Determination of Amino Sugars in Mixtures containing Glucosamine, Galactosamine and Muramic acid

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A colorimetric method is described whereby the direct quantitative determination of glucosamine, galactosamine and muramic acid can be achieved without previous treatment of the cell-wall hydrolysate, for example by column chromatography. Molar ratios of hexosamines in cell-wall preparations, from a number of bacterial species, determined by this method were found to be in general agreement with previously published results.

The procedure of Elson & Morgan (1933) has been widely used to determine the hexosamine content of biological preparations. The method was based on the reaction of amino sugars with acetylacetone under alkaline conditions, forming a chromogenic material. On treatment of this material with ethanolic *p*-dimethylaminobenzaldehyde in an acid solution a pink chromophore was formed. Modifications of this procedure have been proposed and many of these were quoted by Rondle & Morgan (1955).

One disadvantage in the Elson & Morgan (1933) method is that the presence of sugars and amino acids interferes with the quantitative determination of glucosamine and galactosamine. However, Schloss (1951) found that the colour due to sugars and amino acids fades after a comparatively short time. It was also shown that a number of volatile and non-volatile chromogens were formed by heating hexosamines with acetylacetone in alkaline medium. Cornforth & Firth (1958) suggested that in this reaction 3-acetyl-4-hydroxy-2-methyl-5-(tetrahydroxybutyl)pyrrole was formed by condensation of hexosamine with pentane-2,4-dione (acetylacetone); the lateral chain and the acetyl group were removed at the alkaline pH, yielding a 2-methylpyrrole. Cessi (1952) and Cessi & Piliego (1960) developed a method based on the separation of the volatile 2-methylpyrrole for the determination of amino sugars in the presence of amino acids and glucose.

In all these methods muramic acid is unusual with respect to the formation of maximum colour intensity. Crumpton (1959) showed that the colour intensity at the wavelength of maximum extinction (505–510 m μ) increased with time and for this reason readings were taken after 24 hr. Rondle & Morgan (1955) found that with glucosamine and

galactosamine the wavelength of maximum extinction did not alter during the 24 hr. period, though a slow diminution in the colour intensity at this wavelength was observed. Crumpton (1959) pointed out that the colour intensity of muramic acid did not increase at the wavelength of maximum extinction for glucosamine (530 m μ) and consequently in hexosamine mixtures muramic acid might be determined as glucosamine at this wavelength. The amount of colour formed by muramic acid at 530 m μ was equivalent to $27.1 \pm 1.6\%$ of that produced by an equal weight of glucosamine base; this introduces a large error in the determination of muramic acid.

None of the simple colorimetric methods so far proposed allows accurate quantitative determination of glucosamine, galactosamine and muramic acid in a sample. The object of the present work was to develop a method of determining quantitatively the amounts of these hexosamines in bacterial preparations.

METHODS

Strains of bacteria

Escherichia coli 8196, *Pseudomonas aeruginosa* 6750, *Proteus vulgaris* 4175, *Staphylococcus aureus* 6571, *Streptococcus faecalis* 2400, *Micrococcus lysodeikticus* 2665 and *Mycobacterium phlei* 8151 were obtained from the National Collection of Type Cultures, London.

Amino sugars

D-Glucosamine hydrochloride and galactosamine hydrochloride were obtained from British Drug Houses Ltd., Poole, Dorset; synthetic muramic acid [2-amino-3-O'-(D-1'-carboxyethyl)-2-deoxy-D-glucose] was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of bacterial cell walls

E. coli, *Mic. lysodeikticus*, *Prot. vulgaris*, *Ps. aeruginosa* and *Staph. aureus* were grown on meat extract-agar (Stewart-Tull, 1965) in Roux flasks; *Strep. pyogenes* was grown in Hartley's digest broth containing 0.2% (w/v) of NaHCO_3 and 0.5% (w/v) of glucose (Roberts & Stewart, 1961); *Myc. phlei* was grown as a surface pellicle on a modified Sauton medium (Stewart-Tull & White, 1967).

Harvested cells were washed in 0.85% NaCl and centrifuged at 2500g. A thick cooled suspension of cells was mixed with an equal volume of no. 12 Ballotini beads in a homogenizer bottle and shaken in a Braun cell homogenizer MSK at 4000 rev./min. The machine and the cells were cooled during shaking by passing through the apparatus a stream of CO_2 from a cylinder. The disintegrated cells were separated by filtration through sintered glass. The filtrate was centrifuged at 1000g for 15 min. to remove any unbroken cells; the supernatant fluid was spun at 20000g for 30 min. The deposit of cell walls was washed twice with 0.85% NaCl and freeze-dried.

Cell-wall hydrolysates

Portions (20–25 mg.) of the cell-wall samples were hydrolysed in sealed tubes with 1 ml. of 6N-HCl at 105° for 18 hr. The hydrolysates were filtered to remove insoluble humins, evaporated to dryness over a boiling-water bath and finally resuspended in 1 ml. of water.

In the hexosamine determinations half of the hydrolysate was used in the modified Cessi & Piliego (1960) method and half was used in the Cessi & Serafini-Cessi (1963) method.

Determination of glucosamine plus galactosamine content and muramic acid content by using a modified Cessi & Piliego (1960) method

Acetylacetone reagent. Redistilled acetylacetone (b.p. 138–140°) was dissolved (1 ml.) in 100 ml. of bicarbonate buffer, pH 9.8. (This buffer contains 23.02 g. of Na_2CO_3 , 2.76 g. of NaHCO_3 and 5.84 g. of NaCl in 1 l. of water.) The pH was checked after the addition of acetylacetone and readjusted if necessary. This reagent was always prepared just before use.

p-Dimethylaminobenzaldehyde reagents. Reagent A was prepared by dissolving recrystallized p-dimethylaminobenzaldehyde (0.8 g.) in 100 ml. of ethanol containing 3.5 ml. of conc. HCl. Reagent B was prepared by dissolving recrystallized p-dimethylaminobenzaldehyde (0.8 g.) in 30 ml. of ethanol and adding 30 ml. of conc. HCl (A.R., sp.gr. 1.18). Reagents A and B were almost colourless and could be stored in a refrigerator for up to 14 days.

Distillation apparatus. Distillations were carried out in an apparatus consisting of a 100 ml. spherical flask connected to a 20 cm. condenser by a short glass adapter (Cessi & Piliego, 1960). The apparatus was heated directly over a bunsen flame and the contents were distilled at the rate of 1 ml./min.

Procedure. The solution (2 ml.) containing hexosamine or cell-wall hydrolysate was mixed with 5.5 ml. of acetylacetone reagent in glass-stoppered tubes. The stoppers were replaced, and the tubes were sealed with adhesive tape and heated in a boiling-water bath for 20 min. The mixture was cooled to room temperature and poured into a 100 ml.

distillation flask. The glass-stoppered tube was washed three times with 2 ml. of water and the washings were added to the mixture in the distillation flask. The distillate (4 ml.) was collected in a 10 ml. cylinder containing 6 ml. of p-dimethylaminobenzaldehyde reagent A. The extinctions were measured 30 min. later at 545 m μ to determine glucosamine with galactosamine.

The mixture (9 ml.) remaining in the distillation flask was cooled to 50° and added to 1 ml. of p-dimethylaminobenzaldehyde reagent B in a 10 ml. cylinder. The extinctions were measured 24 hr. later at 510 m μ to determine muramic acid.

Determination of galactosamine in the presence of glucosamine and muramic acid (Cessi & Serafini-Cessi, 1963)

The hexosamine solution or cell-wall hydrolysate (0.1–0.5 ml.) was placed in a test tube fitted with a ground-glass joint and dried *in vacuo* at 50°. The acetylacetone reagent (0.5 ml.), methanol-triethylamine-acetylacetone-pyridine (7:3:2:0.6:0.1, by vol.), was added to each tube. The stoppered tubes were incubated for 16 hr. in an oven at 55°. After this time the volatile reagents were removed *in vacuo* in as little time as possible (about 1 hr.). To the deposit was added 6 ml. of borate buffer, pH 8.0, at 35°. (This buffer contains 50.2 g. of $\text{K}_2\text{B}_4\text{O}_7 \cdot 4\text{H}_2\text{O}$ and 40.6 g. of H_3BO_3 in 1 l. of water.) The stoppered tubes were then heated in a boiling-water bath for 20 min. and subsequently the mixtures were cooled to 35°. The contents of a tube were transferred to the distillation apparatus together with two tube washings of 2 ml. of water; 4 ml. of distillate was collected in a 10 ml. cylinder containing 6 ml. of p-dimethylaminobenzaldehyde reagent (preparation as for reagent A of previous method). After 30 min., to allow development of the colour, extinctions were read at 550 m μ .

RESULTS

Preparation of standard hexosamine curves. The curves for glucosamine and muramic acid were prepared by using the modified Cessi & Piliego method; the curve for galactosamine was prepared by using the Cessi & Serafini-Cessi method. The extinction value after 30 min. of the colour obtained with the volatile chromogen of glucosamine in p-dimethylaminobenzaldehyde reagent A was maximal at 545 m μ . The extinctions for pure muramic acid standard under these conditions were not significant, the readings obtained being probably due to traces of contaminating hexosamine in the sample (two minor impurities amounting to 0.4%; Sigma Chemical Co. data); it was evident that the chromogen of muramic acid was not volatile. Therefore the mixture remaining in the distillation flask, usually 9 ml., was added to 1 ml. of p-dimethylaminobenzaldehyde reagent B (Rondle & Morgan, 1955) and the extinction read after 24 hr.; the maximum value was at 510 m μ . A mixture of glucosamine and muramic acid was examined by using the modified Cessi & Piliego method (Fig. 1).

It was noticed that the extinction value for the colour developed in reagent A (i.e. glucosamine) was less at 545 m μ after 24 hr., whereas the value of the colour developed in reagent B (i.e. muramic acid) increased at 510 m μ after 24 hr.

Fig. 1. Absorption spectrum of a mixture of glucosamine and muramic acid. In the modified Cessi & Piliego method glucosamine was determined in *p*-dimethylaminobenzaldehyde reagent A (●) and muramic acid was determined in reagent B (■). The maximum extinction value for muramic acid was at 510 m μ and for glucosamine was at 545 m μ . —, Readings taken after 30 min.; ----, readings taken after 24 hr.

The extinction value after 30 min. of the colour obtained with the volatile chromogen of galactosamine in the *p*-dimethylaminobenzaldehyde reagent of Cessi & Serafini-Cessi (1963) was maximal at 550 m μ . No extinction values were obtained for glucosamine or muramic acid in this test and these

Fig. 2. Standard hexosamine curves. Glucosamine and muramic acid were determined in *p*-dimethylaminobenzaldehyde reagents A and B respectively by using the modified Cessi & Piliego method. Galactosamine was determined by using the Cessi & Serafini-Cessi method. The proportionality factors were: for glucosamine 196, for galactosamine 234 and for muramic acid 450.

Table 1. *Determination of known amounts of hexosamines*

Determinations were carried out by the modified Cessi & Piliego method and the Cessi & Serafini-Cessi method, as described in the Methods section.

	Calc. wt. of hexosamine (μg.)			
	Modified Cessi & Piliego method		Cessi & Serafini-Cessi method	
	Reagent A Total glucosamine + galactosamine	Reagent B Muramic acid	Galactosamine	Glucosamine†
Muramic acid (50 μg.)	0.58	51.3	0	0.58
Glucosamine (50 μg.)	48.0	0	0	48.0
Galactosamine (50 μg.)	50.0*	0	50.3	0
Muramic acid (50 μg.) + glucosamine (50 μg.)	48.2	46.5	1.4	
Muramic acid (50 μg.) + galactosamine (50 μg.)	48.0*	49.0	49.1	
Muramic acid (50 μg.) + glucosamine (50 μg.) + galactosamine (50 μg.)	94.2	49.0	42.5	51.5

* Extinction for galactosamine corrected relative to standard glucosamine curve by multiplying the extinction value by the correction factor 0.73.

† The modified Cessi & Piliego test (reagent A volatile chromogens) represents total hexosamines, i.e. glucosamine and galactosamine. To calculate glucosamine values it is necessary to subtract the extinction value for galactosamine, corrected relative to the standard glucosamine curve, from that of the Cessi & Piliego test, i.e.:

$$E \text{ value (Cessi \& Serafini-Cessi)} \times \frac{234}{196} = x$$

$$[E \text{ value (Cessi \& Piliego)} - x] \times 196 = \mu\text{g. of glucosamine}$$

hexosamines did not interfere with the determination of galactosamine in mixtures.

From the standard curves, by plotting extinction values against hexosamines (Fig. 2), proportionality factors were calculated for glucosamine (196), galactosamine (234) and muramic acid (450). The intensity of the colour produced by an equivalent weight of muramic acid was less than that produced by galactosamine, which in turn was less than that produced by glucosamine.

Determination of standard weights of hexosamine by using the proportionality factors. Standard amounts (50 $\mu\text{g.}$) obtained from a 1 mg./ml. solution with the Agla micrometer syringe, of glucosamine, galactosamine and muramic acid were used, individually or as mixtures, in both the modified Cessi & Piliego method and in the Cessi & Serafini-Cessi method. The amounts of hexosamine in the samples calculated by this combined method are shown in Table 1. By this method the glucosamine standard was calculated to be 48 $\mu\text{g.}$ from the *p*-dimethylaminobenzaldehyde reagent A, and none was found in reagent B or in the Cessi & Serafini-Cessi test. With galactosamine the calculated amount for the Cessi & Piliego method was 50 $\mu\text{g.}$, after correction of the extinction value relative to the standard glucosamine curve (see Table 1); for the Cessi & Serafini-Cessi method the amount was 50.3 $\mu\text{g.}$ The muramic acid sample contained 0.58 $\mu\text{g.}$ of contaminating hexosamine from reagent A and 51.3 $\mu\text{g.}$ of muramic acid from reagent B. The results obtained with hexosamine mixtures were in general equivalent to those obtained by using single hexosamines in the tests. The slight variations might be due to errors in measurement of the hexosamines solution with the Agla micrometer syringe.

Determination of molar ratios of hexosamines in cell-wall samples. Cell-wall hydrolysates were examined by the modified Cessi & Piliego method and the Cessi & Serafini-Cessi method. The weights of the individual hexosamines were determined by multiplying the maximum extinction values by the respective proportionality factors. In the modified Cessi & Piliego method in reagent A both glucosamine and galactosamine are measured. Therefore to calculate the amount of glucosamine present in a cell-wall hydrolysate it was necessary to subtract the extinction value for galactosamine, corrected relative to the standard glucosamine curve, as follows:

$$E \text{ value (Cessi \& Serafini-Cessi)} \times \frac{\text{Proportionality factor for galactosamine}}{\text{Proportionality factor for glucosamine}} = x$$

$$[E \text{ value (Cessi \& Piliego: reagent A)} - x] \times 196 = \mu\text{g. of glucosamine}$$

The weights of muramic acid and galactosamine were directly calculated.

Molar ratios of glucosamine and galactosamine were calculated relative to muramic acid as 1.0 in all the cell-wall samples examined; the results are shown in Table 2. The values obtained for glucosamine, galactosamine and muramic acid were uncorrected for destruction during hydrolysis.

DISCUSSION

The method described for the quantitative determination of glucosamine, galactosamine and muramic acid uses a modified Cessi & Piliego method combined with the Cessi & Serafini-Cessi method. With the former method it was found that glucosamine and galactosamine formed a volatile chromogen after reaction with acetylacetone whereas muramic acid did not. This characteristic allowed the direct quantitative determination of muramic acid present in the mixture in the distillation flask after removal of the volatile chromogens of glucosamine and galactosamine. The Cessi & Piliego (1960) method was modified by increasing the amount of *p*-dimethylaminobenzaldehyde from 80mg. to 800mg. and by increasing the amount of distillate collected from 2ml. to 4ml. The increased volume collected ensured that the volatile chromogens were completely removed from the mixture so that there was no interference in the determination of muramic acid. In addition, any non-volatile chromogens of glucosamine and galactosamine remaining in the distillation mixture that would react with reagent B were too little to affect the measurement of muramic acid (Table 1). As shown by Crumpton (1959), the colour intensity for muramic acid at 510m μ increased to give a maximum extinction value after 24hr.; it was found that any colour produced by amino acids had faded after this time (Schloss, 1951).

In the Cessi & Serafini-Cessi (1963) method galactosamine can be determined in the presence of glucosamine. The method is based on the condensation of hexosamine with acetylacetone to a pyrrole retaining a substituent with some of the specific configuration of the sugar. Subsequently, the lateral chain is split by a specific complex-forming agent, borate (Tracey, 1955), and the 2-methylpyrrole evolved is allowed to react with *p*-dimethylaminobenzaldehyde. A volatile chromogen is not formed with glucosamine or muramic acid.

A comparison of the molar ratios of glucosamine

and muramic acid obtained by the method described in this paper (Table 2) with those published by other

Table 2. *Molar ratios of hexosamine in bacterial cell-wall preparations*

These results were determined by using the methods described in this paper. The size of each sample was 20 mg.

Cell-wall sample	Amount (μ mole/sample)			Molar ratios		
	Muramic acid*	Glucosamine*	Galactosamine*	Muramic acid	Glucosamine	Galactosamine
<i>E. coli</i>	0.351	0.920	0.005	1	2.62	0.015
<i>Mic. lysodeikticus</i>	0.318	0.356	0.007	1	1.11	0.022
<i>Myc. phlei</i>	0.269	0.610	—	1	2.20	—
<i>Prot. vulgaris</i>	0.290	0.428	0.013	1	1.47	0.04
<i>Ps. aeruginosa</i>	0.288	0.630	0.203	1	2.18	0.70
<i>Staph. aureus</i>	0.385	0.615	0.033	1	1.59	0.086
<i>Strep. pyogenes</i>	0.282	0.296	0.002	1	1.04	0.009

* Uncorrected for destruction during hydrolysis.

Table 3. *Quantitative determinations of glucosamine and muramic acid in bacterial cell-wall preparations*

This Table represents a compilation of previously published data for comparison with results obtained by using the described method.

Cell-wall preparation	Molar ratios		Reference
	Muramic acid	Glucosamine	
<i>E. coli</i>	1	1.2-2.0	Rogers (1962)
<i>Mic. lysodeikticus</i>	1	1.27	Czerkawski, Perkins & Rogers (1963)
<i>Myc. phlei</i>	1	1.8	Takeya, Hisatsune & Inoue (1963)
<i>Prot. vulgaris</i>	1	1.4	Rogers (1962)
<i>Staph. aureus</i> Oxford	1	1.15	Rogers & Jeljaszewicz (1961)
<i>Staph. aureus</i> H ₄	1	1.51	Strominger, Park & Thompson (1959)
Group C streptococci formamide-extracted cell-wall mucopeptide	1	1.05	Karakawa, Lackland & Krause (1966)

workers using different methods (Table 3) shows that there is reasonable agreement between the two sets of results. Perkins (1963) has suggested that galactosamine may be a component of essentially polysaccharide constituents distinct from the mucopeptide; no published results were found of the molar ratio of galactosamine in the cell walls of the species examined. In most species the molar ratio of galactosamine was very small; however, in *Staph. aureus* it is noteworthy that 5% of the hexosamine, other than muramic acid, occurred as galactosamine. In *Ps. aeruginosa* the ratio of muramic acid to galactosamine was 1.0:0.7 and this might indicate that in this species galactosamine may be a major structural component of the cell wall. Clarke, Gray & Reaveley (1967a) detected galactosamine in the cell walls of *Ps. aeruginosa* N.C.T.C. 1999 by paper chromatography, but no quantitative determinations of galactosamine were obtained. After extraction of the cell walls with aqueous phenol Clarke, Gray & Reaveley (1967b) found 1.55-2.23% of galactosamine in material from the aqueous layers.

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