XXXVIII. ON THE DIAZO REACTIONS OF HISTIDINE AND TYROSINE.

By GINSABURO TOTANI.

From the Biochemical Laboratory, Cambridge.

(Received August 19th, 1915.)

The test for histidine based on its property of forming a diazo compound, first described by H. Pauly [1904], is one of the most important reactions in the investigation of protein decomposition products. This diazo reaction can be carried out without any modification whether histidine is in the free state or bound up in a protein molecule. Its importance is, however, lessened considerably as it is not quite specific, since tyrosine, too, is capable of coupling with diazobenzenesulphonic acid in alkaline solution and yields a colouration so very similar to that given by histidine, that the differentiation of the two amino acids from each other by means of this reaction is a matter of extreme difficulty. On addition of diazobenzenesulphonic acid in sodium carbonate solution, histidine gives a dark cherry red colouration which, even on dilution with much water, retains its red tone and does not change into yellow: in acid solution it gives a pure orange colouration. As regards tyrosine, the colour in alkaline solution is like that of histidine, but of a less deep red, and, moreover, on dilution, a yellow colour appears, yielding finally an impure yellowish red colour.

Though Pauly was able to describe the difference of the diazo colours produced by histidine and tyrosine, as above, he thought it necessary to mention further, that it is sometimes not easy to distinguish the colours from each other.

Applying the diazo reaction as a test for methyltyrosine, E. Friedmann and S. Gutmann [1910] tried to distinguish the colouration given by this substance from that of histidine by taking into consideration not only the

colours of the solutions themselves, but also the colours with which these solutions dye filter paper. The histidine compound gives either a pure orange or a lemon yellow colour on paper, depending on the reaction of the solution being alkaline or acid respectively. The methyltyrosine compound stains paper red brown in alkaline, yellow brown in acid solution. Even using this method of differentiation for tyrosine, it is still scarcely possible to distinguish it from histidine by the diazo reaction.

Since, according to Pauly, among the protein cleavage products, histidine and tyrosine alone give the diazo reaction, it is quite evident that the reaction will be more useful in its application, if it be made specific for only one of these two amino acids.

Lately K. Inouye [1912] has succeeded with the following useful modification. He noticed that when tyrosine and histidine are submitted to the Schotten-Baumann reaction, the former is converted into its dibenzoyl derivative and loses its property of coupling with diazotised sulphanilic acid, while the latter, though forming its monobenzoyl compound, still maintains unaltered its ability of yielding a red colouration in the presence of the diazonium acid. He therefore treated the solution to be tested by means of the diazo reaction with benzoyl chloride and sodium carbonate, in order to prevent the tyrosine from combining with the diazo acid, and thus made the diazo reaction specific for histidine.

According to a recent paper by A. Kossel and S. Edlbacher [1915], when histidine loses its free carboxyl group, as, for instance, in its methyl ester, the imidazole ring to which the diazo reaction owes its appearance is readily opened on benzoylation with formation of a tribenzoyl-triamino-acid.

As regards histidine in a protein molecule, where it is to be found in a peptide linking united through its carboxyl group with other amino acids, it is most probable that by treatment with Schotten-Baumann's reagent an analogous transformation of histidine will take place, just as in the case of its methyl ester, resulting in the disappearance of the property of giving the diazo reaction.

In order to apply Inouye's modification of the histidine test to protein it is therefore necessary, before performing the test, to hydrolyse the protein by means of acid or trypsin, so as to obtain the histidine in the free state.

Recently, in applying some new methods of investigation to protein decomposition products, I met with some difficulty in distinguishing these two amino acids by means of the diazo reaction, since, owing to the special circumstances, neither Inouye's test for histidine, nor Millon's reaction could

be successfully used. At any rate it seemed to me of interest to discover a method of differentiating the diazo reaction as applied to tyrosine and histidine without recourse to benzylation.

I find a ready distinction may be made by reducing the coloured solutions obtained by Pauly's reaction from these two amino acids. Although the two solutions obtained with diazobenzenesulphonic acid in sodium carbonate solution are at first almost identical in colour, yet on acidifying with hydrochloric acid and reducing with zinc dust, and then adding excess of ammonia, the resulting fluids are quite different in appearance.

The test is applied as follows: a few cubic centimetres of the solution to be tested are placed in a beaker and made alkaline with excess of sodium carbonate solution. A few centigrams of the freshly prepared diazobenzenesulphonic acid are dissolved in a few cubic centimetres of sodium carbonate solution. This solution must always be freshly prepared just before performing the test, as if it be allowed to stand longer than a quarter of an hour, the result of the reaction is sometimes dubious. The use of too large an excess of the reagent should be avoided, otherwise the solution remains slightly yellowish in colour after reduction and the test is occasionally rendered less distinct.

The reagent is now added to the amino acid solution in the beaker. Immediately, or not later than three minutes afterwards, the fluid assumes a dark red colouration if histidine or tyrosine be present. I will refer to this well-known diazo colouration as the primary colouration for the sake of distinguishing it from the colouration produced after reduction. As I have mentioned above, it is extremely difficult to judge by means of this primary colouration, whether the result is due to histidine or tyrosine.

To the coloured solution concentrated hydrochloric acid is added to make it distinctly acid, and this, in each case, changes the colour to a rather yellow shade of orange. The acid solution is reduced by zinc dust for a quarter of an hour or longer, a colourless solution always resulting. A few cubic centimetres of the reduced solution are now transferred to a test-tube by decantation, or, if necessary, by filtering, and then made strongly alkaline by addition of ammonia of about 25 % strength to the extent of approximately twice the bulk of the solution. Of course, the amount of ammonia necessary depends on the quantity of hydrochloric acid and of zinc used, but the above quantities will generally be found sufficient.

If the primary diazo reaction be due to histidine, the fluid yields immediately, or within a few minutes, a very characteristic golden yellow

colouration of great intensity which is permanent for a long time and is unaltered even on boiling. By the action of hydrogen peroxide on this golden yellow solution, the shade is turned into a beautiful lemon yellow.

The colouration produced by tyrosine in the above reaction is yellowish brown with a small quantity of ammonia, and on gradual addition of ammonia to the amount mentioned above is readily changed to a beautiful bright rose red colouration. This colour, too, is unaltered on boiling, but when left for a while becomes markedly yellow in tint and gradually changes to a reddish brown, which, however, may be reconverted into the former colour by further addition of ammonia or by shaking. Unlike that of histidine, the rose red colouration of tyrosine is remarkably unstable to the action of hydrogen peroxide, so that after oxidation by this substance, the solution will be colourless or, at most, of a faint impure rust colour.

The colouration obtained by the addition of ammonia after reduction of the primary coloured compounds will be called the secondary colouration. The intensity of the colour in the secondary reaction is always proportional to that of the primary colouration.

Converting thus the closely resembling primary colourations of the histidine and tyrosine diazonium compounds into the secondary, which differ so markedly in appearance—the one being golden yellow and the other rose red—one can now very readily distinguish the two amino acids by the diazo reaction followed by the reaction giving rise to the secondary colourations.

As regards histidine, the primary diazo reaction can be applied to a solution as dilute as one part of the substance to 100,000 parts of water; the secondary colouration, too, is obtained as a pale yellow even at this dilution. The peculiarly characteristic deep yellow, however, can be obtained only with a solution stronger than 1:20,000.

Glycerol, alanine, cystine, taurine, α -aminobutyric acid, valine, arginine, leucine, lysine, phenylalanine, tyrosine, tryptophane, histidine, proline, aspartic acid, glutamic acid and hippuric acid have been submitted to the test. Serine and oxyproline were not at my disposal.

Not one of these amino acids except histidine was able to produce a golden yellow colouration in the secondary reaction. It appears, therefore, that, at present, this secondary golden yellow colouration may be considered as being given by histidine alone among the protein decomposition products.

The secondary diazo reaction of tyrosine with production of a rose red colouration is so delicate that it can be carried out in a dilution of 1:10,000. With a dilution of 1:50,000 a faint rose red colouration is obtained. The

rose red colour, however, is not specific for tyrosine. Cystine, which was carefully purified and freed from tyrosine either by precipitating with phosphotungstic acid or by the method of R. H. A. Plimmer [1913] (treatment with alcohol and hydrochloric acid gas), gives the same colouration as tyrosine, though it is much less intense. Alanine and phenylalanine also slowly produce a faint pink colouration, but the solution must be rather strong. The same colour reaction is also given by asparagine with a delicacy of 1:1000. All these colourations are destroyed by oxidation with hydrogen peroxide.

Some organic compounds other than protein cleavage products, for instance, hydroxy-acids such as lactic acid and malic acid, alcohols such as ethyl alcohol, glycol and glycerol, and also acetone, ether and oxamide, behave in a similar manner and yield the pink colouration, with or without the formation of a definite primary colour.

The rose red colouration due to tyrosine in the secondary reaction, therefore, is not specific for this amino acid, but taking into consideration the primary colouration in the diazo reaction which, in the intensity and rapidity of colour formation, is peculiar to tyrosine and histidine, one can be certain that the substance to be tested is really tyrosine when both the first and the second diazo colourations are produced in the particular manner described above. The diazo reaction of tyrosine has sometimes an advantage over Millon's reaction, for the former can be applied with ease, even where the latter is impossible, as for example in the presence of much chloride.

Making use now of the secondary diazo reaction it can be demonstrated clearly that tyrosine and histidine have different affinities for diazobenzenesulphonic acid.

Equimolecular quantities of these two amino acids were mixed together. To one portion of the solution an equimolecular proportion and to another twice this amount of pure diazobenzenesulphonic acid were added in sodium carbonate solution. When the secondary reaction was carried out, the former yielded a golden yellow, and the latter a yellowish brown colouration.

N/100 Histidine in cc.	2	2	2
N/100 Tyrosine in cc.	2	2	2
N/100 Diazobenzenesulphonic acid in cc.	2	4	6
Primary colouration	Brownish yellow	Brown yellow	Yellowish brown
Secondary colouration	Golden yellow	Yellowish brown	Yellowish brown
Colouration after oxidation with hydrogen peroxide	Lemon yellow	Lemon yellow	Lemon yellow

The different colourations produced in this experiment show distinctly that histidine has a greater affinity for diazobenzenesulphonic acid than

has tyrosine, so that it is the first to couple with the diazo acid. If more than sufficient diazo acid be present to combine with the histidine, the tyrosine enters into reaction and this results in the production of a colouration which is a mixture of the shades produced by the two pure amino acids.

According to the assumption of P. Clemens [1899] that the substance capable of forming the most intensely coloured diazo compound has the greatest affinity for the diazo acid, histidine and tyrosine should possess a greater affinity for this reagent than any other amino acids. Furthermore, of the two, histidine must be the one more readily able to combine with the diazo acid, as is proved above.

Hence the golden yellow colouration in the histidine reaction can be obtained even in the presence of many other amino acids without their interfering with the result, provided that only a small quantity of the diazobenzenesulphonic acid just sufficient to combine with the histidine be used. If, however, the diazo acid be added in amount more than equivalent to the quantity of histidine an impure mixed brown or reddish yellow colouration is obtained in the secondary reaction owing to the presence of tyrosine or other amino acids. Hydrogen peroxide may then be added to the solution to obtain the pure lemon yellow, characteristic of histidine, the reddish shade being removed by oxidation.

For the detection of histidine in a protein molecule one of the above mentioned methods can be employed. Although the test works well without the necessity of previously hydrolysing the protein, it is nevertheless better to do so.

Two grams of caseinogen were boiled with 6 cc. of concentrated hydrochloric acid under a reflux condenser for seven hours. The solution, after cooling, was almost neutralised by caustic soda solution and then made alkaline with sodium carbonate solution. It was then diluted with water to 100 cc. The following diazo reactions were carried out with 5 cc. of this solution using N/100 diazobenzenesulphonic acid as reagent.

2 % Hydrolysed caseinogen in cc.	5	5	5	5	5	5
N/100 Diazobenzenesulphonic acid in cc.	1	2	3	4	6	10
Primary colouration	brown	brown	brown	dark brown	dark brown	dark red brown
Secondary colouration	pale yellow	yellow	golden yellow	golden yellow with red- dish tint	brown yellow	brown yellow
Colouration after oxidation with 20 % hydrogen peroxide	pale lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow

This experiment shows that when the diazo reagent is used in small quantity the secondary colouration is yellow owing to the presence of histidine: as the amount of the reagent increases, the formation of the reddish colour commences and this mixes with the yellow to yield a brown. This phenomenon may be explained by the different degrees of affinity of the amino acids for the diazo reagent, as discussed above. If the pure golden yellow end colouration for histidine be masked by being accompanied by the reddish colouration brought about chiefly by tyrosine, the oxidation process by means of hydrogen peroxide may be used in conjunction with the test, in order to get rid of the interfering tints. In this case a pure lemon yellow colouration of strong intensity indicates the presence of histidine.

I propose to endeavour to apply this modified diazo reaction to the quantitative estimation of histidine in the protein molecule.

SUMMARY.

1. It is easy to distinguish between tyrosine and histidine by means of the diazo reaction by making use of the different colourations produced by reducing their similarly coloured diazo compounds with zinc dust in hydrochloric acid solution, subsequently rendering the solution alkaline with excess of ammonia: tyrosine gives a rose red, histidine a golden yellow colouration.

2. The golden yellow colouration produced by histidine is specific for this amino acid among the protein cleavage products. The reaction may be carried out even with a solution of 1:100,000, but the peculiarly characteristic colour comes into appearance only with a solution stronger than 1:20,000.

3. The rose red colour of tyrosine is not specific for this amino acid, as the same colouration may be produced by some other amino acids, but taking into consideration the fact that the original diazo reaction is given only by tyrosine and histidine, it seems possible to detect the presence of tyrosine by means of these colour reactions. The delicacy of the modified tyrosine reaction is 1:10,000. The reaction has sometimes an advantage over that of Millon as in the case when chlorides are present in large amount.

4. The reddish colourations produced by tyrosine or other amino acids in the secondary diazo reaction are readily destroyed by oxidation with

hydrogen peroxide, resulting in the formation of nearly colourless or faintly rust coloured solutions, while the golden yellow colouration of histidine is little altered, changing only to a lemon yellow.

5. Using a small quantity of diazobenzenesulphonic acid sufficient only to couple with the histidine, or applying the process of oxidation by means of hydrogen peroxide in conjunction with the reduction, the presence of histidine in proteins can be easily detected by reason of the characteristic colouration formed in the secondary diazo reaction.

In conclusion, I wish to express my indebtedness to Professor Hopkins for his valuable advice and criticism and to Mr Foreman for his kindness in supplying me with some of the amino acids used in this investigation.

REFERENCES.

- Clemens, P. (1899), *Deutsch. Arch. klin. Med.* **63**, 108.
Friedmann, E., and Gutmann, S. (1910), *Biochem. Zeitsch.* **27**, 491.
Inouye, K. (1912), *Zeitsch. physiol. Chem.* **83**, 79.
Kossel, A., and Edlbacher, S. (1915), *Zeitsch. physiol. Chem.* **93**, 396.
Pauly, H. (1904), *Zeitsch. physiol. Chem.* **42**, 516.
Plimmer, R. H. A. (1913), *Biochem. J.* **7**, 311.