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The specificity of the reaction for amino sugars discovered by Elson & Morgan (1933) has been increased by several workers through knowledge of some of the intermediates involved. The interference by amino acids and glucose was overcome in different ways (Schloss, 1951; Immers & Vasseur, 1952; Cessi, 1952; Cessi & Piliego, 1960). The separation of a volatile fraction among the products of condensation of amino sugars with pentane-2,4-dione (acetylacetone) (Schloss, 1951), later identified as 2-methylpyrrole (Cornforth & Firth, 1958), led to a procedure (Cessi, 1952; Cessi & Piliego, 1960) by which unsubstituted amino sugars can be differentiated from their 3-derivatives, as Johansen, Marshall & Neuberger (1960) pointed out.

This paper deals with the estimation of D-galactosamine in the presence of D-glucosamine, a problem also studied by Tracey (1955) and other workers.

The mechanism of the Elson–Morgan reaction is still obscure. Cornforth & Firth (1958) suggested that an intermediate (I):

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\begin{align*}
\text{OH} & \quad \text{HC} - \text{CH} \cdot \text{CO} \cdot \text{CH}_3 \\
\text{HO} \cdot \text{CH}_3 & \quad \text{[CH(OH)]}_3 \cdot \text{HC} \cdot \text{C} \cdot \text{CH}_3 \\
\text{N} & 
\end{align*}
\]

(I)

is formed by condensation of hexosamine with acetylacetone, which in alkaline medium subsequently loses the polyhydroxy chain and the acetyl group to yield 2-methylpyrrole, the more effective chromogen in the reaction with p-dimethylaminobenzaldehyde. The assumed intermediate is very likely to have a short life in the conditions of the Elson–Morgan reaction, but it seemed that when formed in milder conditions it might be sufficiently stable and retain enough of the configuration of the hexose to allow reactions specific for different amino sugars. A reaction for D-galactosamine that is not affected by D-glucosamine has been obtained (Cessi & Serafini-Cessi, 1962) by dividing the procedure into steps: (1) transformation of the amino sugar hydrochlorides into the free bases with triethylamine in anhydrous conditions; (2) condensation with acetylacetone (as this is catalysed by triethylamine it is carried out in the same operation as step 1); (3) transformation of the condensation product into 2-methylpyrrole in a suitable buffer; (4) reaction of the steam-distilled 2-methylpyrrole with p-dimethylaminobenzaldehyde. The presence of amino acids with and without glucose does not interfere with the colour produced by D-galactosamine. Nevertheless, when small amounts of amino sugar are to be estimated in hydrolysates containing large amounts of amino acids, the concentration of acetylacetone is lowered by condensation with the latter and the sensitivity decreased, a difficulty easily overcome by increasing the concentration of acetylacetone in the reagent.

EXPERIMENTAL

Reagents. All reagents were analytical grade. Anhydrous methanol specified for the Karl Fischer reaction was used. D-Galactosamine hydrochloride was obtained from Mann Ltd. (New York) and a sample of D-mannosamine was kindly presented by Dr H. R. Perkins.

Acetylacetone–triethylamine reagent. This consists of (by vol.) 25% of acetylacetone, 25% of triethylamine and 1% of pyridine in anhydrous methanol and should be prepared freshly. The concentration of 6% of acetylacetone reported previously (Cessi & Serafini-Cessi, 1962) was found to be too low when large amounts of amino acids were present as in protein hydrolysates.

Borate buffer. A buffer containing K₂B₄O₇·4H₂O (50·2 g/l.) and H₃BO₃ (40·6 g/l.), pH 8·0, was found to give the best differentiation between the amino sugars; it was made with warm water and stored at 35°. When the effect of concentration of borate or of pH was investigated appropriate dilutions were made from the above solution and pH was adjusted with H₂BO₃ or KOH.

Ehrlich reagent. The concentrated solution of p-dimethylaminobenzaldehyde (0·8%, w/v) in ethanol containing 3·5% (v/v) of conc. HCl, proposed by Johansen et al. (1960), was used throughout.

Analytical procedure

Samples containing 10–50 μg. of amino sugar hydrochlorides, in a volume not exceeding 1 ml. and preferably 0·5 ml., are dried at 50° under reduced pressure in test tubes (e.g. 16 mm. × 160 mm.)
fitted with ground-glass joints, connected to a water pump and individually shaken in the water bath. Excess of hydrochloric acid in hydrolysates is conveniently removed at this step and neutralization is not required. A portion (1 ml) of the acetylacetone—triethylamine reagent is added and the stoppered tubes are incubated for 16 hr. in an oven at 55°C. The bulk of the reagents and methanol are removed under reduced pressure as described above to make the subsequent steam-distillation of 2-methylpyrrole easier. Small residues of acetylacetone and triethylamine do not affect the following steps. The stoppered tubes are heated for 20 min. with 6 ml of borate buffer in a boiling-water bath. The tubes are cooled to 35°C, the contents transferred to a distillation apparatus and the first 2 ml of the distillate is collected in a 10 ml volumetric flask containing 8 ml of the p-dimethylanilinobenzaldehyde reagent. The distillation apparatus shown in Fig. 1 was found satisfactory. Since all the 2-methylpyrrole distills with the first few drops, 2 ml is sufficient for quantitative recovery and rinsing of the condenser. In our experience a supplementary rinsing with water was found necessary only after distillation of very concentrated samples. The colour soon develops, reaching a maximum in 30 min., and is stable for some hours. The extinction measured at 545 mμ is a linear function of the quantity of amino sugar taken and is reproducible. The extinction values expected if the colour from 1 m-mole of amino sugar was read in 1 ml of final solution would be 14 200 for D-galactosamine and 180 for D-glucosamine.

Optimum conditions for the reaction. It was observed that the addition of basic catalysts to the reagent proposed by Pauly & Ludwig (1922) for the condensation of D-glucosamine with acetylacetone to 3-acetyl-2-methyl-5-tetrahdroxybutylpyrrole left the reaction unchanged. Under the same conditions D-galactosamine condensed to one or more intermediates from which a volatile chromogen could be obtained in the subsequent step. Different basic catalysts at different concentrations were tested to find the optimum yield of colour and to increase the specificity. The best results were obtained with 25% of triethylamine. The addition of a small amount of pyridine also increased the sensitivity of the reaction. The colour yield was found to depend upon the concentration of acetylacetone. Fig. 2 shows the values of the extinctions as functions of concentration of acetylacetone in the reagent.

The intensity of the colour produced from D-galactosamine increased with the time of incubation at 55°C (steps 1 and 2). A standard time of 16 hr. was chosen. Longer times increased the sensitivity of the method, but affected the specificity. In Fig. 3 the extinction is plotted against incubation time. Optimum temperature is 55°C. The accuracy of ±1° obtained in common laboratory ovens allows reproducible results.

The condensation of D-glucosamine with acetylacetone under the conditions described still led to a small interference due to some precursor of the chromogen. The transformation of this intermediate to a volatile chromogen was found to be inhibited by borate, whereas the transformation of the products of condensation of D-galactosamine was not (step 3). The inhibition by borate of the colour produced from D-glucosamine was tested at

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Fig. 1. Distillation apparatus for the analytical recovery of 2-methylpyrrole.

Fig. 2. Effect of the concentration of acetylacetone on the colour developed by 0.2 μmole of D-galactosamine hydrochloride alone (●) and in the presence of 50 μmoles of lysine (○).
various concentration of the buffer. The upper limit of the concentrations tested was that of the solution described above (Reagents), which is almost saturated at 35°. This buffer gave the best differentiation between the amino sugars. Fig. 4 gives the dependence of colour intensity upon the concentration of borate. Changes of pH between 7.5 and 8.5 do not affect the yield of colour much.

**Interference by other compounds.** N-Acetyl-D-glucosamine was tested with the method described and found to give no interference. In the course of a very helpful discussion Dr Perkins stressed the importance of D-mannosamine as an interfering compound and kindly gave us a sample of N-acetyl-D-mannosamine. As Carrol & Cornforth (1960) pointed out, N-acetyl-D-glucosamine epimerizes to N-acetyl-D-mannosamine on alkaline treatment and this event must be taken into account when alkali is employed during preparation. We repeated the experiment, already performed by Dr Perkins by the method of Cessi & Serafini-Cessi (1962), with the present method and we found, for D-mannosamine, 1400 as extinction referred to 1 m-mole/ml of final solution, i.e. one-tenth of the colour given by D-galactosamine. The same sample gave two-thirds of the colour of D-galactosamine with the method of Cessi & Piliego (1960).

A number of amino acids were tested in the presence of glucose and the extinctions calculated from the observed values, referred to 1 m-mole of compound/ml. of final solution, are listed in Table 1. When the estimation of D-galactosamine was carried out with the 6% acetylation reagent described previously (Cessi & Serafini-Cessi, 1962) in the presence of fairly high concentration of amino acids an inhibition of colour formation was observed. It was easily explained on the basis of the decreased concentration of acetylacetone due to the keto-imine formation with amino acids (Critchfield & Johnson, 1957). In the conditions described the acetylacetone derivatives of several amino acids were prepared and acetylacetone was quantitatively recovered by hydrolysis. The inhibition disappeared when the concentration of acetylacetone was increased, as shown in Fig. 2.

**Table 1. Extinctions from various amino sugars and amino acids at 545 mμ**

Values are referred to 1 m-mole of compound/1 ml. of final solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Galactosamine</td>
<td>14 200</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>180</td>
</tr>
<tr>
<td>D-Mannosamine</td>
<td>1 400</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>140</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
</tr>
<tr>
<td>Alanine</td>
<td>2</td>
</tr>
<tr>
<td>Lysine</td>
<td>3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>10</td>
</tr>
</tbody>
</table>
The presence of Na\(^+\), K\(^+\), Cl\(^-\) and formate ions in amounts up to 1 m-equiv. do not interfere; NH\(_4\)\(^+\) ion gives a faint pink colour with maximum extinction at 520 m\(_\mu\) when present in amounts as high as 0-2 m-equiv. Amounts of NH\(_4\)\(^+\) ions higher than 0-2 m-equiv. inhibit the production of colour.

**Isolation of intermediates of the reaction**

In an attempt to elucidate the mechanism of the reaction some products have been isolated. Although the presumably more interesting precursors of 2-methylpyrrole originating from D-galactosamine have not been isolated, some information is available that stresses the different behaviour of D-galactosamine and D-glucosamine in the conditions given. From D-glucosamine 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole and the keto-imino derivative of the amino sugar have been isolated. 3-Acetyl-2-methyl-5-tetrahydroxybutylpyrrole was prepared from 1 g. of D-glucosamine hydrochloride by condensation with 50 ml. of the acetylace tone (25\%, v/v) reagent at 55° overnight (cf. Pauly & Ludwik, 1922). After evaporation of the reagent the residue was washed with light petroleum, dissolved in the minimum amount of water and cooled in an ice-box overnight. 3-Acetyl-2-methyl-5-tetrahydroxybutylpyrrole (1 g.) separated as a crystalline powder and was recrystallized several times from ethanol; m.p. 133°, uncorr., [\(\alpha\)]\(_D\)\(^{20}\) = 57° (c 0-59 in water) (Found: C, 54-5; H, 7-1; N, 5-7). Calc. for C\(_{11}\)H\(_{19}\)NO\(_5\): C, 54-3; H, 7-0; N, 7-8\%). \(E_{\text{max}}\) in water 246 and 290 m\(_\mu\) (c 6820 and 5330 respectively) were in good agreement with the values given by González, Gómez, Gutiérrez & Sánchez (1961). It gave a faint reaction with p-dimethylaminobenzaldehyde after heating in hydrochloric acid and produced neither 2-methylpyrrole nor other volatile chromogens on heating at 100° in water or alkaline solution.

To the mother liquors absolute ethanol was added until opalescence appeared. After the solution was left in the cold overnight an additional fraction separated. It contained a high proportion of another crystalline condensation product of D-glucosamine and acetylace tone. It was made free from 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole by chromatography on Amberlite IRA-400 (same conditions as given for the chromatography of the products of condensation of D-galactosamine, see below) and recrystallized from absolute ethanol. It was also prepared by a modification of the above method, the identity of the two products being proved by the ultraviolet spectra and the unchanged mixed melting point: D-glucosamine hydrochloride was heated with the acetylace tone (6\%, v/v)–triethylamine (6\%, v/v) reagent at 100° under reflux for 5 min. The excess of reagent was evaporated under reduced pressure, the residue washed with light petroleum and dissolved in absolute ethanol. The insoluble residue was discarded and a crystalline product separated on standing overnight; m.p. 107°, uncorr., [\(\alpha\)]\(_D\)\(^{18}\) = 21° (c 1-37 in water), \(E_{\text{max}}\) in water 312 m\(_\mu\) (c 18 400) (Found: C, 47-4; H, 7-5; N, 5-2. C\(_4\)H\(_9\)NO\(_4\) requires C, 47-3; H, 7-6; N, 5-0\%). The values for extinction are fairly close to those reported for the spectra of several keto-imino compounds by Cromwell, Miller, Johnson, Frank & Wallace (1949) and by Weinstein & Wyman (1958). It gave no colour with the Ehrlich reagent and we were unable to transform it into 2-methylpyrrole. The structure (II):

\[
\begin{align*}
\text{HO-CH}_4\text{CH}-[\text{CH(OH)}]_3\text{CH}\cdot\text{NH}\cdot\text{CH-OH} \\
\text{H}_3\text{C}\cdot\text{C}=\text{CH} \cdot \text{CO} \cdot \text{CH}_3
\end{align*}
\]

is proposed for the compound and is supported by the following observations: it gives a positive test with ferric chloride under the conditions described by Weinstein & Wyman (1958) for the keto-imines, and is easily hydrolysed in 0-01 N-hydrochloric acid at 25° for 30 min., giving the expected quantities of amino sugar (by the reaction of Cessi & Piliego, 1960) and acetylace tone. The latter was recovered by distillation, gave a positive test with nitroprusside and showed ultraviolet spectra identical with those of a sample of pure acetylacetone both in neutral and alkaline solution. The easy splitting of keto-imino compounds in dilute hydrochloric acid was reported by Combes & Combes (1892). On heating in water, 0-05 M-sodium carbonate or borate buffer, pH 9-2, the keto-imine gives 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole. This reaction probably occurs in the conditions of the condensation proposed for the analytical procedure.

The attempt to isolate intermediates arising from the condensation of D-galactosamine with acetylace tone and leading to the chromogen has so far been unsuccessful. 3-Acetyl-2-methyl-5-tetrahydroxybutylpyrrole is produced in a lower yield (25–30\%) than from D-glucosamine. Another condensation product was found to be unstable in water, passing very easily to 2-methylpyrrole on heating. An attempt was made to purify this substance that is probably responsible for the development of colour. The product of reaction with the acetylace tone–triethylamine reagent for 16 hr. at 55° was absorbed on a column (1 cm. \(\times\) 60 cm.) of Amberlite IRA-400 (formate form; 400 mesh) and eluted with water. Three distinct fractions soon emerged, the third being identified as 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole. The first, very
scanty, gave a positive direct reaction with Ehrlich reagent; its absorption spectrum showed a maximum at 245 m\(\mu\), suggesting the presence of a pyrrole ring, but not 2-methylpyrrole. The second peak was still a complex mixture with maximum absorption at 312 m\(\mu\), which disappeared on standing, while the band at 245 m\(\mu\) appeared. This fraction gave a positive Ehrlich test after heating in alkali. Paper chromatography with butanol-water-ethanol (5:1:4, by vol.) resolved the mixture into three spots. The major spot (\(R_f\) 0.55), when eluted from the paper, showed an adsorption maximum at 312 m\(\mu\) and was transformed into 2-methylpyrrole by heating with alkali. Hydrolysis with 0.01-0.2 N-hydrochloric acid for 30 min. at 100° yielded no amino sugar. The minute amount of substance obtained did not permit its identification.

**DISCUSSION**

A differential method for the estimation of D-glucosamine based on the depression of colour by borate in the Elson-Morgan reaction was proposed by Tracey (1955). In his method the colour obtained with D-glucosamine is about half that yielded by D-galactosamine. The present method stems from the different behaviour of the two amino sugars when condensed with acetylatedone under mild anhydrous conditions. The experiments on the mechanism of the reaction explain why D-glucosamine gives no colour with the proposed procedure. This amino sugar condenses with acetylatedone to give quantitatively 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole, which has been recovered as a crystalline product in 90 % yield. It is a fairly stable compound and gives no volatile chromogen in the subsequent steps. Under slightly different conditions the keto-imine derivative of D-glucosamine is obtained, which is very easily transformed into 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole by heating in neutral or alkaline solution. Neither compound can be transformed into 2-methylpyrrole under the conditions given and they cannot account for the colour produced from D-glucosamine on heating in water (Fig. 4). The precursors of the chromogen involved in this case differ from those of D-galactosamine in their sensitivity to borate, which inhibits the transformation into a volatile pyrrole.

It is not yet clear which products are formed by condensation of D-galactosamine and acetylatedone under the same conditions. A compound related to 5-tetrahydroxybutylpyrrole is formed only in poor yield, and one or more unstable products are formed which change very easily to 2-methylpyrrole by heating in mild alkaline solution. The used of borate provides good and reproducible rates of transformation, but it is not essential. The yield of 2-methylpyrrole from D-galactosamine is higher with the present method than with the procedure of Cessi & Piliego (1960).

**SUMMARY**

1. The determination of D-galactosamine is carried out by condensation of the amino sugar with acetylatedone in anhydrous methanol in the presence of triethylamine and pyridine, transformation of the intermediate formed into 2-methylpyrrole in borate buffer, reaction of the distilled chromogen with p-dimethyaminobenzaldehyde and spectrophotometric measurement of the product at 545 m\(\mu\). Interference by D-glucosamine is less than 2 %.

2. Some compounds formed during the condensation have been isolated. The mechanism of the reaction is discussed.

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**REFERENCES**


