CVI. A TEST FOR THYMINE, WITH OBSERVATIONS ON THE KETO-ENOLIC TYPE OF DIAZO-TEST.

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FOR quantitative colorimetric purposes there is probably no other reagent so sensitive and so well adapted for precise measurements, and at the same time applicable in so many fields as diazotised sulphanilic acid. The present paper contains some observations, now some years old, on certain substances in many respects often widely different but which under similar conditions yield colours with this reagent. The group has the common character that all its members are capable of keto-enolic tautomerism for which reason I venture to speak of a keto-enolic type of diazo-test.

The reagents required for the tests are:

1. The diazo-reagent made according to Koessler and Hanke [1919].
2. 1.1 % sodium carbonate solution.
3. 3.0 *N* sodium hydroxide solution.
4. 20 % hydroxylamine hydrochloride in water.

Pyrimidines and diazo-tests.

The behaviour of pyrimidines with diazo-compounds has been most closely studied by Johnson and Clapp [1908-9]. Uracil, cytosine and especially thymine were found to give colours when mixed with diazobenzenesulphonic acid in presence of excess of sodium hydroxide. No reaction occurred with 3-methylpyrimidines, whence it was suggested that the grouping —CO—NH— must be present in the 2:1- or 2:3-positions of the pyrimidine ring to allow coupling. That coupling does not occur in the 4- or 5-positions was shown by the fact that 4:5-dimethyluracil gives a red colour.

Johnson and Clapp tried without success to isolate the reaction product of thymine and diazobenzenesulphonic acid. The nature of the linkage thus remains obscure although the available evidence favours the view that coupling takes place in position 3, in which case the reaction products would be *N*-azo-compounds. Johnson and Clapp further state that 6-hydroxy- and 6-amino-pyrimidine do not couple; Evans [1893] obtained a red colour with 2-oxy-4:6-dimethylpyrimidine, and Pauly [1904] an intense colour with 4-methyluracil. Steudel [1904] first observed that thymine couples with diazo-compounds in presence of sodium hydroxide.

The writer has confirmed the results of Johnson and Clapp with uracil, cytosine and thymine. It should be noted however that the colours are obtained only with relatively large amounts of the pyrimidines in question. The sensitivity of the tests can be raised slightly by the application of heat to the solutions, but it is unnecessary to emphasise this in view of the much more delicate test for thymine which will now be described.

The new test for thymine.

The test is performed as follows.

To 2.5 ml. of sodium carbonate solution add 1 ml. of the diazo-reagent. At the end of 1 min. add 0.5 ml. of a solution containing about 0.1 mg. thymine. Keep for 5 min., after which period a faint yellow colour is present. Add 1 ml. of 3 *N* NaOH and keep for 1 min. Then add 1 drop of the hydroxylamine solution and mix quickly. An intense red colour rapidly develops. It is stable for several hours.

The high sensitivity of the test is shown by the fact that a marked colour is still obtainable with less than 0.01 mg. thymine present in the test solution.

The test is not given by as much as 0.5 mg. uracil or cytosine, although yellow colours are produced by these in contact with the reagent and sodium carbonate.

It is necessary that coupling take place in sodium carbonate solution, since no colour is obtained at the above dilutions with excess of sodium hydroxide even on the addition of hydroxylamine.

It is difficult to assess the relevance of the present test to previous observations on the behaviour of pyrimidines with diazo-reagents. With Johnson and Clapp's procedure uracil and cytosine give positive tests though less intense than thymine, whilst the test now described quite definitely distinguishes thymine from uracil or cytosine. In the present test uracil and cytosine may couple, but if they do, the product is incapable of undergoing reduction to an intensely coloured complex as in the case of the thymine-coupled product.

The keto-enolic diazo-test.

Hanke and Koessler [1922] showed that if (in place of thymine in the test described above) 0.25 mg. acetone, acetaldehyde or acetoacetic acid were added, a purplish colour developed after the addition of hydroxylamine. The coupling was supposed to take place with the keto-form of these substances, the hydroxylamine playing an essential part in the production of the final red colour which was regarded as a hydrazoxime derivative.

The principle of the Hanke and Koessler test derives from the work of Ehrlich [1882], Penzoldt [1883, 1, 2] and Petri [1883] who independently found that under certain conditions the urine of diabetic patients gave a marked diazo-reaction which was traced to the presence of glucose.

According to Penzoldt 2 ml. of glucose solution (containing more than 0.1 %) were made strongly alkaline with KOH, and 2 ml. of 1.67 % diazobenzene-sulphonic acid, previously made slightly alkaline, added. In presence of a large amount of sugar a purplish colour developed after 15 min., but with small amounts of glucose only after an hour. A slight excess of nitrous acid did not interfere.

Penzoldt and Fischer [1883] showed that the test is a general one for aldehydes. Easily oxidisable aldehydes behaved like glucose, but aromatic aldehydes reacted only in presence of sodium amalgam, by the use of which a much greater colour was also obtained from glucose and acetaldehyde.

Positive tests were obtained by Penzoldt and Fischer with acetaldehyde, acetone, ethyl acetoacetate, valeraldehyde, furfuraldehyde, glyoxal and benzaldehyde. The same test was also given by phenol, resorcinol and pyrocatechol, if care were taken to bring them in contact with the diazo-compound in presence of excess of alkali so that the known dyes are not formed.

Petri [1883; 1883-84; 1884] added to 5 ml. of urine 2 ml. of 30 % KOH and 5 ml. of a diazo-reagent, made with approximately the theoretical amount of

potassium nitrite. He found that colour development was hastened by warming and was given only with fixed alkalis. Reduction with zinc in the absence of oxygen could replace reduction with sodium amalgam but over-reduction with zinc produced a colourless solution, which on exposure to oxygen rapidly became coloured again. Petri later found that peptones and proteins by the use of zinc give the same fuchsin-red colour as is obtained from aldehydes.

It appears probable that the glucose test is not given by glucose as such, but by substances of the type of acetaldehyde produced by relatively long contact with the strong alkali, although it is possible that enolisation [Nef, 1910] may cause the reaction to be given by glucose itself.

It thus appears that the tests described by Penzoldt and Fischer and by Petri for aldehydes are fundamentally the same as that described by Hanke and Koessler for acetone *etc.*, and that the hydroxylamine of the last workers replaces sodium amalgam or zinc as the reducing agent which is necessary for all the tests, except when, as with acetaldehyde, the substance itself has strong reducing properties.

The writer has confirmed the main findings of Penzoldt and of Petri, as well as those of Hanke and Koessler. It appears that the best conditions for the test with such a substance as acetone lie between those adopted by Penzoldt and by Hanke and Koessler. It is true that coupling does take place, especially with the aid of heat, in the presence of a large excess of strong alkali; but it is at best very slow and incomplete presumably owing to the fact that there is little active coupling diazo-compound present under such conditions. On the other hand in a dilute sodium carbonate solution of acetone there is probably very little of the enolic tautomeride, which according to Dimroth and Hartmann [1908] is the only form capable of coupling. The greater colour produced by acetone on prolonging the time of coupling in presence of dilute sodium carbonate, as found by Hanke and Koessler, or by the use of 10% sodium carbonate instead of 1.1% and keeping the coupling time 5 min. as found by the writer, is readily explicable on this hypothesis. A high concentration of weak alkali thus provides the best conditions for coupling as it does not greatly diminish the active diazo-compound and at the same time tends to increase the proportion of enolic form present.

The great variations in the extent of coupling of different substances of this type under such specific conditions as those adopted by Hanke and Koessler is also in accordance with the above explanation, the proportion of enolic form present under definite conditions being very variable for different substances.

It has further been found that many reducing agents may replace hydroxylamine in the production of the final colour. The addition, instead of hydroxylamine, of zinc, aluminium, tin or a drop of very dilute stannous chloride is quickly followed by the appearance of the red colour. In the case of stannous chloride the colour intensification with acetaldehyde is practically as great as that obtained with hydroxylamine. With the former, however, the colour is unstable. On keeping the test-tube, the colour fades rather rapidly except on the top layer. By shaking the contents it quickly returns, apparently to its full extent. The process may be repeated many times.

It is thus apparent that, although hydroxylamine may have more than a purely reducing function in the Hanke and Koessler test, yet it is not a necessary constituent in the general type of reaction under consideration. The Hanke and Koessler formulation is thus inadequate as an explanation of the mechanism of the reactions concerned.

It is probable that these proceed on the lines indicated by Dimroth and Hartmann [1908] for the coupling of diazonium salts with tribenzoylmethane.

According to this view the enolic compound would first form a yellow *O*-azo-derivative; migration of the arylazo-group to the neighbouring carbon atom would convert the latter compound into a deeply coloured *C*-azo-derivative which is unstable and passes spontaneously into the isomeric hydrazone (colourless).

The above series of reactions would represent the complete course of events in the absence of a reducing agent. The effect of such an agent may be explained by supposing that it converts the hydrazone into a hydrazide which is oxidised in turn by atmospheric oxygen with regeneration of the coloured *C*-azo-compound.

In the absence of direct proof of the existence of the above-mentioned intermediate products this explanation must be regarded as tentative; it appears however adequately to cover the observed facts.

Tyrosine and tyramine.

Hanke and Koessler [1922] discovered that tyrosine and tyramine give in a very beautiful fashion, and indeed quantitatively, the type of diazo-reaction under discussion. *p*-Hydroxyphenylpropionic and *p*-hydroxyphenyllactic acids behave as normal phenols, the presence of NH_2 in the side-chain giving rise to this remarkable difference in behaviour.

The writer has tested *p*-nitrophenol and *p*-aminophenol but with negative results. He has also found that tyrosine gives the Penzoldt aldehyde test but only in the presence of a metallic reducing agent. The use of stannous chloride in place of hydroxylamine hydrochloride in the Hanke and Koessler method gives the characteristic intensification of colour. As with the keto-enolic substances, the colour fades rather rapidly except on the surface layer and is quickly regenerated by shaking. As before the process can be repeated many times.

The parallel behaviour of tyrosine with the keto-enolic tautomerides thus leaves little doubt that the red compound produced in both cases is of the same type, and in the case of tyrosine is explicable by a set of reactions similar to that outlined for acetone. The initial formation of an *O*-azo-compound is in conformity with the conceptions of Chattaway and Hill [1922] of the mechanism of the coupling of phenols, but in the case of tyrosine, through the influence of the NH_2 -group in the side-chain, the migration of the arylazo-group is in some way effected, or the hydrazone structure is the more stable form as in the mixed azo-compounds. No azo-derivative of tyrosine has yet been described.

Thymine.

The test for thymine has already been described. Here again, the final colour behaves with stannous chloride as does that from acetone or tyrosine, and the colour produced by hydroxylamine is very stable.

The fact that uracil and cytosine do not give the test is perhaps no more remarkable than that *p*-hydroxyphenylpropionic acid and similar substances without an amino-group in the side-chain do not behave like tyrosine. The methyl group in the 5-position of thymine has an effect on the reaction apparently analogous to that of the amino-group in the side-chain of tyrosine. In the case of thymine, we must also suppose that the labile hydrogen migrates between a carbon and a nitrogen atom.

Vitamin B₁.

It is mentioned by Kinnersley *et al.* [1935] that their vitamin B₁ gives the keto-enolic type of diazo-test. As noted by these workers their test is given in a weak manner by acetone. It is more marked with acetaldehyde and glyoxal but

negative tests are obtained with tyrosine, thymine and acetoacetic acid. By their particular technique acetaldehyde gives a colour in presence of formaldehyde but none when the latter is replaced by hydroxylamine. The colour obtained from acetaldehyde by the so-called formaldehyde-azo test is less than that by the procedure described here. It is not stated by the Oxford workers if the colours obtained from vitamin B₁ by the two techniques are comparable in intensity.

SUMMARY.

1. A sensitive diazo-test for thymine has been described by which this pyrimidine may be distinguished from cytosine or uracil.

2. The term "keto-enolic type" of diazo-test is suggested to embrace a diversity of diazo-tests previously described as given by such substances as glucose, acetoacetic acid, acetaldehyde, acetone, tyrosine and now thymine, on evidence submitted that the tests all rest fundamentally on the same chemical principles.

3. The essentials for such a test appear to be (a) the capacity for keto-enolic tautomerism in the substance to be coupled; (b) the necessity for a reducing agent in the development of significant colour from the product of coupling.

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