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The Determination of Amino Sugars in the Presence of Amino Acids and Glucose

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The presence of other sugars and amino acids interferes with the method for quantitative estimation of glucosamine and galactosamine described by Elson & Morgan (1933). The procedure, which is based on the reaction of amino sugars with acetylacetone in an alkaline medium and subsequent development of a red colour by chromogens formed with *p*-dimethylaminobenzaldehyde, has been modified by several authors. Many of these modifications are quoted by Rondle & Morgan (1955) in their paper dealing with the improvement of the estimation. Immers & Vasseur (1952) have examined the interference arising from mixtures of amino acids together with glucose under various conditions. Schloss (1951) proposed a method which overcomes such interference by taking advantage of the fact that colour due to amino acids and glucose fades after a comparatively short time. In his study of the mechanism of the reaction Schloss (1951) stated that a number of chromogenic products are formed by heating hexosamines with acetylacetone in alkaline media; partial fractionation of the reaction mixture yielded one volatile liquid and two or more non-volatile solid chromogens. The use of a homogeneous fraction giving a colour with *p*-dimethylaminobenzaldehyde would seem safer than a complex mixture for an analytical procedure. One of us (Cessi, 1952) developed a method based on the separation of the volatile chromogen(s) for the estimation of amino sugars in strongly coloured solutions. The procedure has been now adapted to the quantitative estimation of amino sugars in the presence of amino acids and glucose.

EXPERIMENTAL

Materials and methods

Amino sugars. D-Glucosamine hydrochloride was obtained from Hofmann–La Roche and Co. (Basle, Switzerland) and recrystallized from water by addition of conc. hydrochloric acid. D-Galactosamine was a kind gift from Professor W. T. J. Morgan. Aqueous solutions were made from hydrochlorides, and weights are referred to free sugars.

Acetylacetone reagent. Colourless, redistilled acetylacetone (b.p. 138–140°) was stored in the refrigerator and dissolved (1 ml.) in 100 ml. of 0·5N-sodium carbonate–sodium bicarbonate buffer containing 0·1M-sodium chloride. A buffer of pH 9·8, as adopted in the proposed method, was obtained by dissolving 23·02 g. of sodium carbonate, 2·76 g. of sodium bicarbonate and 5·84 g. of sodium chloride/l. of solution. The pH was checked after the addition of acetylacetone with a glass electrode standardized against 0·05M-sodium tetraborate and adjusted if necessary. The reagent

was stable for 1 day in the refrigerator. The pH was unchanged after boiling in stoppered, all-glass tubes.

p-Dimethylaminobenzaldehyde reagent. Recrystallized *p*-dimethylaminobenzaldehyde (80 mg.) was dissolved in 100 ml. of absolute ethanol containing 3·5 ml. of conc. HCl. The solution was almost colourless and could be stored in the refrigerator for several days.

Distillation apparatus. Distillations were performed in an all-glass apparatus assembled with a 100 ml. spherical flask with short neck and a 20 cm. condenser. The apparatus was heated directly over a gas burner, brought to boiling in about 1 min. and distilled at the rate of 1 ml./min.

Spectrophotometer. The extinctions of the solutions were measured with a Beckman DU spectrophotometer, with cells of 1 cm. light path. Wavelength calibration was checked with reference to a sodium source.

Procedure

Colour reaction. A portion (2 ml.) of solution containing 5–50 μg. of amino sugar, or mixtures of the amino acids listed and glucose (see Table 1) were mixed with 5·5 ml. of acetylacetone reagent, pH 9·8, in glass-stoppered tubes and heated in a boiling-water bath for 20 min. After cooling in cold water the reaction mixture and three washings of 2 ml. of water were transferred from each tube to the distillation apparatus. Portions (2 ml.) were distilled into 10 ml. volumetric flasks containing 8 ml. of *p*-dimethylaminobenzaldehyde reagent. The extinctions were determined 30 min. later at 545 mμ. For comparison the same quantities of amino sugars and of amino acids and glucose were tested by the procedure given by Rondle & Morgan (1955). Lysine yielded a strong colour with the latter method and very little after the distillation method, for which a quantity ten times as large was therefore taken.

Table 1. *Comparison of extinctions obtained by the present method and that of Rondle & Morgan (1955)*

Extinctions were measured for 1 m-mole of compound in 1 ml. of final coloured solution. The reactions were carried out with: amino sugars, 5–50 μg.; methylamine, 5 μmoles; choline, 20 μmoles; amino acids, 20 μmoles in the presence of 25 μmoles of glucose. Lysine was tested by taking 1–5 μmoles with the first method and 10–50 μmoles with the second, in the presence of 25 μmoles of glucose.

Compound	Method of Rondle & Morgan (1955) ($E_{530\ m\mu}$)	Present method ($E_{545\ m\mu}$)
D-Glucosamine	7200	6800
D-Galactosamine	7200	6600
Lysine	3700	4
Alanine	360	8
Histidine	320	7
Valine	280	8
Serine	260	8
Glycine	230	9
Cystine	220	3
Arginine	200	4
Phenylalanine	190	8
Glutamic acid	100	4
Leucine	100	5
Tyrosine	100	10
Aspartic acid	90	3
Hydroxyproline	60	26
Methionine	40	2
Tryptophan	30	4
Methylamine	2600	570
Choline	0	0

RESULTS

Influence of pH. The procedure described was tested at different pH values of the acetylacetone reagent. The colour intensities obtained were independent of pH between pH 9·5 and 10·0 (Fig. 1);

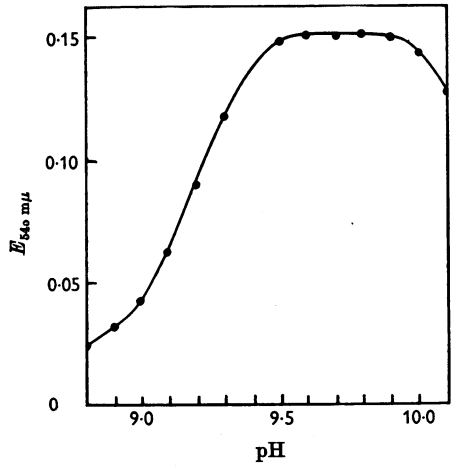

Fig. 1. Influence of pH during the reaction between amino sugars and acetylacetone on the intensity of colour. Each point was obtained with 40 μg. of D-glucosamine.

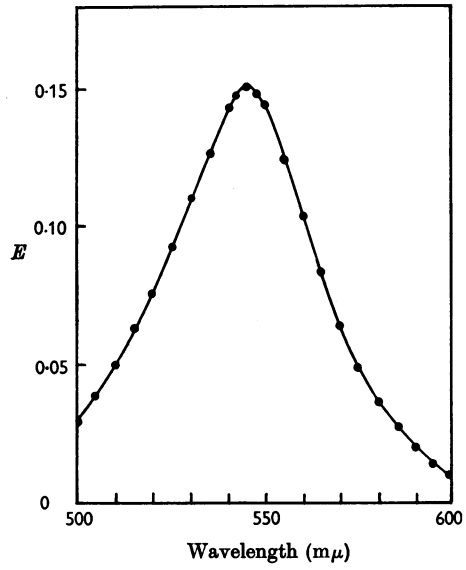

Fig. 2. Absorption spectrum of colour obtained by reaction with *p*-dimethylaminobenzaldehyde and chromogen(s) separated by distillation from the reaction mixture between 40 μg. of D-glucosamine and acetylacetone in alkali.

pH 9·8 has been chosen as standard value for all other experiments.

Absorption maximum. The absorption spectrum of the colour obtained with the described procedure showed a maximum at 545 mμ (Fig. 2). The colour was fully developed after 30 min. at room temperature and its intensity was unchanged after 24 hr.

Interference from amino acids and glucose. Table 1 gives a comparison of the extinctions of amino sugars and of amino acids in the presence of 25 μmoles of glucose with the procedure described and with the procedure of Rondle & Morgan (1955). With the former method lysine was found to give the most serious interference; alanine, valine and glycine interfered less. All interferences were practically completely overcome with the present method. The only seriously interfering substance was methylamine. Hydroxyproline was found to give a molar extinction about 0·3% of that of glucosamine. The addition of 0·02 M-sodium sulphite to the acetylacetone reagent decreased the colour due to hydroxyproline to one-tenth. Since the addition of sulphite reduces the sensitivity of the method it should not be added if hydroxyproline is known not to be present in large amounts. The method was tested with a hydrolysate of crystalline insulin that was known not to contain any amino sugar; 2 mg. of hydrolysate was used for the reaction. In one experiment 25 μmoles of glucose were added to the same quantity of hydrolysate. In no instance was colour observed.

Range, sensitivity and accuracy. The extinctions of the coloured solutions were found to be proportional to the amounts of amino sugars taken within the range 5–50 μg., and by decreasing the volume of the final solution it has been possible to estimate 2 μg. of hexosamine. The sensitivity could be increased by taking advantage of the concentration effected by distillation of the volatile chromogen(s), but this point has not been extensively investigated. The accuracy of the method was tested by determining the extinctions of 30 samples each containing 20 μg. of glucosamine, the determinations being carried out on three different days. The observed standard deviation was 2·4%.

DISCUSSION

The method described for the quantitative estimation of amino sugars uses the volatile fraction of chromogens formed by heating these compounds with acetylacetone in an alkaline medium. Among several substances investigated only methylamine was found to give interference. It is possible, however, that other volatile amines do so and their occurrence should be taken into account. The important interference by amino acids and glucose, relevant for lysine, is overcome with the present method. The procedure, which yields a stable colour, has been applied to a number of determinations of amino sugars in mucoproteins (C. Cessi, unpublished work; Johansen, Marshall & Neuberger, 1960).

SUMMARY

1. A method is described for the quantitative determination of amino sugars in the presence of amino acids and glucose. The procedure is based upon the observation of Schloss (1951) that amino sugars form volatile chromogen(s) when heated with acetylacetone in alkaline solution.

2. The volatile fraction is separated by distillation and a red colour is formed by reaction with *p*-dimethylaminobenzaldehyde.

3. The method overcomes the interferences due to amino acids in the presence of glucose, which form only non-volatile chromogens.

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