XV. A STUDY OF SOME BIOCHEMICAL COLOUR TESTS. No. I. THE THIOPHENE TEST FOR LACTIC ACID. A COLOUR TEST FOR ALDEHYDES.

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A CHARACTERISTIC of biochemistry is its great heritage of colour tests which have been devised to meet the demand for means of identifying substances of complex or unknown constitution where the more exact methods of pure organic chemistry were of no avail.

Some of these biochemical tests are of a degree of delicacy and specificity almost comparable to biological reactions; but, owing to the difficulties of micro-analysis, the mechanisms of many of these tests are as yet imperfectly understood.

This is not a practical disadvantage as long as the specific nature of the test is assured, but occasionally doubtful cases arise when the knowledge of the rationale of a reaction is essential, and it was from experience of such cases that I was led to investigate some of the well-known colour tests, laying stress on the nature and limiting conditions of the test apart from the chemistry of the substances involved in it.

Hydrocnic Colour Reactions.

Many colour tests are only obtained in the presence of an excess of strong acid, generally sulphuric. In some cases, addition of water after the colour has appeared merely dilutes the tint, but there are certain colour reactions the products of which lose their colour at once if a few drops of water be added.

I have ventured to suggest the term *Hydrocnic*, derived from "Hudōr" (water) and "Okneō" (I shrink), to describe these tests in which the colour is unstable towards water.

These reactions have several points in common, even when they are due to very dissimilar compounds; such as in the Hopkins lactic acid test, Salkowski's cholesterol test, and many of the aldehyde reactions for tryptophan.

A study of *hydrocnic* phenomena calls attention to various important factors capable of modifying or inhibiting colour reactions, as, for example, the local heating due to the addition of the concentrated acid, and the significance of certain impurities in the acids employed.

The Thiophene Test for Lactic Acid.

This test has been selected as it is a typical *hydrocnic* reaction and, though it involves no complex chemical substances, its mechanism has not yet been fully explained.

The test was introduced by Hopkins and was first described in Cole's *Physiological Chemistry* in 1904.

The test is very sensitive to the presence of water in two ways. If the sulphuric acid used be at all dilute or a little water be added with the lactic acid, the solution on warming becomes yellow and does not give the cherry-red colour on the addition of the thiophene. Again, if a few drops of water be added after the red colour has developed it is at once discharged.

In his description of the test, Hopkins [Hopkins and Fletcher, 1907] observes that it is also given by acetaldehyde and by glyoxylic acid.

It is well known that lactic acid when heated with sulphuric acid is decomposed into acetaldehyde and formic acid, or, if an oxidising agent be present, into pyruvic acid and water.

G. Denigès [1909] has employed alcoholic guaiacol to show this formation of aldehyde, and Ryffel [1909] has used it for the estimation of lactic acid in urine.

The results of the action of sulphuric acid in varying proportions on lactic acid were investigated.

Expt I. 20 cc. lactic acid and 20 cc. pure concentrated sulphuric acid, with a crystal of copper sulphate, were mixed. The liquid gradually darkened and gas was evolved. When the reaction had subsided the mixture was distilled under reduced pressure, from a water-bath at 80°.

The distillate was collected in 20 cc. water. It consisted of formaldehyde, acetaldehyde chiefly, formic acid, acetic acid. The gas evolved was CO₂ with some CO.

Expt II. 10 cc. lactic acid and 20 cc. sulphuric acid as before. This time there was a considerable amount of acetaldehyde in the distillate. Not much gas was evolved.

Expt III. 20 cc. moist lactic acid (containing about 10 % water) and 10 cc. sulphuric acid. The distillate contained only a trace of aldehyde but gave a fairly distinct red coloration with ferric chloride.

There was a great evolution of gas (chiefly CO) during the reaction.

The thiophene lactic acid test was then performed on a drop of each of the distillates. I and II gave the cherry-red, III gave a faint orange.

Each of the substances identified in the distillate was then tested separately, when it was found that both formaldehyde and acetaldehyde gave a colour with thiophene in the presence of excess of sulphuric acid.

With regard to the poor production of aldehyde in the last experiment, it was found that acetaldehyde is much less stable in moderately dilute sulphuric acid than in strong acid, consequently it is necessary in performing the lactic acid test to see that there is no appreciable amount of water present at the outset, otherwise the acetaldehyde will be destroyed as it is produced and the thiophene will give no coloration.

It is this decomposition of aldehyde that probably accounts for the 10 % error in Ryffel's method for estimating lactic acid in urine.

A Colour Test for Aldehydes.

Working along the lines of the previous test, the following reaction for aldehydes was devised.

A couple of drops of a 0.2 % alcoholic solution of thiophene are added to 5 cc. concentrated sulphuric acid (which must be free from nitrous and nitric contaminations) and mixed. Then, if a drop of a weak solution of an aldehyde be added, a red colour develops and spreads through the acid. A few drops of water discharge the colour but it returns on the addition of more sulphuric acid.

The test is very delicate with most aldehydes. 1 part CH_2O in 10,000 parts H_2O gives a deep plum-coloured ring; 1 part in 100,000 gives a distinct band.

The colour varies slightly with the different aldehydes. Formaldehyde gives a purple red, acetaldehyde a cherry-red, acrolein a rose carmine. The test is given also with the substituted aldehydes, such as chloral, *p*-hydroxy-benzaldehyde, etc. All these colours are typically hydrocnic.

By the aid of this test, the presence of aldehydes in moist ether can be demonstrated. If some aqueous ether be left in the sunlight for a few days it

gives an aldehyde reaction with the sulphuric acid and thiophene. This fact is of considerable importance since ether is frequently used in the extraction of lactic acid. The ether employed should be fresh and dry and the subsequent evaporation should not be done on a water-bath but at ordinary temperature. It is well known that moist ether, when warmed, readily oxidises with production of acetaldehyde and other substances.

The Interaction of Thiophene and Aldehydes.

Amongst the early work on thiophene two condensations with chloral and bromal, in the presence of glacial acetic acid and sulphuric acid, are recorded by V. Meyer [1883] and A. Peter [1884]. The formula ascribed is

for the chloral derivative.

Töhl and Nahke subsequently [1896] described a condensation between thiophene and benzaldehyde with the formation of dithienylphenylmethane;

On mixing thiophene and acetaldehyde in light petroleum in the molecular proportions corresponding to a body, $\text{CH}_3 \cdot \text{CH} : (\text{C}_4\text{H}_3\text{S})_2$, and adding phosphorus pentoxide, the pentoxide turned deep purple and a reaction began. The products were extracted with alcohol, ether, and light petroleum, respectively. The extracts were neutralised, washed, dried and fractionated, or fractionated straight away.

From two of the extracts a liquid fraction was obtained which gave a deep crimson with sulphuric acid.

The fraction was an orange liquid which boiled with decomposition at 121° , was insoluble in water, and had a peculiar smell.

The determination of the molecular weight and the composition of this substance was deferred until an adequate supply of thiophene could be obtained.

SUMMARY.

1. It is proposed to introduce the term *hydrocnic* for biochemical colour reactions, the products of which are decolorised by the addition of small quantities of water.

2. The thiophene reaction for lactic acid is due to the production of formaldehyde and acetaldehyde from the lactic acid, which interact with the thiophene in the presence of excess of sulphuric acid to give the cherry-red colour.

3. A new colour test for aldehydes is described depending on the fact that they interact with thiophene in the presence of sulphuric acid to give coloured substances.

4. A preliminary reference is made to the reaction between aldehydes and thiophene.

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