XLV. A METHOD FOR THE COLORIMETRIC ESTIMATION OF CARNOSINE.

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THE idea of estimating carnosine colorimetrically is based on the fact that histidine when treated with diazobenzenesulphanilic acid and sodium carbonate gives rise to an intense red colour and this led to an attempt by v. Fürth and Hryntschak [1914] to evolve a colorimetric estimation of carnosine nitrate.

They took the base prepared according to the method of Gulewitsch and precipitated it as a copper salt. A known amount was then suspended in water and decomposed with hydrogen sulphide. Using the colorimetric method of Weiss and Ssoblew [1914] they then compared the intensity of the red colour obtained on diazotising the carnosine solution with that given by a standard histidine solution when treated in the same way. The results were:

0.195 g. carnosine copper salt gave 0.173 g. carnosine (theoretically 0.145 g.).

0.6378 g. carnosine copper salt gave 0.430 g. carnosine (theoretically 0.473 g.).

From this it may be seen that the method was decidedly inaccurate and could not be used with confidence if the amount of carnosine were unknown.

These observers then worked on extracts of horse flesh, precipitated the carnosine as the copper salt, decomposed it, and then, by colorimetric means, obtained an average of 0.27 % in the extract. Dietrich [1914], however, had by precipitation methods found the percentage to be 0.17. The discrepancy was possibly due to the numerous precipitations involved, each probably being a source of loss of carnosine.

In 1919 Koessler and Hanke [1919] described a colorimetric method for estimating iminazole derivatives based on the diazo-reaction. The chief modification was the substitution of the standard histidine solution by a standard consisting of a mixture of methyl orange and Congo red (CR. MO), which gave a colour matchable with diazotised histidine.

Since their method had been worked out for histidine, histamine and other iminazole derivatives, it was probable that it could be used for the direct estimation of carnosine in muscle extracts.

Experimental method used.

The colorimetric estimation of carnosine to be described was, accordingly, based on Koessler and Hanke's method for the estimation of pure solutions of iminazole compounds. Several modifications were found necessary before the method could be used to determine the amount of carnosine present in a muscle extract, which naturally is more complex in composition than a pure iminazole solution and contains substances which inhibit the diazo-reaction.

The modifications introduced were as follows:

(1) *Modification with regard to mixing reagents.*

The first alteration was in regard to the mixing of the reagents.

Koessler and Hanke [1919] mixed their solutions directly in the colorimeter cup and laid stress on the necessity for this procedure. Using their suggested amounts, however, it was found impossible to set the test cylinder at the proposed depth of 20 mm. since the colorimeter was larger than theirs.

Double quantities of the reagents were thoroughly mixed in a beaker and were then transferred to the colorimeter cup. No difference in the final colour was obtained whether the reagents were mixed in the cylinder or in the beaker. The depth of colour was also unaltered whether the mixture was transferred directly after mixing in the beaker or at any other stage up to the development of a maximum red colour.

The temperature of the laboratory, within ordinary limits, was also without effect on the reaction. Estimations carried out in winter and in summer gave parallel results, as also did those performed with the mixing beaker near a Bunsen flame.

(2) *Modification with regard to linear relation of readings.*

Koessler and Hanke obtained a perfect linear relation between the amount of histidine diazotised and the readings of the standard CR. MO cylinder.

Thus they stated that:

0.00002 g. histidine	matched at 10 mm.
0.00003 " "	15 "
0.00004 " "	20 "
0.00005 " "	25 "

On attempting to repeat the results of these observers, no such linear relation could be found. The depth of colour formed by diazotising histidine and carnosine was not proportional to the amount of solution diazotised, though for any given amount the readings were constant.

Some actual figures obtained using a standard solution of histidine matched against a CR. MO indicator solution were:

Amount of iminazole diazotised	Actual reading of standard cylinder	Theoretical reading assuming linear relationship
0.00002 g. histidine	10.75 mm.	10.75 mm.
0.00003 " "	18.70 "	16.10 "
0.00004 " "	28.1 "	21.5 "
0.00005 " carnosine	13.7 "	13.7 "
0.0001 " "	30.0 "	27.4 "

From these results found experimentally it was decided that linear relationship between depth of colour and amount of carnosine diazotised could not be accepted. It was therefore decided to base all results on a constant reading of the standard cylinder.

In all cases the amount of solution diazotised was altered till, when set at 20 mm., it gave a colour matching the indicator cylinder when set at 30 mm. This reading was chosen since a match with the indicator at 30 mm. corresponded to 0.0001 g. of carnosine and calculations were simplified.

(3) *Number of extractions needed.*

Three portions of a sample of English veal were taken and treated as follows:

- (a) Extracted once with 50 cc. of water; yield 1.15 % carnosine.
- (b) Extracted twice with two separate 50 cc. water; yield 1.12 % carnosine.
- (c) Extracted three times with three separate 50 cc. water; yield 1.15 % carnosine.

With three portions of rabbit muscle treated similarly the results were 0.15, 0.15, 0.14 % respectively.

Accordingly, for further estimations it was considered that one extraction was sufficient to give a full yield of carnosine.

This fact indicates that carnosine is in very loose combination with the muscles and the possibility that the substance estimated is histidine and not carnosine is practically ruled out since acid hydrolysis is needed to split histidine from a protein.

(4) *Modification with regard to the presence of proteins.*

Since proteins had been found by Koessler and Hanke to lower the readings of a standard histidine solution by 30 % it was obviously necessary to obtain a precipitant for soluble proteins when estimating carnosine in a watery extract of muscle.

Alcohol and ammonium sulphate cause the formation of an unmatchable yellow colour if added to histidine or carnosine before diazotising and therefore could not be used to free the extracts from protein.

Potassium dihydrogen phosphate up to a concentration of 5 % was stated by Koessler and Hanke to cause no alteration in the colour of a diazotised histidine solution. This led to a trial of metaphosphoric acid as a protein precipitant according to the method of Folin and Denis [1916].

A solution of carnosine was made of such a strength that when 1 cc. was diazotised and set at 20 mm. it gave a match with the CR. MO at 25 mm.

30 cc. of this solution were taken, 10 cc. of 20 % metaphosphoric acid added and the mixture was allowed to stand for three hours.

No precipitate was formed and therefore the metaphosphoric acid removes no carnosine from the solution.

The acid carnosine solution, which it was unnecessary to filter, was then made neutral to litmus with 10 % caustic soda and made up to 50 cc. with distilled water.

1.62 cc. of this solution gave a match with the indicator at 25 mm. against the theoretical 1.66 cc.

This experiment showed that metaphosphoric acid has no precipitating effect on carnosine and that it does not affect the red colour of diazotised carnosine.

It then remained to prove that metaphosphoric acid would successfully remove proteins from a solution without carrying down carnosine as an adsorption compound, or in any other physico-chemical manner.

A 5 % solution of egg albumin was made up and found to give a strong yellow colour with the diazo-reagent. No trace of red was present. To 30 cc. of this protein were added 50 cc. of the carnosine solution previously used (*i.e.* 1 cc. when diazotised gave a match with the CR. MO at 25 mm.) and 10 cc. of 25 % metaphosphoric acid. After standing overnight the carnosine protein mixture was filtered, neutralised with 10 % caustic soda and made up to 100 cc.

2 cc. of this filtrate gave a match with the CR. MO at 24.9–25.1 mm.

Since the mixture was 50 % carnosine and 50 % albumin, acid and water, 2 cc. equalled the original 1 cc. which on treatment gave a match at 25 mm.

Similar results were obtained using gelatin and blood albumin as the protein added to the carnosine solution.

These experiments showed that metaphosphoric acid effectually removed the proteins without in any way interfering with the diazotisation of carnosine. They also showed that metaphosphoric acid does not split off histidine from the proteins when used as a precipitant, since the result on diazotising a standard carnosine protein solution was unaltered by precipitation with metaphosphoric acid.

A further test was made using metaphosphoric acid to precipitate the proteins in "Marmite," a commercial preparation of yeast.

The resultant filtrate when diazotised gave a deep yellow colour without a trace of the typical carnosine red.

A standard carnosine solution was taken of which 1 cc. gave a match with the indicator at 29.95 mm. 13.9 g. of "Marmite" were dissolved in 30 cc. of water. 50 cc. of the carnosine solution were added, together with 10 cc. of 20 % metaphosphoric acid. This mixture was allowed to stand overnight. It was then filtered, neutralised to litmus and made up to 100 cc.

2 cc. of this filtrate (*i.e.* the same amount of carnosine as previously gave a reading of 29.95 mm.) gave a match at 29.95–29.97 mm. The additional yellow tinge caused by the diazo-reagent on the "Marmite" gave a more perfect match against the CR. MO standard than when pure carnosine was diazotised.

Similar results were obtained when cod extract was used in place of "Marmite." 13.96 g. of cod were extracted with 50 cc. of water and 10 cc. of 25 % metaphosphoric acid were used to precipitate the protein.

After filtration and neutralisation 40 cc. of the mixture were diluted to 100 cc. On diazotisation no pink colour was formed, but again a deep yellow resulted.

A further 14 g. of cod muscle was treated in exactly the same way, except that besides the water and acid 60 cc. of carnosine solution were added. This carnosine was of such a strength that 1 cc. when diazotised gave a match with the CR. MO at 27.8–28.2 mm.

The fish-carnosine mixture gave a match with the CR. MO at 28.0–28.3 mm. when 2 cc. of the filtrate were treated with diazobenzenesulphanilic acid and sodium carbonate.

Here again there was no difference in the colour obtained when proteins in the solution were first precipitated by means of metaphosphoric acid, though when free in the diazotised liquid they decreased or even destroyed the colour formation.

It was also obvious that no histidine was liberated from commercial protein or fresh cod muscle by the experimental methods used, otherwise on diazotisation a deeper red colour than could be accounted for by the added carnosine would have been obtained.

Metaphosphoric acid neither destroys nor interferes with the reaction and the proteins are effectually removed by it, since the final solution gives negative results with Millon's, the xanthoproteic and glyoxylic tests. In carnosine-free solutions the biuret reaction is also negative.

Therefore it was decided that metaphosphoric acid up to a concentration of 10 cc. of 20 % solution per 50 cc. of fluid has no effect on the colour formed by adding diazobenzenesulphanilic acid and sodium carbonate to a carnosine solution.

Final method adopted.

The final method adopted for the colorimetric estimation of carnosine in muscle extracts is as follows:

A weighed amount of finely minced tissue is extracted for 15–60 minutes at 60–90° in a known volume of water. This is then filtered through a dry paper into a dry beaker and the pulp well squeezed out. 2–10 cc. of 20 % metaphosphoric acid (according to the amount of tissue taken) are added and the mixture is left to stand 2–24 hours.

The solution is then filtered from the precipitated protein. An aliquot part of the filtrate is placed in a graduated flask, neutralised to litmus with 10 % caustic soda and made up to the mark with distilled water. This neutralised solution is treated as a carnosine solution. 10 cc. of 1.1 % sodium carbonate are placed in a clean dry beaker together with 2–X cc. of distilled water, 4 cc. of the diazo-reagent are run in from a pipette, and the whole

mixed by shaking the beaker. Exactly one minute later X cc. of the extract are added.

After mixing the diazotised solution is placed in one cup of a Duboscq colorimeter which is then set at 20 mm. On the other side is Koessler and Hanke's [1919] CR. MO standard. This standard cylinder is moved till it matches the unknown when the colour of the latter has gained its full intensity (about 5 minutes from mixing). Readings are taken and the value of X altered till, with the unknown set at 20 mm., the CR. MO cylinder matches at 30 mm.

As this is so when $X = 1$ cc. of a 0.01 % carnosine solution, the amount of extract required to give this colour is taken to contain 0.0001 g. carnosine.

An actual experiment was as follows:

13.56 g. rabbit muscle were extracted with 50 cc. of distilled water at 60° for 25 minutes.

The solution was then filtered and the pulp squeezed out. To the filtrate were added 10 cc. of 20 % metaphosphoric acid. The mixture was allowed to stand 24 hours and was then filtered.

30 cc. of this filtrate were taken, neutralised with 10 % soda, and made up to 100 cc. in a graduated flask.

Different amounts were then diazotised and compared with the CR. MO indicator.

$X = 1.0$ cc. gave a match with the indicator cylinder at 30 mm., whereas 0.54 was insufficient and 1.5 cc. too strong.

Therefore 1 cc. contained 0.0001 g. of carnosine.

100 „ „ 0.01 „ „

And since half the original filtrate was taken the 100 cc. contained half of the carnosine of the extracted muscle.

Therefore percentage = $\frac{2 \times 0.01 \times 100}{13.6} = 0.15$ % carnosine.

In another experiment 8.5 g. lean imported beef treated in a similar manner gave 0.36 % carnosine.

Parallel estimations should agree to 0.05 mm. Thus in the above experiment the readings obtained for separate amounts of 0.94 cc. each were:

29.96, 30.0, 29.98, 29.99, 29.96, 30.1, 30.0.

This method has been used for over one hundred estimations of carnosine in various muscles and is the basis of an investigation now in progress of the distribution of carnosine in the animal kingdom.

SUMMARY.

A modification of Koessler and Hanke's colorimetric method for estimating iminazole derivatives has been devised which applies to the estimation of carnosine.

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