CX. THE CARBAMIDO DIACETYL REACTION: A TEST FOR CITRULLINE

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THE carbamido-acid citrulline, isolated by Koga & Odake in 1914 from the water melon, attracted no general attention until Wada [1930] established its constitution as α -amino- δ -carbamidovaleric acid, and showed [1933, 1] that it also occurred among the products of the tryptic digestion of caseinogen.

The importance of the acid in nitrogen metabolism was revealed when Krebs & Henseleit [1932] demonstrated that citrulline is an intermediate in the mechanism whereby urea is formed in the liver.

These discoveries raise subsidiary questions regarding the distribution of citrulline in natural proteins, the origin and significance of carbamido-acids, and the nature of the mechanism involving the elaboration of citrulline from its precursors, ornithine, ammonia and carbon dioxide, and its subsequent conversion into arginine. An approach to the study of such problems is begun in the present paper by the introduction of a colour test by which citrulline can be detected in proteins, and its formation traced during a reaction cycle.

Colour reactions of urea and substituted ureas

Since citrulline may be regarded as a substituted urea, a survey was made of urea colour tests applied to proteins. The most familiar of these tests depend on condensation between the urea and an aldehyde in strongly acid solution. The reagents employed include (i) furfuraldehyde [Schiff, 1877], (ii) furfuraldehyde and stannous chloride [Nakashima & Maruaka, 1923], (iii) methylfuryl [Fenton, 1903], (iv) *p*-dimethylaminobenzaldehyde [Barrenscheen, 1923].

Although these reagents yield colours with monosubstituted ureas, including citrulline, they are unsatisfactory as tests with most proteins, owing to the presence of tryptophan, which combines with the aldehyde to form pigments that are intensely coloured and completely obscure any reaction given by the citrulline.

A study of the colour reactions between ketones and substituted ureas gave more promising results, for it was observed that diacetyl in strongly acid solution yielded products which, on careful oxidation, were converted into orange or red pigments. Under these conditions tryptophan gave no colour nor did the substituted guanidines, the latter observation indicating that the reactions of diacetyl in acid solution display a selectivity that differs from the reactions in alkaline solution, such as the familiar tests for arginine and creatine, discovered by Harden.

The scope and mechanism of the reactions of diacetyl in alkaline solutions have been investigated by Harden & Norris [1911], O'Meara [1931], and Lang [1932]. O'Meara (personal communication) has observed that diacetyl yields a yellow colour with urea in presence of conc. H_2SO_4 , and Smith [1935] has described a similar series of colour reactions between diacetyl and many azides,

including semicarbazide. To avoid confusion, it is proposed to classify these various types of test as follows:

(1) *Carbamido diacetyl reactions*, which occur in acid solution, and are given by compounds containing the system $R.NH.CO.NH_2$, or, in certain instances, $R_1.NH.CO.NH.R_2$.

(2) *Guanidino diacetyl reactions*, which occur in alkaline solution, and are given by compounds containing the system $R.NH.C(:NH).NH_2$, or

While seeking a stable form of the diketone for use as a class reagent, both the monoxime and the dioxime were examined. The dioxime, as might be expected from its greater stability, was unsatisfactory, but the monoxime proved to be superior in range and sensitivity to ordinary diacetyl. For example, symmetrical dimethylurea yields a deep purple colour with the monoxime reagent, but very little colour with diacetyl itself. In applying the test to substituted ureas, the solution is strongly acidified with HCl, a few drops of 3% diacetyl monoxime are added, and the mixture is boiled for 3–4 min., during which time an orange colour develops if the test be positive. The colour deepens to red on standing or after addition of a drop of 1% potassium persulphate.

The scope of the carbamido diacetyl reaction

Using diacetyl monoxime, followed by an oxidizer, the test is positive with: urea, methylurea, butylurea, phenylurea, β -naphthylurea, dimethylurea (symmetrical and asymmetrical), allantoin, semicarbazide, citrulline and all higher proteins examined (ovalbumin, serum albumin, seroglobulin, caseinogen, lactalbumin, fibrin, edestin, gluten, mucin). Commercial peptones and gelatins also give the reaction with varying degrees of intensity.

The test is negative with: ammonium salts, hydrazine, carbamate, cyanate, acetamide, acetylurea, diphenylurea (asymmetric), guanidine, methylguanidine, creatine, creatinine, glycoeyamine, uroxamic acid, uric acid, indole and all amino-acids examined, other than citrulline (glycine, glycine ester, sarcosine, alanine, cystine, tyrosine, tryptophan, arginine, histidine, lysine, proline, hydroxyproline, asparagine, aspartic acid).

Reaction with urea. Urea is the only compound so far observed which yields a bright yellow colour, deepening to orange on oxidation. The test is very sensitive, and will reveal 0.1 mg. of urea in 2 ml. of solution, and may be used to show the presence of urea as a contaminant in the monosubstituted ureas, which give a red colour with the reagent. With semicarbazide, the reaction is exceptional in that pigment formation proceeds rapidly in the absence of an external oxidizer, and the compound gradually separates out as a dark red precipitate. Conversely, semicarbazide provides a useful reagent for the detection of diacetyl in aqueous extracts of butter and similar materials.

Reaction with citrulline. The test will give a well-marked red colour with citrulline in concentrations of $M/100$ (0.175%), and will detect the carbamido-acid in concentrations as low as 0.01%. The colour changes from madeira to garnet red during oxidation and closely matches the colour given by a tryptic digest of caseinogen, but is redder than the colour given by unhydrolysed caseinogen. It can be matched within ordinary limits by the colour given by pure methylurea or butylurea, uncontaminated by ordinary urea.

The carbamido diacetyl reaction with proteins

About 2 ml. of the protein solution are treated with at least twice as much conc. HCl and 3–5 drops of 3% aqueous diacetyl monoxime. The mixture is boiled for 30 sec., allowed to cool for 2 min., and then carefully oxidized by addition of 1–3 drops of dilute (1%) potassium persulphate or very dilute (0.01%) hydrogen peroxide. A carmine colour develops if citrulline or a similar carbamido-compound be present. The colour deepens on standing or on warming gently. A slight excess of the oxidizer rapidly bleaches the pigment in hot solution.

Notes on the test. (1) H_2SO_4 , H_3PO_4 or trichloroacetic acid may be used as condensing agents instead of HCl, but appear to offer no advantages. By adding conc. H_2SO_4 to a mixture of protein and diacetyl monoxime the reaction may be obtained as a "ring" test. In this form it is slow, and several hours are required for colour development at room temperature.

(2) The quantity of acid used should be sufficient to redissolve any protein precipitated by the first addition of acid, previous to heating the mixture.

(3) For maximum colour development, oxidation must be carefully controlled. While almost any of the commoner oxidizing agents may be used, persulphate appears to be the least destructive to the formed pigment. The reaction is inhibited by copper salts, and for this reason is not given by copper citrullinate.

(4) The time of boiling must be curtailed when dealing with proteins rich in carbohydrate residues but relatively poor in citrulline, such as the albumins, or protein preparations containing carbohydrate, such as dried milk powder. When boiled with excess of acid, furfuraldehydes are liberated from the carbohydrate and react with tryptophan in the protein to form violet pigments (Liebermann-Cole reaction). The effect of this accompanying reaction is to change the tint of the citrulline colour from red to purple. Furfuraldehyde pigment formation can be suppressed by avoiding prolonged boiling and the use of excess of protein. Where there is uncertainty, a control test should be carried out in which the diacetyl reagent is omitted. After oxidation, the control test may develop a lilac colour, but neither the intensity nor the shade is comparable with the citrulline colour. When dealing with materials free from tryptophan, such as gelatin, boiling may be continued for several minutes, and the colour allowed to develop spontaneously by atmospheric oxidation. Samples of crude caseinogen prepared from sour milk may contain diacetyl or its precursor, acetylmethylcarbinol (acetoin) in quantities sufficient to give a distinct carbamido-test in the absence of the reagent.

(5) Caseinogen and egg albumin in 1% solutions give a bright purple-red colour with the reagent. Gelatin in 1% solution gives a very feeble reaction unless boiling be continued for at least 5 min. As none of these colours exactly matched the colour given by free citrulline, an endeavour was made to determine approximately the citrulline content of caseinogen by applying the test to a 10% solution that had been subjected to prolonged tryptic digestion. After treatment with charcoal, 1 ml. samples of the digest were submitted to the carbamido-test and the colours compared with that given by a standard ($M/100$) solution of citrulline. Good comparisons were obtained, the colorimetric readings indicating a citrulline value for the caseinogen of the order of 2.0–2.5%.

The significance of the carbamido diacetyl reaction with proteins

Citrulline is readily obtained from arginine by alkaline hydrolysis or by bacterial degradation [Ackermann, 1931; Horn, 1933], and its appearance in tryptic digests does not necessarily indicate that it is a preformed component of the original protein molecule, although Wada has claimed that citrulline may be isolated from hydrolysed caseinogen under conditions that do not lead to the conversion of arginine into the carbamido-acid. The present test, it is believed,

affords direct evidence for the existence of citrulline in natural proteins; nevertheless, several possible sources of error must be considered, especially when dealing with proteins hydrolysed in alkaline solution. Some preparations of trypsin, notably commercial pancreatic extracts and autolysed pancreas, are very rich in material giving a positive carbamido-reaction indistinguishable from citrulline, and thus may contribute to the citrulline value of a digest.

Free arginine is readily hydrolysed in alkaline solution (e.g. $N/2$ NaOH) at room temperature, and after a few days yields a gradually intensifying series of carbamido-reactions, and the citrulline value of an apparently sterile tryptic protein digest may increase considerably with time. Lastly, carbamido-acids other than citrulline may be present in the original protein or may arise during hydrolysis. Prolysine, discovered by Wada [1933, 2], is rapidly decomposed by weak alkalis with production of α -amino- α' -carbamidopimelic acid, which gives a positive carbamido-reaction.

Indeed, the instability of prolysine in presence of alkalis is such that one is led to question Wada's formula, and regard the compound as containing an ester linkage rather than the conventional ureide structure.

Prolysine, however, is much more stable in acid solutions, and is unlikely to cause a fallacy when the carbamido-test is applied to intact proteins.

A note on the hydrolysis of arginine

The citrulline required for the present work was prepared from arginine by alkaline hydrolysis, according to the method of Fox [1938]. The arginine used was the purest carbonate obtainable from Messrs Schuchardt, and gave no reaction with the carbamido-test. The citrulline was isolated as the copper salt, and was subsequently recrystallized twice. The crystalline carbamido-acid began to melt at 218° and was completely melted at 223° (uncorrected). During trial preparations of citrulline some observations were made on the course of arginine hydrolysis in $0.1\text{ }N$ and $5\text{ }N$ NaOH. The mixtures were boiled under a reflux condenser fitted to an aspirator and acid trap to retain the liberated ammonia. Within the first 15 min. citrulline could be detected by means of the carbamido-test in samples from the mixture. Urea accompanied the citrulline, as was suggested by the colour of the carbamido-reaction, which was yellower in shade than that given by pure citrulline, and the presence of urea was confirmed by the urease test. After 4 hr. hydrolysis in presence of $5\text{ }N$ NaOH, the mixture contained in addition cyanate, derived no doubt from the decomposition of urea, and also tetramethylenediamine, derived from the ornithine.

Since urea is not formed from citrulline under these conditions of hydrolysis, it must arise from the arginine directly, the decomposition of which can be represented as following two independent paths.

Reaction A is catalysed by the arginine desimidase, described by Horn [1933]. Reaction B is catalysed by arginase. During alkaline hydrolysis both reactions occur, but the conditions that determine their relative extents have not yet been elucidated; increase in the concentration of alkali promotes reaction A. For simplicity, the compounds are not represented as zwitterions or as resonance hybrids in the above formulation.

Chemistry of the carbamido-diacetyl reaction

From inspection of the formulae of the reactants, it appears that the test is positive with compounds containing the system $R_1 \cdot \text{NH} \cdot \text{CO} \cdot \text{NH} \cdot R_2$, where R_1 is either hydrogen or a simple aliphatic radicle, and R_2 is not an acyl radicle. Thus, phenylurea and symmetrical dimethylurea both form pigments, whereas acetylurea and symmetrical diphenylurea do not. In the absence of an oxidizing agent, diacetyl unites with substituted ureas in acid solution to form cyclic derivatives or diureins investigated by Biltz [1907]. These compounds are colourless and do not yield pigments on oxidation. Their formation involves both ketone groups of the diacetyl. It is suggested that the pigments obtained in the carbamido-reaction are derivatives of a pyrimidine ring, the closure of which is brought about by oxidative condensation involving one methyl group of the diacetyl. The compound obtained from diacetyl and semicarbazide has already been obtained by Thiele [1898], and shown to be a triazine derivative.

SUMMARY

A colour test is described for citrulline and other substituted ureas. The test is capable of being applied directly to proteins, and affords evidence that citrulline exists preformed in caseinogen, egg albumin, and other natural proteins.

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