LXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT—A CRITICAL STUDY.

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In a recent communication [Hunter, 1921] a colorimetric method for the estimation of carnosine in muscle extract was generally outlined. This was based on a method of estimation of iminazoles devised by Koessler and Hanke [1919, 1]. This method depended on the coupling of carnosine with diazotised sulphanilic acid in alkaline solution to form an azo dye. The colour produced was measured against a standard solution of a mixture of Congo red and methyl-orange. Pure solutions of carnosine were found to react very satisfactorily, and in different extracts of the same muscles the colour values were consistent. The accuracy of the method could not be doubted, but the certainty that carnosine was responsible for the total colour production was called in question.

The problem—of the extent to which carnosine is responsible for the production of the azo colour in muscle extracts—may be attacked in two ways: 1. By the elimination of all interfering substances. 2. By a direct method of confirming the carnosine content.

I. The Elimination of Interfering Substances.

Potentially interfering substances. It is well known that aromatic amines and phenols readily couple with a diazotised aromatic amine and are thus potentially interfering substances with the reagent here employed for the estimation of carnosine.

The iminazoles form another well defined group—of which carnosine is only one member—giving the diazo reaction.

The purines which contain the iminazole ring are generally described as substances giving a positive diazo test—this applies at least to the members adenine, hypoxanthine, and xanthine [*v.* Plimmer, 1918].

There are various other substances diversely referred to throughout biochemical literature as giving the diazo test. Of these may be mentioned thymine [Thierfelder, 1908], bilirubin [Neubauer-Huppert, 1913], urochro-

mogen and urobilinogen (Neubauer-Huppert), besides the "neutral-sulphur" compounds of urine (Neubauer-Huppert).

An exhaustive investigation into the behaviour of all substances—confined even to biochemical literature—towards the diazo reagent would probably be endless as well as fruitless. Attention will thus be confined only to those reagents ordinarily employed in the preparation of muscle aqueous extracts and to substances that may be present with a fair degree of probability in the extracts.

A. *Reagents.* Soluble chlorides, sulphates, nitrates, phosphates and sodium acetate, have been tested in relatively high concentrations and found to have no effect on the diazo reagent.

Ammonia and its salts not only interfere with the production of colour from other substances such as histidine [Koessler and Hanke, 1919, 2] but when added alone to the diazo reagent give a yellow colour.

Soluble sulphides give a colour in low concentration. The presence of sulphur in cystine probably accounts for the similar behaviour of that substance towards the diazo reagent.

Ethyl alcohol was found to give no colour with the reagent though Koessler and Hanke find that it inhibits colour production.

Hydrogen peroxide, formaldehyde and acetone all give yellow colours.

Tannic acid in very low concentration gives a marked colour. This is accounted for by its phenolic constituents.

All solutions in which carnosine is to be estimated by means of the diazo reagent should thus be free from ammonium salts, sulphides, and tannic acid. Formaldehyde or phenols, such as thymol, should not be used as preservatives in this connection. Muscle tissue may be preserved in alcohol and extracts by a layer of toluene.

B. *Muscle constituents.* Certain normal muscle constituents which may possibly act as interfering substances in the extracts require a more detailed examination, as these are much less within the control of the worker than substances which may be used in the preparation of the extracts.

The presence of bilirubin, urochromogen and urobilinogen in muscle extracts is too unlikely to claim further consideration for those substances. Thymine may also be dismissed on account of the difficulty with which it is liberated by hydrolysis from nucleic acid [*v.* Jones, 1920].

(1) *Phenols and aromatic amines.* The presence of these substances in protein-free extracts has not been shown by any of the tests used to detect them. Extracts from several types of muscle were shaken in acid solution with ether. The ether extracts were evaporated to dryness and the slight residues taken up in small quantities of water. These gave no diazo reaction nor has Millon's test been found positive either in the extracts themselves or in the ether fractions.

It is known that aromatic amines couple with the diazo reagent in acid solutions, whereas iminazoles and phenols require a weak alkaline medium before

a colour is produced. Thus if a little 1. 2. 4-diaminotoluidine is added to the reagent without the addition of sodium carbonate, a strong orange colour is developed. If an iminazole or phenol is added under the same conditions no colour is produced[1].

Various extracts have been tested with the acid reagent but all with negative results. It may thus be concluded that there are no aromatic amines contributing to the azo colour developed in meat extracts[2].

(2) *Tyrosine.* This substance, which is not extractable by ether, has received considerable attention from the point of view of the diazo reaction. Totani [1915] devised a method to distinguish the azo colour developed by histidine from that developed by tyrosine. The colours were first reduced with zinc and hydrochloric acid, twice the volume of 25 % ammonia added and in both cases a golden yellow colour was obtained. In the case of histidine the addition of hydrogen peroxide changed the colour to lemon yellow whereas the colour was destroyed in the case of tyrosine. A dilution greater than 1 : 20,000 histidine was necessary to get the characteristic final colour.

Among the various substances considered by Totani no mention is made of iminazoles other than histidine, nor of purines, nor of aromatic amines nor of phenols other than tyrosine.

On repetition of Totani's procedure with histidine alongside carnosine, the two substances went through approximately the same colour changes. The final colour was not destroyed and so the presence of tyrosine could not be detected by this method.

Tyrosine gives a positive Millon's test; histidine and carnosine are negative to Millon's. Tyrosine gives a faint Millon's test at a dilution of 1 : 25,000.

A still more delicate test for tyrosine—on which is also founded a method of estimation—has recently been devised by Hanke and Koessler [1922]. This depends on a further modification of the diazo reaction and is approximately as delicate as that reaction is for carnosine. For quantitative purposes the procedure is the same as that adopted for iminazoles and phenols. The test cylinder is allowed to stand for exactly 5½ minutes after the tyrosine solution has been added to the alkaline reagent. This gives rise to a primary yellow colour the intensity of which is not directly proportional to the amount of tyrosine used.

2 cc. of 3*N* sodium hydroxide solution are now added and the contents of the cylinder mixed. This gives rise to a colour intensification with a change of tint towards the red.

One minute after the addition of the sodium hydroxide 0·10 cc. of a 20 % solution of hydroxylamine hydrochloride is added and rapidly mixed. After

[1] The aromatic amines also give a colour in alkaline solutions.

[2] This test would also eliminate bilirubin which gives a blue colour in acid solution [Ehrlich, quoted by Neubauer-Huppert].

a latent period of 5 to 10 seconds an intense bluish red colour rapidly develops. This colour is stable and is directly proportional to the amount of tyrosine present.

Hanke and Koessler note that this intensification is also given by substances capable of a keto-enol tautomerism such as acetaldehyde, acetone and aceto-acetic acid.

It was decided to test this new modification on muscle extracts. It was first observed that the addition of sodium hydroxide and hydroxylamine hydrochloride in the manner above described to the azo colour developed by histidine or carnosine had the effect merely of a proportional dilution. Thus a solution of carnosine gave by the ordinary method a reading of 22·5 mm.; by the new method a reading of 18·3 mm., *i.e.* approximately the same as would be obtained by adding 2·1 cc. water to the cylinder. The following results were obtained on the extracts.

	Reading with diazo reagent mm.	Reading with diazo reagent, NaOH and NH_2OH . HCl mm.	Calculated reading—assuming proportional dilution mm.	Intensification %
Ox muscle	27·2	21·4	21·6	None
Cat ,,	18·0	16·5	14·3	15·4
Rabbit muscle	10·8	11·3	8·5	33·0
Otter ,,	15·5	18·5	12·3	50·4
Salmon ,,	13·7	21·5	10·8	100·0

The percentage of intensification of these extracts is in the order of the yellowness of their azo colours. Thus ox muscle extracts give a colour which matches the carnosine colour standard. The colour developed from cat muscle extract is generally slightly more yellow; that from rabbit almost matches the histidine colour standard; that from otter is still more yellow and that from salmon is so yellow as to be almost unmatchable.

The intensification colours were pink. They did not show the purplish tinge given by tyrosine. No Millon's reaction was obtained in concentrated extracts of salmon muscle and it is concluded that tyrosine is not responsible for the intensification obtained in that tissue.

The specific cause of this intensification has not been determined.

(3) *Purines.* These are normally present in muscle extracts so that a consideration of the individual members is rendered necessary. Adenine and guanine were prepared from commercial plant nucleic acid. This was hydrolysed by suspending in methyl alcohol and passing dry hydrochloric acid gas according to the method employed by Levene [1921] for animal nucleic acid. This process was found to work very satisfactorily. The precipitated chlorides of adenine and guanine were filtered off and separated according to methods described by Jones [1920].

Hypoxanthine nitrate and xanthine were prepared from parts of the adenine and guanine respectively by deaminisation and subsequent purification.

Cytosine and uracil were also prepared from the residues freed from methyl alcohol by further hydrolysis in the autoclave at 160° with 25 % sulphuric acid for five hours according to Jones [1920].

Neither of these pyrimidines gave a colour with the diazo reagent.

0·05 % solutions were made of adenine sulphate, of guanine chloride, of hypoxanthine nitrate and of xanthine—all in 1·1 % sodium carbonate solutions.

1 cc. of each of these was tested in the ordinary way. Guanine and xanthine gave marked colours. The adenine solution showed only a slight reaction and there was no colour in the case of hypoxanthine. With a tenth of the above amounts guanine and xanthine were still strongly positive whilst adenine and hypoxanthine were entirely negative. The intensities of the colour were approximately the same in like concentrations of guanine and xanthine. With 0·025 mg. of guanine hydrochloride in the cylinder a reading of 8·5 mm. was obtained with the histidine colour standard. With 0·05 mg. the reading was 13 mm. Xanthine in the same amounts gave the respective readings 8 mm. and 12·5 mm.[1] The colour production is not directly proportional to the amounts of guanine and xanthine in the cylinder; nor do guanine and xanthine, mixed with a known amount of carnosine, give proportional colours. Thus 1 cc. of a mixture of 1 cc. 18·6 mm. per cc. carnosine solution with 1 cc. 13·5 mm. per cc. guanine solution gave a reading of 13·2 mm. with the test cylinder set at 20 mm. This is 2·8 mm. short of the calculated reading of 16 mm. A similar result was found with xanthine. Adenine and hypoxanthine do not give a colour or inhibit colour production at this concentration, but they tend to make the colour due to carnosine too yellow. Thus with 1 cc. of a mixture of carnosine solution of the above concentration with 1 cc. 0·05 mg. per cc. of adenine sulphate solution, the calculated reading of 9·3 mm. was obtained. Hypoxanthine behaves similarly.

Guanine and xanthine are about half as sensitive towards the diazo reagent as carnosine. If present in appreciable amounts in muscle extract they must seriously affect the estimation of carnosine.

Uric acid in excess gives a slight yellowness to the diazo reagent.

To what extent are purines likely to interfere with the estimation of carnosine in muscle extracts? On the assumption that there is present in extract of meat free purine nitrogen to the extent of 0·045 % as quoted by Lusk [1921] it is unlikely that more than 0·020 % nitrogen represents purines that give a colour with the diazo reagent. If the nitrogen be taken as representing 42 % of the purine molecule—an average figure from adenine, guanine, hypoxanthine, xanthine and uric acid—the purines affecting the diazo reagent amount to about 0·05 %. In muscles with a carnosine content of less than, say 0·1 % the presence of such a proportion of purines would make the results worthless.

[1] The readings are only approximate as the colours are much more yellow than the standard.

To determine the actual purine interference under the conditions of extraction previously recommended, it was then considered necessary to carry out some quantitative fractionations[1].

A quantity of ox muscle was extracted at 70° with small amounts of water until the filtrate no longer gave a positive diazo test. The proteins were precipitated with excess of lead acetate and the excess of lead was removed with disodium phosphate. The filtrate was neutralised with sodium hydroxide and its colour value measured with the carnosine colour standard. This was found to be 1050 mm. per cc.

50 cc. of the extract were taken in a small beaker, made just acid with nitric acid, and silver nitrate was added till a drop of the solution on a tile gave a brown colour with baryta. The test drops were washed back into the beaker.

After settling, the contents of the beaker were filtered by suction through washed asbestos in a small Hirsch funnel. The precipitate was carefully washed with water and after sucking dry was transferred with the asbestos to a small beaker, stirred up with a little water and hydrogen sulphide passed to precipitate the silver. The contents were again filtered through asbestos in the same way and the hydrogen sulphide removed by prolonged aeration with the help of a pump. The filtrate was neutralised. This is the purine fraction.

To the filtrate from the purine fraction excess of solid finely ground baryta was added. The precipitate was allowed to settle, filtered as above, and washed with saturated baryta water. The silver was removed with hydrogen sulphide and the barium with slight excess of sulphuric acid. The slightly acid solution was aerated to remove the hydrogen sulphide and the solution was then neutralised with sodium hydroxide. This is the carnosine fraction.

The filtrate from the carnosine fraction was freed from barium and hydrogen sulphide and neutralised as above. This is the final filtrate.

The exact volume of each of the three fractions was noted. The colour value per cc. of each of the fractions was then measured and their total colour values calculated. The sum total colour value was then obtained and compared with the original, *i.e.* 50 × 1050 or 52,500 mm.

[1] All the carnosine can be extracted from muscle with much smaller proportions of water than recommended by the writer in his previous paper on this subject. A filtrate of about 50 cc. per g. of muscle used was there recommended mainly because the proteins are easily precipitated by slight acidification with acetic acid and heat under those conditions. In concentrated extracts this simple process is not effective and the use of lead acetate or other precipitant is necessitated. The number of washings, however, should not be reduced. There is no need to reduce the proportion of water to muscle when the extract is to be tested only for colour value unless the carnosine content is less than 0·1 %. With that amount in muscle a reading of 12 mm. will still be obtained with the 50 cc. per 1 g. proportion. But for precipitation purposes and other tests it is desirable to keep the filtrate small rather than have to evaporate it to a low volume.

The distribution of colour value is shown by the total colour values in mm. of the three fractions:

Purine fraction	...	1,560
Carnosine fraction	...	47,587
Final filtrate ...	...	2,200
Sum total ...	...	51,347

The unrecovered colour value is thus 1153 mm. or approximately 2 %. The purine fraction accounts for about 3 % and the final filtrate for about 4·5 %. The carnosine fraction represents almost 91 % of the original colour value.

The relatively small error due to purines by the gentle method of extraction recommended is further seen in the table to follow.

Three portions were taken from the same muscle and two extracted as above. The third was extracted at a temperature just under boiling point.

Aliquot portions of the filtrates from the lead precipitates were taken for total nitrogen estimation and for further fractionation into purine and carnosine fractions. The final filtrates were rejected without evaluation. The total nitrogen was also estimated in the carnosine fractions. In the table carnosine, nitrogen and purine (calculated as carnosine) are shown as percentages of the original muscle. The colour values of the purine fraction are at best only a rough estimate on account of the difficulty in comparing with a much redder standard. The error due to the purines in ox muscle is however small.

Meat No.	Carnosine from lead acetate filtrate	Total N from lead acetate fraction	Carnosine from carnosine fraction	Total N from carnosine fraction	Colour value in purine fraction	Difference in % carnosine in lead acetate filtrate and in carnosine fraction
1	0·517	0·530	0·498	0·246	0·004	0·019
2	0·512	0·546	0·495	0·230	0·012	0·017
3	0·533	0·653	0·487	0·258	0·014	0·046

Before considering other substances likely to interfere with the estimation it may be noted that the more drastic extraction process of muscle No. 3 results in a higher percentage colour value. The total nitrogen in this extract is also raised. In the carnosine fraction the percentage falls again into line with the other two samples. The loss is not accounted for by a higher purine value. Though the colour value of the lead acetate filtrate is raised by the more rigorous extraction, the actual percentage of carnosine is lowered. The lowering of colour value becomes obvious even in the lead acetate filtrate if boiling is prolonged. Thus two samples of muscle treated in the usual way showed an average of 0·639 % carnosine in the lead acetate filtrate, whilst three samples of the same muscle after boiling for about 30 minutes showed an average value of only 0·614 % or a fall of 0·025 % reckoned as carnosine.

In muscles 1 and 2 approximately 96 % of the colour is recovered in the carnosine fraction. Calculated as carnosine this accounts for about 50 % of

the nitrogen in the same fraction. There is no reason to doubt that carnosine is responsible for all the colour production in the carnosine fraction from ox muscle. In the case of rabbit muscle and notably salmon muscle, fractionation does not appreciably improve the match. Thus two portions of a rabbit muscle showed from the lead acetate filtrate colour values of 0·092 % and 0·099 % reckoned as carnosine. The carnosine fraction from the same muscles showed each a value of 0·072 % as carnosine. In the latter case the azo colour developed was still very yellow. The colour from salmon muscle extract is slightly more red in the carnosine fraction than in the lead acetate filtrate, yet it is far from satisfactory.

The precipitation process occupies much time and to obtain reliable quantitative results from it, great care must be exercised. With the additional possibility that the carnosine fraction may still contain other colour-giving substances, it would appear of little use to attempt the elimination of interfering substances by the silver-baryta precipitation except in very special cases.

(4) *Iminazoles.* Of this group only histidine and carnosine are known with certainty to occur in animal tissue. Histamine has been found in the intestinal mucosa by Barger and Dale where its presence is attributed to bacterial action [Barger, 1914]. Its isolation from the pituitary gland by Abel is accounted for by Hanke and Koessler through Abel's use of a commercial product. Hanke and Koessler [1920, 2] show that it is absent from fresh hypophysis cerebri.

In protein-free extract from fresh muscle there is no evidence that histidine is present provided the extraction process has been gentle. The ease with which carnosine may be hydrolysed (*v.* later), along with other factors to be considered, renders it important that there should be some ready means of detecting histidine in the presence of carnosine.

As far as the writer is aware there is no known method of separating these substances. They both precipitate in the carnosine fraction in the "arginine separation." Though histidine is more readily precipitated with ammonium-silver than carnosine, yet the latter also precipitates in such relatively dilute solutions as 0·5 % carnosine. Nor could any test be found in the literature to distinguish the two substances.

Various colour tests for histidine were performed on solutions of carnosine. The main differences were found in their behaviour towards the biuret and Knoop tests.

The biuret reaction was found to be entirely negative even in about 30 % solution of carnosine and after standing at least one hour. This difference in behaviour is however of little value for discrimination purposes as relatively concentrated solutions of histidine must be used to get a positive biuret. Thus 1 % solution of histidine monohydrochloride gives the reaction in the cold only after standing for about $1\frac{1}{2}$ hours.

The writer has found Knoop's test to be the most useful way of detecting histidine in the presence of carnosine. Carnosine is quite negative to Knoop's

test. By a modification of the test as explained in the preceding paper, the writer has been able to increase both its delicacy and certainty as a test for histidine. The presence of histidine may be detected with certainty at a dilution of 1 : 10,000.

Though this reaction has not yet the delicacy that might be desired, its very specific nature makes it valuable for work of this kind. Knoop [1908] states that the reaction is also positive for histamine. No other substance, as far as the writer is aware, gives the reaction.

Certain muscle extracts suspected of histidine, were then tested by the modified Knoop's test. Extracts of rabbit and salmon which give notably yellow colours were tested in sufficient concentration but without giving an indubitable Knoop's test. It is thus concluded that histidine is not a factor in causing the yellow colour of those extracts.

In various cases of purchased butcher's meat a positive Knoop's test has been obtained. If the meat is allowed to become just noticeably putrid a very marked reaction is given. Histamine may in part be responsible for the positive test in such cases.

The test may perhaps be used to most advantage as an indirect test for carnosine where the presence of that substance is doubtful in any tissue or fluid. If a protein-free extract is at first negative to Knoop's test, and after hydrolysis (*v.* later) is positive to the test, it appears necessarily to follow that the unhydrolysed liquid contains carnosine.

The test can also be applied to elucidate some other problems that had arisen in the course of this work. The effect of heat on carnosine solutions will first be considered.

The effect of heat on solutions of carnosine.

In the writer's preliminary communication on the estimation of carnosine, a slight fall was noted in the colour values of solutions of carnosine heated on the water-bath for one hour. Similar experiments have since been repeated but the period of heating has been extended to four hours. Thus a number of solutions of carnosine nitrate with an original colour value of 22 mm. per cc. showed after four hours on a boiling water-bath an average value of 19·4 mm. per cc. or a fall of 2·6 mm. per cc.

10 cc. of a 0·5 % solution of carnosine as nitrate was brought to dryness three times in an open basin on a steam-bath. The total period of heating was about four hours. It was finally taken up in about 2 cc. of water and Knoop's test applied. A decided brown colour was developed indicating that in the process the carnosine had been partly hydrolysed, giving rise to histidine. This observation confirms the fall in colour value.

Though four hours is a relatively long period of heating, the fact that carnosine is thus destroyed in pure neutral solution indicates that care should be exercised in the process of extraction from muscle.

The fall in colour value as estimated in carnosine from 0·639 % to 0·614 %

on prolonged extraction of the muscle, previously noted (p. 646), may be accounted for by the destruction of carnosine. The occurrence of histidine in muscle extracts will thus arise more likely from the hydrolysis of carnosine than from the hydrolysis of histidine-containing proteins. For if the latter process occurred to a marked degree the colour values would tend to rise rather than to fall.

It will thus be observed that any process of evaporation at atmospheric pressure must result in at least a partial destruction of the substance.

Solutions of carnosine whether pure or in muscle extracts may be evaporated *in vacuo* to dryness with very little destruction. Thus 10 cc. of a neutral dilute meat extract were evaporated to dryness at 20 mm. pressure of mercury from a 200 cc. distilling flask on a water-bath about 60°. Exactly 10 cc. of water were then pipetted into the flask and rinsed round to dissolve the dried residue. The colour value of the original solution was 25·6 mm. per cc. whilst that of the dried substance taken up in 10 cc. of water was 25 mm. per cc. (The volume of solid in the original 10 cc. was regarded as negligible.) Repetition of this experiment still showed a loss in colour value of about 2 %.

This experiment at the same time goes to show that a very small proportion, if any, of the colour-producing substances in muscle extract is volatile. It serves to confirm the results obtained by ether extraction and Millon's test.

(5) *Other substances.* The effects of cystine, leucine and arginine on the diazo reagent have been measured by Hanke and Koessler [1920, 1]. These workers find that there is no interference so long as the ratio of cystine to histidine does not exceed 6 : 1, which is higher, they note, than has yet been found in any protein. Cystine may thus be dismissed as a probable interfering factor in muscle extracts.

The effects of arginine and leucine are less marked than those of cystine.

Creatine in large amounts gives a slight yellowness to the reagent.

Creatinine, urea and lactic acid have been found to have no effect on the reagent.

Such a process of elimination as has here been attempted must yield mainly negative conclusions. None of the substances most likely to interfere with the colour values has any very marked effect. In the case of ox muscle it has been shown that most probably less than 5 % of the colour value is not due to carnosine. In cat and frog extracts, judging merely from the colour development, the non-carnosine colour value is likely to lie within the same limit, but of salmon little can be said except that it contains carnosine. Any elimination method must necessarily be unsatisfactory unless the substance can be obtained pure; and many factors militate against that possibility in the case of carnosine. Among those may be noted the high solubility of the substance in water, its facility for adsorption, the inadequacy of the methods for its fractional precipitation and the ease with which it is hydrolysed. It is upon this last property that the writer has sought a more direct method for confirming the values.

II. Hydrolysis Experiments.

(1) *The stability of histidine.* Histidine has been observed by Koessler and Hanke [1919, 2] to be remarkably stable towards hot concentrated hydrochloric acid. They observed that when 2 cc. of a 1 % solution of histidine dihydrochloride were heated on a boiling water-bath for 10 hours with 25 cc. concentrated hydrochloric acid the histidine was quite unchanged, as shown by the colour values recovered. Over a shorter interval the writer has confirmed those findings as shown by the following experiment.

Into each of five numbered test tubes there was introduced 1 cc. of a solution of histidine along with 3 cc. concentrated hydrochloric acid. These were then set on a boiling water-bath and one removed every 15 minutes. As each test tube was removed from the water-bath it was cooled, about 1 sq. mm. of litmus paper introduced, and the contents neutralised with sodium hydroxide. The contents were again cooled and poured into a 25 cc. flask. The test tube was repeatedly washed into the measuring flask, then water was added up to the mark. 1 cc. of this was taken for measuring the colour value against the histidine colour standard. The original value of 23 mm. was obtained by directly diluting 1 cc. of the original to 25 cc. and taking 1 cc. for the test. The colour values after periods of boiling lasting 15, 30, 45, 60 and 75 minutes were found respectively to be 23·2, 23·1, 23, 22·9 and 23 mm. High concentrations of sodium chloride affect neither the tint nor the intensity of the colour developed.

(2) *Hydrolysis of carnosine—theoretical considerations.* It thus seemed very probable that if carnosine were hydrolysed with hydrochloric acid, the colour value would continue to fall until the reaction was complete. If the diazo value of the solution were measured before hydrolysis and again after hydrolysis, the ratio of the colour values thus obtained should be that of their molecular colour values. The molecular colour value of histidine was previously found to be 117 million mm. as compared with 114 million mm. determined by Koessler and Hanke. A revised determination of the molecular colour value of carnosine has led the writer—mainly from increased skill in matching the histidine and carnosine colour standards—to the conclusion that the molecular colour value of carnosine is somewhat greater than the 152 million mm. previously published, viz. 161 million mm. This value is checked by the following considerations.

The histidine colour standard is a mixture of 0·22 cc. methyl-orange with 0·20 cc. Congo red in 100 cc. water. For convenience call this S_H.

The carnosine colour standard is a mixture of 0·10 cc. methyl-orange with 0·25 cc. Congo red in 100 cc. water. Call this S_C.

These amounts were taken from 0·1 % stock methyl-orange and from 0·5 % stock Congo red [*v.* Hunter, 1921].

On comparing in the colorimeter it is found that 24 mm. S_C = 28·5 mm. S_H.

From the table previously published [Hunter, 1921] 0·040 mg. of carnosine in the test cylinder set at 20 mm. showed a reading of 24 mm. S_C.

0·04 mg. of carnosine should thus give a reading of 28·5 mm. S_H.

The molecular colour value of carnosine is thus

$$\frac{28{\cdot}5}{4} \times 226 \times 10^5,$$

or approximately 161 million mm. S_H.

Now, carnosine yields by theory on hydrolysis 68·5 % of histidine, *i.e.* 0·04 mg. of carnosine yields on hydrolysis 0·0274 mg. of histidine.

0·04 mg. of histidine monohydrochloride gives a reading of 22·4 mm. S_H.

[N.B. The molecular colour value of histidine is thus

$$\frac{22{\cdot}4}{4} \times 209 \times 10^5,$$

i.e. approximately 117 million mm. S_H.]

Histidine monohydrochloride contains 74 % histidine, *i.e.* 0·04 mg. of histidine monohydrochloride contains 0·0296 mg. of histidine.

0·01 mg. of histidine will thus give by calculation a reading of $\frac{22{\cdot}4}{2{\cdot}96}$ or 7·57 mm. S_H, and

0·0274 mg. of histidine will give 20·7 mm. S_H.

That is, a solution of carnosine giving an original reading of 28·5 mm. S_H will give after hydrolysis a reading of 20·7 mm. S_H.

The ratio of the final reading to the original reading should be the same as the ratio of the molecular colour value of histidine to the molecular colour value of carnosine.

Thus $$\frac{20{\cdot}7}{28{\cdot}5} = \frac{117}{161} = 0{\cdot}73.$$

For convenience this figure will be termed the *hydrolysis quotient.*

(3) *Experimental.* The results obtained from the actual hydrolysis of carnosine solutions are less satisfactory than one might expect from the above considerations. Thus a series of carnosine solutions were treated in exactly the same way as that described for histidine. 0·1 % solution of carnosine was used. The original value of 1 cc. from 25 cc. dilution was 28·5 mm. S_H. After hydrolysis for periods of 15, 30, 45, 60 and 75 minutes 1 cc. showed the respective colour values 20·4, 19, 18·2, 18·1 and 18 mm. S_H. After 15 minutes' hydrolysis the colour was more yellow than S_H.

Carnosine is thus very readily hydrolysed and under the above conditions part of the histidine is destroyed. After the initial sudden fall from 28·5 mm. to 20·4 mm. continued boiling lowers the value at a much diminished rate. After 30 minutes' boiling the values remain almost constant but the colours are too yellow—indicating some destruction. It would appear that in the course of the hydrolysis the histidine passes through an unstable phase in which the iminazole ring is readily disrupted. The histidine that emerges from that hypothetical intermediate condition resists further boiling.

Other hydrolytic agents, such as sodium hydroxide and acetic acid, besides

different conditions of temperature and concentration were tested, in the attempt to overcome the difficulty. Finally an approximately constant-boiling mixture of hydrochloric acid was used and the hydrolysis conducted at 90°. Under these conditions a strictly quantitative conversion is not yet attained, but a comparison of the results from various extracts under standard conditions would appear to be of some weight. The extracts hydrolysed were of such a concentration that 1 cc. of the final dilution gave approximately the same values. To the amount of extract to be hydrolysed exactly half its volume of concentrated hydrochloric acid was added. The following are the results from a solution of pure carnosine and from muscle extracts of cat, of ox, and of salmon. This ox extract was slightly positive to Knoop's test. All the readings were taken with the histidine standard.

Time mins.	Carnosine mm.	Cat muscle extract mm.	Ox muscle extract mm.	Salmon muscle extract mm.
Original	28·5	29·0	28·5	27·0
15	25·4	24·3	26·0	26·5
30	23·3	23·0	23·5	24·0
45	21·5	21·8	22·7	22·5
60	21·0	21·2	22·0	22·1
75	20·9	20·7	21·7	21·4
Hydrolysis quotients after 75 mins.	0·73	0·71	0·76	0·79

The carnosine solution and the cat muscle extract agree very well in both their rate of fall and in their hydrolysis quotients. The ox muscle quotient is slightly high, probably owing to the presence of either histidine or histamine in the original. The salmon extract quotient is still higher, but it is remarkable in face of the very yellow azo colour given by the original that the rate of fall accords so well with the others.

Although this method of hydrolysis is not sufficiently sensitive to show that a definite percentage of the colour is due to carnosine, it at least gives one a sense of assurance in the use of a very unspecific reagent for the estimation of carnosine in such a complex solution as muscle extract. It would further seem to indicate that carnosine is responsible for a very high percentage of the colour as measured from fresh muscle extracts treated only with lead acetate.

The specific cause of the yellow colour produced in such cases as salmon has not been found. In the case of rabbit muscle, it has been observed that the colour is more red when the carnosine content of the muscle is high and yellow when low. Some cat and frog muscles with a low carnosine content also showed a yellow colour. This is not surprising as the chances of interference are greatly increased when the test portions are less dilute.

Though purines certainly give rise to a small error and exact quantitative results cannot be got when the colour is not of the right tint, yet the total error in the lead acetate filtrate is too small to eliminate. With a better hydrolytic agent it might yet be possible to evaluate the error.

Carnosine Content of some Muscles.

With the method of extraction and treatment of the extract as previously described, the following results have been obtained. Each result represents the percentage colour value reckoned as carnosine in the fresh skeletal muscle. Further, each result represents the average of at least two results obtained from different pieces of the same muscle.

For convenience the amounts found are given from lowest to highest carnosine contents. The contents for four members of each species of animal are given.

Rabbit muscles	0·026	0·064	0·090	0·101 % carnosine	
Frog ,,	0·107	0·128	0·142	0·280 ,,	,,
Cat ,,	0·123	0·203	0·336	0·380 ,,	,,
Ox ,,	0·340	0·400	0·515	0·640 ,,	,,

The results show that the carnosine content of muscle varies not only with the species of animal but varies greatly in different animals of the same species. The highest values obtained in the one species are two to four times greater than the lowest values in the same species. This finding is at variance with that of Clifford [1921, 2]. This worker finds, for example, ox muscle to have an almost constant carnosine content of 1·1 %. But apart from the constancy of the results found, the writer is compelled to question their accuracy. In a previous paper [1921, 1] Clifford finds that 0·02 mg. of histidine gives a value of 10·75 mm. measured with the Koessler and Hanke histidine standard. With these data—and assuming the author means 0·02 mg. of histidine *dihydrochloride*—the molecular colour value of the histidine is

$$\frac{10{\cdot}75}{2} \times 228 \times 10^5,$$

which is approximately 122 million mm. S_H.

With the same colour standard Clifford records that 0·1 mg. of carnosine gives a reading of 30 mm. The molecular colour value of Clifford's carnosine is thus $30 \times 226 \times 10^4$ or approximately 68 million mm. As previously stated, the writer finds the molecular colour value of carnosine against this same colour standard to be 161 million mm. Assuming this figure represents 100 % pure carnosine, the carnosine employed by Clifford is thus only about 43 % pure. The carnosine content of the various muscles tested by Clifford should from this point of view be about 43 % of the values actually given.

Summary.

1. Extracts to be tested for carnosine should be free from ammonium salts, sulphides, phenols and aldehydes.

2. The degree of yellowness of the azo colours developed from muscle extracts is in the order of the percentages of the intensification of the azo colours as given by sodium hydroxide and hydroxylamine.

3. In ox muscle purines are responsible for about 3 % of the colour value reckoned as carnosine. In the same tissue there is probably about other

2 % of the colour not due to carnosine. The error in the method due to colour-producing substances other than carnosine is probably about 5 % in ordinary muscles.

4. Histidine under certain circumstances may be present in muscle extracts.

5. A test has been found to distinguish histidine from carnosine.

6. A more certain means than the diazo reagent is given for the identification of carnosine in any tissue.

7. The fall in colour value of hydrolysed meat extracts agrees with that of pure solutions of carnosine.

8. The carnosine content of muscle varies with the species of animal and with different members of the same species.

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REFERENCES.

Barger (1914). Simple Natural Bases.
Clifford (1921, 1). *Biochem. J.* **15**, 400.
Clifford (1921, 2). *Biochem. J.* **15**, 725.
Hanke and Koessler (1920, 1). *J. Biol. Chem.* **43**, 527.
—— —— (1920, 2). *J. Biol. Chem.* **43**, 557.
—— —— (1922). *J. Biol. Chem.* **50**, 235.
Hunter (1921). *Biochem. J.* **15**, 689.
Jones (1920). Nucleic Acids.
Knoop (1908). *Beiträge*, **11**, 356.
Koessler and Hanke (1919, 1). *J. Biol. Chem.* **39**, 497.
—— —— (1919, 2). *J. Biol. Chem.* **39**, 521.
Levene (1921). *J. Biol. Chem.* **48**, 177.
Lusk (1921). Science of Nutrition.
Neubauer-Huppert (1913). Analyse des Harns.
Plimmer (1918). Practical Organic and Biochemistry, 293–296.
Thierfelder (1908). Chemische Analyse, 164.
Totani (1915). *Biochem. J.* **9**, 385.