VI. COLOUR STANDARDS FOR USE IN THE DETERMINATION OF IMINAZOLES.

By GEORGE HUNTER.

From the Department of Pathological Chemistry, University of Toronto, Canada.

(*Received December 8th, 1924.*)

THE use of a mixture of Congo red and methyl-orange solutions was recommended by Koessler and Hanke [1919] when these workers published their method for the determination of iminazoles. Suitable mixtures of the two pigments gave colours which matched closely the colour developed by mixing solutions of histidine or other iminazole with the diazo reagent in a sodium carbonate solution.

It is a good rule in colorimetric work to use a standard made from the substance to be measured rather than to employ an artificial standard; but in the case of iminazoles the use of an artificial standard is almost indispensable, chiefly because it is impracticable to make up the standard and test solutions exactly at the same time, a procedure which the relative instability of the colours obtained would seem to demand. Further, iminazoles are as a rule difficult to prepare, and with the exception of histidine are not on the market. A reliable artificial standard will thus always serve as some check on the purity of iminazole solutions of different workers.

The stock solutions of Congo red and methyl-orange recommended by Koessler and Hanke [1919] were stated to keep indefinitely. This seems to be true. Recently, however, mainly to test this point, the writer had occasion to revise his standards. Previously, vacuum dried Grübler's Congo red and methyl-orange had been used as recommended originally by Koessler and Hanke. In this instance it was thought desirable to recrystallise the Grübler products and compare the standard made from these with the old standard. The new standard was found to have about 25 % greater colour value than the old standard. This finding was not, as at first suspected, due to a diminution of colour in the old stock solutions through standing, but was due to impurities in the Grübler's Congo red and methyl-orange.

The Congo red of four different manufacturers was tested and ranged from 40 % to 75 % of the colour value of the purified Congo red. Different methyl-orange products showed a like range of variation.

This difference in colour value is borne out by the residues obtained on ignition from the purified and commercial products. Both Congo red and methyl-orange are sodium salts, and thus leave a residue on ignition. The theoretical amount of sodium, weighed as sodium sulphate, can be calculated from the formulae. Thus Congo red, $C_{32}H_{22}O_6N_6S_2Na_2$, contains 6·62 % of sodium or 20·44 % as sodium sulphate. The formula of methyl-orange is $C_{14}H_{14}O_3N_3SNa$, which yields 21·71 % sodium sulphate.

Before dealing with the ash values the method of purification of the pigments will first be given.

1. *Congo red.* About 10 g. of Grübler's Congo red with 150 cc. of water are heated in a beaker placed in a boiling water-bath and stirred until the sediment is at a minimum, when the contents of the beaker are filtered through a Buchner previously heated by steam. The filtrate is reheated on the water-bath and 150 cc. of 95 % alcohol added. It is then placed in an ice chest over night. The crystal mass is filtered by suction, washed with alcohol, and allowed to dry in the open. The yield (about 7 g.) is ground in a mortar, redissolved in 100 cc. of hot water, filtered and reprecipitated by an equal volume of alcohol. The final air dry product is ground and heated in a water oven until constant in weight, when it is assumed to be water free: Yield 4–5 g.

2. *Methyl-orange.* A beaker containing 200 cc. water and 15 g. of Schuchardt's methyl-orange, which was found to give a rather higher colour value than Grübler's, is placed in a boiling water-bath. The solution is filtered through a hot Buchner, and the filtrate allowed to cool, when the methyl-orange immediately separates out. The solution is allowed to stand at 0° overnight. The methyl-orange is then filtered off, washed with cold water, and dried in the open. About 10 g. of dry material are then redissolved in 100 cc. of water and the procedure, as described, repeated. The final air dry product is ground and brought to constant weight in the water oven. A final yield of almost 8·0 g. is obtained.

It is advisable to keep the substance in an evacuated calcium chloride desiccator rather than in one containing sulphuric acid, as Congo red will darken in colour in presence of the acid.

The residue on ignition as sodium sulphate is perhaps the most convenient index of the degree of purity of Congo red or of methyl-orange. The only disadvantage of ashing is that a little of the sodium appears to volatilise on combustion of the carbon before the addition of the sulphuric acid.

The following percentages of sodium sulphate were found in Grübler's products and in the Congo red and methyl-orange twice recrystallised. The high residue on ignition from Grübler's Congo red is due mainly to sodium sulphate and from the methyl-orange to sodium chloride.

	Congo red % Na_2SO_4	Methyl-orange % Na_2SO_4
Grübler's	41·95	49·40
Twice recrystallised	19·42	20·45
Theoretical	20·44	21·71

The above findings necessitate a new basis for the making of the colour standards used in the estimation of iminazoles. If the quantities of stock Congo red and methyl-orange recommended by Koessler and Hanke are taken from pure solutions of these pigments the molecular colour values of the different iminazoles are materially lowered and a correction of all the work previously done is required. This, however, is unnecessary if a new colour standard is made to give a molecular colour value of 114 million mm. for histidine—the value originally given by Koessler and Hanke. The writer [Hunter, 1921] previously found a rather higher molecular colour value for histidine so that his colour standard was probably more dilute than that used by Koessler and Hanke. For this reason the molecular colour value of carnosine is now appreciably lowered. This does not affect the writer's results previously published for carnosine as the molecular colour value was accurate according to the colour standard then used.

The stock solutions are made as follows:

Stock methyl-orange. This consists of 0·1 g. of pure methyl-orange made to 100 cc. with water.

Stock Congo red. This consists of 0·2 g. of pure Congo red made to 100 cc. with water.

Koessler and Hanke originally recommended 0·5 % Congo red, but with pure Congo red this is too concentrated for accurate pipetting. These authors also used 10 cc. of alcohol per 100 cc. This appears quite unnecessary as Congo red is more soluble in water than in alcohol. Alcohol, indeed, gives the solution a turbid appearance for at least some days after mixing.

Histidine Colour Standard (S). About 80 cc. of distilled water are taken in a 100 cc. flask and 0·40 cc. stock Congo red added. After mixing, 0·06 cc. stock methyl-orange is added and the flask filled to the mark with water.

This colour standard, which contains a rather greater proportion of Congo red than that originally recommended by Koessler and Hanke [1919], matches excellently the colour produced by histidine and at the same time keeps the molecular colour value of this substance at about 114 million mm.

The colour produced by carnosine is slightly redder than that obtained from histidine but may be easily read against the above histidine colour standard. For this reason the writer has discontinued the use of a separate colour standard of carnosine.

The molecular colour values of histidine and carnosine were determined as follows: aqueous solutions were made containing 0·03 mg. histidine, 0·05 mg. histidine monohydrochloride, 0·03 mg. carnosine and 0·05 mg. carnosine nitrate per cc. respectively.

From each of the four test solutions thus prepared five readings were obtained using amounts differing by 0·2 cc. The reading given by 0·01 mg. of the substance is thus obtained and the molecular colour values calculated as shown below. In each case the molecular weight $\times$ the reading for 0·01 mg. $\times$ 10^5 = the molecular colour value in mm.

1. *Solution containing* 0·03 *mg. histidine per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	22·1	20
0·8	17·5	,,
0·6	13·2	,,
0·4	8·7	,,
0·2	4·4	,,

From these readings 0·01 mg. histidine gives a reading of approximately 7·4 mm.; whence the molecular colour value of histidine is

$$155 \times 7{\cdot}4 \times 10^5 = 114{\cdot}7 \text{ million mm.}$$

2. *Solution containing* 0·05 *mg. histidine monohydrochloride per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	26·8	20
0·8	21·5	,,
0·6	16·2	,,
0·4	10·8	,,
0·2	5·4	,,

From these readings 0·01 mg. histidine monohydrochloride gives a reading of approximately 5·4 mm.; whence the molecular colour value of histidine is

$$209{\cdot}5 \times 5{\cdot}4 \times 10^5 = 113{\cdot}1 \text{ million mm.}$$

3. *Solution containing* 0·03 *mg. carnosine per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	18·6	20
0·8	15·0	,,
0·6	11·1	,,
0·4	7·4	,,
0·2	3·8	,,

From these readings 0·01 mg. carnosine gives a reading of approximately 6·2 mm.; whence the molecular colour value of carnosine is

$$226 \times 6{\cdot}2 \times 10^5 = 140{\cdot}1 \text{ million mm.}$$

4. *Solution containing* 0·05 *mg. carnosine nitrate per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	24·8	20
0·8	19·5	,,
0·6	14·6	,,
0·4	9·8	,,
0·2	4·9	,,

From these readings 0·01 mg. carnosine nitrate gives a reading of approximately 4·9 mm.; whence the molecular colour value of carnosine is

$$289 \times 4{\cdot}9 \times 10^5 = 141{\cdot}6 \text{ million mm.}$$

The molecular colour value of histidine as determined from the free base and from the monohydrochloride differs by about 1 %. It may be taken at 114 million mm. as measured by the new standard.

With the same colour standard the molecular colour value of carnosine as determined from the free base and from the nitrate is 141 million mm.

SUMMARY.

It has been shown that the best commercial Congo red and methyl-orange are not suitable, without purification, for use as artificial standards in colorimetric work.

A colour standard made from pure Congo red and methyl-orange suitable for the estimation of histidine or carnosine is described.

The purity of other dyes used for colorimetric standards should be ascertained before standardising against particular iminazoles, phenols or other substances measurable by the diazo method.

REFERENCES.

Hunter (1921). *Biochem. J.* **15**, 689.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 497.