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The Determination of Glucosamine and Galactosamine

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An analytical procedure for the quantitative determination of glucosamine and galactosamine was described by Elson & Morgan (1933). The method was based on the observation by Pauly & Ludwig (1922) that in alkaline solution at 100° the amino sugars react with acetylacetone to form a chromogenic material which gives a chromophore or chromophores on treatment in acid solution with ethanolic *p*-dimethylaminobenzaldehyde. The development and use of photoelectric spectrophotometers during the intervening years has increased the sensitivity and accuracy of technique very considerably, but although many modifications of procedure designed to improve the method have been described there has, in fact, been no fundamental change in the procedure originally employed. The method given in this paper has been used for many years and has given satisfactory results. The procedure is now described because we have frequently referred, in publications from this Laboratory, to a modification, whose details have not been published, of the technique described in the original paper. To ensure that a reliable determination of amino sugar is achieved, it is imperative that an established and rigid technique is used, since slight variations in procedure, especially those affecting the pH during the condensation with acetylacetone, lead to marked differences in the colour intensity finally obtained.

In the extensive literature on this reaction papers by Sørensen (1938), Blix (1948), Schloss (1951) and Belcher, Nutten & Sambrook (1954) describe work

done on the estimation of amino sugar itself, and an account of the determination of the amino sugar content of a variety of naturally occurring substances is given by Boyer & Fürth (1935), Jorpes (1935), Palmer & Meyer (1935), Dakin & West (1935), Dakin, Ungley & West (1936), Nilsson (1936), Lüstig & Ernest (1937), Palmer, Smyth & Meyer (1937), Masamune & Nagazumi (1937), Dakin, Young & Krauss (1938), West & Clark (1938), Hewitt (1938), Hamasato & Akakura (1941), Hadidian & Pirie (1948), Ogston & Stanier (1950), Johnston, Ogston & Stanier (1951), Cessi (1952) and Anastassiadis & Common (1953). Reference is made later to work done on other aspects of the determination (see Discussion).

EXPERIMENTAL

Materials and methods

Acetylacetone reagent. Redistilled acetylacetone (b.p. 138–140°, 1 ml.) is dissolved in 50 ml. of 0.5N-Na₂CO₃ solution. The reagent is prepared immediately before each estimation and is stable for 2–3 hr. at 18°.

Ethanol. This is distilled after drying for 24 hr. over freshly heated calcium oxide.

***p*-Dimethylaminobenzaldehyde reagent** (Ehrlich's reagent). Commercial-grade *p*-dimethylaminobenzaldehyde is purified as described by Adams & Coleman (1948) and finally reprecipitated from ethanol by the addition of 50–75% of water. A suitable material is colourless to pale yellow and melts at 73°. The aldehyde (0.8 g.) is dissolved in 30 ml. of ethanol and 30 ml. of conc. HCl (A.R., sp.gr. 1.18) are added. The solution should be free from insoluble matter and pale yellow; it keeps well when stored at –10°.

Glucosamine and galactosamine standard solutions. Aqueous solutions of the amino sugar hydrochlorides, 1% as free base, are convenient for use and stable if stored frozen at -10° .

Apparatus. For the determination of hexosamine in neutral solution, test tubes (approx. 20×1 cm.) graduated at a volume of 10 ml. are used. When the solution available is not neutral or when the volume of solution is not exactly known, an additional graduation at 3 ml. is necessary.

Procedure

To the test solution (1 ml.) contained in the tubes described acetylacetone reagent (1 ml.) is added, the tube contents are well mixed by gentle shaking, and the walls of each tube washed down with 1 ml. of distilled water. If the solution used is acid 1 drop of phenolphthalein is added before the addition of acetylacetone, and the minimum quantity of N -NaOH is run in to give a full pink colour. Dil. HCl (approx. $0.3N$) is then added until the indicator colour is just discharged. In these circumstances, or when less than 1 ml. of test solution is available, the walls of the tubes are washed with sufficient water, after the addition of the acetylacetone reagent (1 ml.), to achieve a final volume of 3 ml., and for this purpose the second graduation is required.

The tubes are closed with long-necked (2–3 cm.) sealed glass ampoules (2–3 ml. volume) each containing 1–2 ml. of water; these act as loose stoppers and as condensers to prevent the loss of acetylacetone. The tubes are heated in a vigorously boiling water bath for 20 min., cooled to room temp. (cold water bath) and ethanol (approx. 5.0 ml.) is added, followed by 1 ml. of Ehrlich's reagent and ethanol to reach the 10 ml. mark. The contents of the tube are thoroughly but gently mixed and warmed for 10 min. in a water bath at 65 – 70° to accelerate liberation of CO_2 . Heating above 70° leads to a decrease in the final colour intensity. After cooling to 18° (water bath), the contents of the tubes are again mixed and the colour intensity at $530 m\mu$. is measured for each tube in a Hilger Uvispek or similar apparatus, 2 cm. cells being used.

In practice the following tubes are set up: (i) Blanks: duplicate, each containing water in place of amino sugar solution. (ii) Standards: three or four different concentrations of amino sugar (in duplicate) are included to cover that range of the test where estimations are required. (iii) Amino sugar to be determined, in duplicate or triplicate.

RESULTS

It has been found repeatedly that the intensities of colour, i.e. $\log_{10} (I_0/I)$, produced in an estimation bear a linear relationship to the amount of glucosamine present over the range 5.0 – $150 \mu g.$ of amino sugar. Beer's law is not obeyed with amounts of glucosamine greater than $150 \mu g.$ (cf. Fig. 1).

Within these limits the 'best' range can be evaluated by the method of Ayres (1949). For the technique described a graphical plot of percentage absorption against log (glucosamine concentration) yields a sigmoid curve whose slope at any point is proportional to the accuracy at that point. Examination of a number of standard curves

similar to that given in Fig. 2 showed that the most accurate range for the test is 20 – $60 \mu g.$ of amino sugar, 'best' estimates of solutions of unknown concentration being obtained at an amino sugar concentration of approx. $30 \mu g./ml.$ of test solution. By means of the technique described a coefficient of variation of 1.3% was obtained for fifteen replicate estimations of $30 \mu g.$ of glucosamine.

Fig. 1. Relationship between colour intensity and glucosamine concentration. Colour intensity read on Uvispek apparatus at $530 m\mu$. with 2 cm. cells.

Fig. 2. Accuracy and range of the modified Elson-Morgan reaction. Details of experiment are as given for Fig. 1.

It is of no consequence to define the range of the estimation more closely than this because standard lines obtained in different experiments are not necessarily identical. A recent series of forty standard lines [ordinate, $\log_{10} (I_0/I)$; abscissa, glucosamine concentration], derived from 10 to 30 points for each line and using the glucosamine range 20 – $60 \mu g.$, was evaluated by the method of least squares (cf. Brownlee, 1949), and showed a range of regression coefficients covering 0.0090 – 0.0130 (standard deviation of scatter 0.004 – 0.020)

and a range of intercepts covering 0.000–0.020. No correlation was observed between the number of points per line, the slope of the line and the standard deviation of the scatter.

The absorption spectrum of the red colour remains unchanged for about 3.0 hr., after which a slow diminution in the intensity of absorption at 530 m μ . is observed. It has been found that between 3 and 30 hr. the decrease in maximum absorption can be expressed as about 0.5 %/hr. for concentrations of amino sugar between 10 and 100 μ g./ml. of test solution.

In one series of experiments, examination of the change in absorption spectrum with time showed that a fall in colour intensity was accompanied by

a slow drift of the peak of maximum absorption towards the blue end of the spectrum. The shift was most marked with the higher amino sugar values, but after 120 hr. a definite peak at approx. 512 m μ . (cf. Schloss, 1951) was discernible only when the concentration of amino sugar was greater than the highest level recommended (cf. Fig. 3).

In agreement with the results of the original workers and those obtained subsequently (Palmer *et al.* 1937; Hamasato & Akakura, 1941; Schloss, 1951), it has been found that the intensity of colour produced by galactosamine is identical with that given by an equal weight of glucosamine, and no significant difference is observed between the absorption spectra of the chromophores of glucosamine and galactosamine within the time interval 0.5–10.0 hr.

DISCUSSION

The method described is suitable for the estimation only of free amino sugar; where a determination is carried out to ascertain the amino sugar content of a polysaccharide or other material of high molecular weight it is essential that the exact conditions that give complete hydrolysis, without destruction of the amino sugar, should be known for each substance investigated. Any amino sugar units remaining as oligosaccharides or substituted amino sugars give less colour per unit weight of amino sugar than that found for the free amino sugar (cf. Jorpes, 1942; Blix, 1948).

In this Laboratory interest has been centred on the amino sugar content of mucoids exhibiting blood-group activity (Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952*a, b*; Gibbons & Morgan, 1954). It has been customary to hydrolyse each test material in a sealed glass ampoule at 100° (mucoid concentration 0.5–1.0 %) for 8 or 16 hr. in 0.5N hydrochloric acid. A known volume of the resultant hydrolysate has been withdrawn from the ampoule, neutralized with an equal volume of 0.5N sodium hydroxide and made up to a convenient known volume. The amino sugar content of the neutralized solution has then been determined in the manner described. From studies of the acid-hydrolysis products of the blood-group mucoids it is considered probable that this treatment results in the complete liberation of amino sugars, although, as indicated above, the conditions described are not necessarily valid for other types of mucoid or polysaccharide. In the application of the technique to naturally occurring substances, the possibility of obtaining spurious results must not be overlooked, for it is well known that materials other than amino sugars can interfere in the test. Large amounts of some non-nitrogenous sugars give a colour in the reaction, as can urea and glyceralde-

Fig. 3. Change in absorption spectrum of chromophore with time. Curves a_1 , a_2 , a_3 determined for 120 μ g. of glucosamine at 24, 50 and 120 hr. respectively. Curves b_1 , b_2 , b_3 similarly determined for 40 μ g. of glucosamine. Curves for 1 hr. time intervals are omitted from the figure, being indistinguishable from the 24 hr. curves on the graph scale used.

hyde, but the most serious errors arise when small quantities of amino sugar are estimated in the presence of large quantities of mixtures of amino acids with non-nitrogenous sugars (Palmer *et al.* 1937; Sideris, Young & Krauss, 1938; Lutwak-Mann, 1941; Yasuoka, 1944; Horowitz, Ikawa & Fling, 1950; Immers & Vasseur, 1950; Storey, Yensen, Lisie & Biliën, 1951). For this reason it is advisable to confirm determinations of 'total amino sugar' by alternative means such as that proposed by Gardell (1953). An account of a method whereby such a confirmation can be obtained will be given later.

SUMMARY

1. A modification of the method described by Elson & Morgan (1933) for the determination of amino sugars is given.

2. The 'best' range, the accuracy and the reproducibility of the determination are illustrated by results obtained by the use of authentic amino sugar samples, and the limitations of procedure, imposed by the instability of the chromophore produced, are given.

3. Factors of importance in the application of the method to naturally occurring substances are discussed.

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The Amino Acid Composition of Mammalian Collagen and Gelatin

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The name collagen is given to a group of related fibrous proteins, found in the mesodermal tissues of animals. The best-characterized forms of collagen occur in the higher animals, where they constitute the main organic components of skin, bone, tendon and loose connective tissue. Gelatin, a derived protein, can be prepared as a breakdown product of

collagen by extracting these tissues with hot water (above 40°). Extraction with minimal degradation can normally be accomplished in neutral solutions after a prolonged pretreatment with cold alkali or by an acid extraction (Ward, 1954). Gelatin is the principal constituent of the commercial products 'gelatine' and glue and is responsible for their gel properties.