LXXXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT. (PRELIMINARY NOTE)

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With a view to the study of the physiological significance of carnosine, it was first necessary to devise a method of estimation applicable to small quantities of material, which, at the same time, would give more accurate results than have hitherto been obtained with this substance [*vide* von Fürth and Hryntschak, 1914]. A general method of estimation of iminazole derivatives by a modification of the diazo reaction described by Koessler and Hanke [1919]—but not applied by those workers to carnosine—has here been tried as a likely means of affording a reliable indication of the carnosine content of muscle[1]. Before the method could be satisfactorily tested, it was first necessary to prepare pure carnosine and for comparative purposes also histidine.

Histidine monohydrochloride was prepared from ox-blood by the method of Fränkel [1907]. M.PT. 253°, with softening and loss of water at 175–180°.

Kjeldahl. Nitrogen. Found 19·79 %. Calculated 20·05 %.

Preparation of Carnosine. This was obtained from Lemco. The most successful method found for the preparation was that adopted by Baumann and Ingwaldsen [1918] with the use of mercury acetate followed by the usual silver-baryta precipitation. A very readily crystallisable product is obtained if, after the addition of excess silver nitrate solution and before filtration of the purine fraction, barium hydroxide solution is added to more than neutralise the nitric acid set free by the use of the silver nitrate reagent. Otherwise, part of the purine group remains dissolved and escapes precipitation. This incomplete separation of the purine fraction from the carnosine fraction probably accounts for much of the difficulty workers have experienced in the isolation of pure carnosine [*vide* Gulewitsch, 1913; Smorodinez, 1913]. On first attempting the preparation this difficulty was met with. On precipitating the carnosine fraction, which refused to crystallise from alcohol, with the above precaution the product was readily purified. The purine part which

[1] Clifford [1921], since the commencement of this research, has applied a modified Koessler and Hanke procedure to the estimation of carnosine. As to both treatment of material and results found, the writer is so much at variance with the above worker that a publication at the present stage of this research has been deemed desirable.

separated here was treated with hydrogen sulphide, concentrated, and a syrup separated on treatment with alcohol. This appears at least presumptive evidence for the validity of the foregoing suggestion of the cause of the difficulty. The substance was first isolated as carnosine nitrate. This agreed in properties with those described by Gulewitsch and others [*vide* Gulewitsch and Amiradžibi, 1900; Gulewitsch, 1913]. M.PT. 220°.

The free base was prepared from part of the above product by reprecipitation with silver nitrate and barium hydroxide, and concentrated without neutralisation. The carnosine thus obtained was similar to that of Gulewitsch; M.PT. 260° with decomposition.

Part of the carnosine was used to prepare the copper-salt according to the directions of Mauthner [*vide* von Fürth and Hryntschak, 1914]. Deep-blue hexagonal crystals about 1 mm. in greatest length were obtained.

Solutions of 0·1 % carnosine were made from the free base and from the carnosine nitrate after these had been dried in desiccator to constant weight. Equal quantities of these tested colorimetrically agreed almost perfectly.

It was further intended to make a standard solution from the copper salt but the difficulty of getting this to constant weight, along with the rapidity with which it took up moisture when dry, led to the acceptance of the value as confirmed by the free base and its nitrate.

The Colorimetric Method.

Working mainly from certain theoretical considerations of the properties of diazonium compounds and their mode of coupling, Koessler and Hanke have, undoubtedly, greatly enhanced the value of the diazo reaction as a method of estimating iminazole derivatives. The use of a relatively stable colour-standard is only a small part of the improvement they have effected on the previously somewhat haphazard way of performing the diazo test.

As a quantitative method for the estimation of histidine the diazo reaction had been used by Weisz and Ssobolew [1914] and their method was adopted for the estimation of carnosine in mammalian muscle by von Fürth and Hryntschak. The much more consistent results obtainable by the Koessler and Hanke procedure testify to its superiority over any previous method.

The method has been adopted here almost *in toto* for the estimation of carnosine and found to give very satisfactory results in pure solutions of that substance. Different proportions of methyl-orange and Congo red had of course to be employed to get a match for the carnosine solutions. A very suitable match for pure carnosine solutions was obtained with 0·10 cc. methyl-orange and 0·25 cc. Congo red per 100 cc. water. These amounts were taken from 0·1 % stock methyl-orange and from 0·5 % stock Congo red.

Molecular colour values. To test the purity of the above-mentioned histidine compound and at the same time to verify the accuracy of the method, the molecular colour value of histidine was determined according to the (CR-MO)

colour-standard employed by Koessler and Hanke. This was found to be approximately 117,000,000 mm. as compared with 114,000,000 mm. found by those workers. The slight discrepancy between these results can be accounted for by experimental error, along with the possibility that there might be slight differences in the quality of the methyl-orange and Congo red employed.

The molecular colour value of carnosine was found to be 152,000,000 mm. against the same indicator.

Question as to the linearity of colour production. Clifford [1921] questions the direct proportionality between colour-production and the amount of iminazole in solution. The linear relationship, as set forth in the ideal tables of Koessler and Hanke, was tested for both histidine and carnosine by the writer and accepted within the limits of experimental error before the publication of the paper above referred to. It was very carefully retested on Clifford's figures coming to his notice, but the conclusion reached was merely a re-confirmation of Koessler and Hanke's results. Histidine was tested only within the limits of 0·01 mg. and 0·05 mg. histidine monohydrochloride in the test cylinder, whilst carnosine was tested between 0·005 mg. and 0·2 mg. The following table shows the results for carnosine, using a difference of 0·005 mg. carnosine between each reading up to 0·05 mg.

Carnosine in test cylinder mg.											
0·005	0·010	0·015	0·020	0·025	0·030	0·035	0·040	0·045	0·050	0·1	0·2
Reading of standard cylinder mm.											
3·2	5·7	8·9	11·8	14·8	17·5	20·8	23·8	26·9	30·0	59·7	120
Calculated reading of standard cylinder mm.											
3	6	9	12	15	18	21	24	27	30	60	120

When 0·1 mg. and 0·2 mg. carnosine were present in the test cylinder, the plunger was set at 10 mm. and 5 mm. respectively, and the reading of the standard cylinder multiplied by 2 or 4 gave the value equivalent to the test cylinder set at 20 mm.

The results for histidine within the limits tested were similar in their linearity.

Clifford finds colour values greater than those given by calculation on the linearity basis, in the more concentrated iminazole solutions. Exactly the opposite results were found by the writer, when the usual concentration of diazonium sulphonate reagent was used for widely differing concentrations of carnosine. Experiments here showed that the time required for maximum colour development increased with the amount of carnosine present so long as that maximum was the theoretical value. This was especially marked when more than 0·045 mg. carnosine was used. To give a few practical examples, it may be noted that with 0·045 mg. the theoretical value was reached only after standing for 12 to 14 minutes; with 0·05 mg. it was almost reached after 20 minutes; with 0·1 mg. and 0·2 mg. the highest readings were obtained only after 15 and 10 minutes respectively, but both of these were short of the

theoretical. With 0·1 mg. this shortage was 7 mm., *i.e.* approximately 12 %. With 0·2 mg. the reading was 24 mm. low, *i.e.* 20 %. If, however, a more concentrated diazonium reagent is used, the time necessary for maximum colour development is decreased, and the actual maximum is then approximately theoretical as shown in the foregoing table.

These results are quite in accordance with the mass action law. In presence of a relatively large amount of carnosine the diazonium sulphonate is in great measure used up, with the result that the speed of coupling under these conditions becomes low, and at a certain point practically nil. All the diazonium compound is not, however, used up with 0·1 mg. carnosine as the behaviour with 0·2 mg. shows. Here, the presence of a higher concentration of carnosine accounts for a greater amount of coupling, yet a similar equilibrium becomes established before complete coupling of the carnosine has taken place.

These facts merely indicate that a definite excess of diazonium sulphonate is necessary to complete the chemical reaction in one direction. They do not at all affect the validity of the linear relationship.

It is difficult to account for the deviation found by Clifford.

The best results were obtained when the test cylinder contained 0·02 mg.–0·04 mg. carnosine. The maximum is reached in 8–12 minutes after mixing, and is stable at those concentrations for approximately another 8 minutes.

No appreciable colour was developed by the interaction of the sodium carbonate used (Merck's) with the diazo reagent alone, even after 20 minutes.

Certain Considerations preliminary to the Estimation of Carnosine in Muscle Extract.

Filter paper adsorbs carnosine. Koessler and Hanke [1919] show that iminazoles are adsorbed by animal charcoal. This fact suggested that the use of filter paper might vitiate the results in such a microchemical method as that here employed. The adsorptive effect of filter paper was shown in the following way.

20 cc. of a cold solution of carnosine with a colour value of 13·9 mm. per cc. were run from a pipette through a 15 cm. diam. dry filter paper into a dry measuring cylinder. 1 cc. of this filtrate was then tested and found to have a colour value of only 11·5 mm. This process was thrice repeated, through three papers, and 1 cc. of the filtrate gave successively colour values of 10·0 mm., 8·5 mm., and 7·7 mm. That is, the total loss in colour value was 6·2 mm. per cc., or approximately 44 %.

In order to determine to what extent this loss was recoverable by washing, 10 cc. solution of carnosine with colour value of 18·0 mm. per cc. were added to four dry filter papers as above but folded into one funnel. The results are expressed in the following table:

Added to filter	Volume of filtrate cc.	Colour value of filtrate per cc. mm.	Total colour value of filtrate mm.
10 cc. carnosine solution	3·2	8·5	27·2
10 cc. water	9·2	5·5	50·6
20 cc. „	19·8	2·5	49·5
40 cc. „	evaporated to 27·0	1·0	27·0

The loss as shown in the first filtrate is 9·5 mm. per cc., *i.e.* approximately 53 %. The sum total of colour values of filtrates is 154·3 mm. The total colour value of the original solution is 180 mm. so that, after washing with water to the extent of seven times the volume of the original carnosine solution, there yet remains 14 % of the original carnosine held by the filter paper.

For these reasons, glass wool, which shows no adsorptive properties, has been adopted for filtration purposes.

The effect of heat on the colour value of pure carnosine solutions. Several clean, dry duroglass test-tubes were constricted to a neck of about 3 mm. diameter about 3 cm. from the bottom. A horizontal mark was made with a file at the constriction. (The object of constricting the tubes was to obtain more accurate measurement.) The tubes were filled to the mark with solutions of carnosine of known colour value and set on a boiling water-bath for 1 hour. They were then removed, cooled, and made up to the mark with water. A number of solutions of carnosine with an original colour value of 19·5 mm. per cc. showed after this treatment an average loss in colour value of 1 mm. per cc. Similarly a number of solutions of carnosine nitrate with an original colour value of 18·5 mm. per cc. showed after the same treatment an average loss of 0·5 mm. No change of tint in the colour developed was observable in these tests.

It is probable from these results that a process of extraction which involves prolonged boiling may be harmful to the carnosine.

Method of Extraction employed.

The most consistent results have been obtained by the following procedure. A small quantity of muscle, say 1·5 g., is extracted with about 20 cc. water on a bath at 70°. After 30 minutes the solution is filtered through glass wool into a small Buchner flask. The meat residue is transferred to a mortar, and ground with glass to a fine pulp. This is again extracted with 20 cc. water for 15 minutes, poured through the same filter and sucked as dry as possible. The top layer of wool and meat residue is re-transferred to beaker, stirred up with a further 20 cc. water and heated another 15 minutes. It is then re-filtered and washed twice with 10 cc. warm water, sucking as dry as possible between washings. After this treatment the final filtrate should give no diazo reaction.

One extraction, found by Clifford [1921] to be sufficient, has never given satisfactory results in the writer's hands. After extracting the finely ground tissue once with a large volume of water, followed by copious washing, the

colour value has shown a loss of 6 to 10 % reckoned as carnosine, as compared with that from the same meat extracted as directed above.

The total milky filtrate—representing about 50 cc. per g. of meat used—is poured into a suitable beaker. The reaction is made slightly acid with dilute acetic acid and the beaker set on the water-bath at 70° for a few minutes. Under these conditions the proteins are very effectively precipitated. The coagulum settles quickly and is readily caught in the glass wool filter. The contents of the filter are washed thrice with a little warm water. The final filtrate should be quite clear and entirely negative to all protein tests.

The results are practically unaltered if instead of the above method excess of lead acetate followed by excess of disodium phosphate is used to precipitate the protein. Thus three samples from a piece of lean muscle, extracted as above, showed on reckoning the total colour value as carnosine 0·355 %, 0·369 % and 0·380 %. In the first two samples the proteins were precipitated with lead acetate and sodium phosphate; in the last by the acetic acid method. The acetic acid method was finally adopted because it is more simple and gives a smaller precipitate by which less carnosine is likely to be adsorbed.

Further it would appear that there are no substances in muscle extract as prepared above which inhibit the diazo colour production; thus 1 cc. meat extract with colour value 22·0 mm. per cc. was mixed with 1 cc. carnosine solution 18 mm. per cc., and 1 cc. of the mixture tested. This was found to have a colour value of 20 mm. which is the theoretical number.

The total colour value of muscle extract as obtained by the methods outlined above, is consistent for the same material to within 3–4 % of the colour value reckoned as carnosine in meat. Thus three pieces of ox muscle yielded 0·388 %, 0·378 % and 0·379 %. Another sample of ox muscle gave 0·614 %, 0·606 % and 0·620 %. These materials were purchased from a butcher. A cat muscle in three portions showed 0·280 %, 0·287 % and 0·293 %.

As yet, however, the method does not appear to be entirely satisfactory for the estimation of carnosine in muscle extract. Carnosine is, undoubtedly, responsible for the production of the great bulk of the colour in these muscle extracts, but the writer has good reason to believe that certain other substances contribute to a considerable degree to the yielding of the total diazo value. Further investigation is in progress on this point.

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