrocnemius	0.500, 0.496	
	Psoas	0.499, 0.496	
	Gluteus max. (right)	0.490	
	„ (left)	0.492	
	Soleus	0.483	
	Tensor fasciae	0.485	
	Triceps brachialis	0.484, 0.483	
	Semi-membranosus	0.495, 0.498	
	Quadriceps	0.480, 0.482	
	Biceps femoris	0.498, 0.500	
2	—	0.517, 0.509, 0.515	Killed 10. iii. 28. In cold room till 12. iii. 28. Muscle in (4) smells of ether
3	—	0.511, 0.513, 0.514	
4	—	0.484, 0.477, 0.485	
5	—	0.482, 0.487, 0.480	Killed 12. iii. 28. Sent that day. Smells slightly of ether

It is, however, mainly with the cat that Hunter and Mitsuda claim to have found differences in carnosine content between muscle and muscle and cat and cat, though they state that the right gastrocnemius has the same amount as the left. A careful dissection of one cat yielded nine muscles in reasonable amounts for estimation, and we obtained, in addition, from the Medical Research Institute, through the kindness of Dr Dale, four carcasses of

cats which had been used for experiments there. From these, portions of muscle were taken, but no careful dissection into separate muscles was made. The results are given in Table IV.

It is hardly necessary to comment on the figures of Table IV. No case can be made from them for a wide variation of the carnosine content of skeletal muscle from muscle to muscle, or from animal to animal.

The difference between the highest and the lowest is but 8 % of the lowest, whereas Mitsuda's figures run (for the gastrocnemius) from 0.684 % to 1.293 % and Hunter's from 0.007 % to 0.433 %.

DISCUSSION.

The results detailed above, though going far to substantiate the adequacy and accuracy of Clifford's method for the estimation of carnosine, do little to clear up the confusion. This method has been used with great accuracy to determine the amount of carnosine in solutions of strength known to one person but not to the estimator. Further, the carnosine content of various solutions, colourless and giving no clue as to their content on inspection, has been shown to be proportional to the amount of tissue from which they came. Care was taken by the one who carried out the weighings that there should be great differences in the weight of tissue used, whereas the final solution handed over to the estimator was always 100 cc. The weight of tissue was varied 600 % without any effect on the final result. Consequently we consider that these figures make out a good case for the estimation of carnosine by Clifford's method, though we admit that we cannot understand or explain why other workers get such discordant results.

SUMMARY.

1. A reinvestigation of Clifford's method of estimation of carnosine shows that the original carnosine upon which the work was based was pure and that the indicators, once standardised, were satisfactory.

2. By this method the carnosine content of a solution of pure carnosine in water can be estimated with an error less than 1 %.

3. Using this method to estimate the chromogenic substances in samples of muscle, it has been shown that they vary in amount directly as the amount of muscle taken, no matter what skeletal muscle is used, or if it be from a different animal of the same species. In other words, for the skeletal muscle of a species the carnosine content is a constant. This constant varies from species to species though not from member to member of that species.

4. This work on ox and cat muscle confirms observations on the muscles of rats, rabbits, calves, sheep and lambs [Clifford, 1921, 2].

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