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The Determination of Galactosamine in the Presence of Glucosamine

APPLICATION TO *ASPERGILLUS NIGER* CELL WALLS

BY I. R. JOHNSTON
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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Methods for the estimation of galactosamine in the presence of glucosamine involve modification of the galactosamine (Scott, 1962) or become increasingly inaccurate for the minor component when it represents less than one-fifth of the total hexosamine (Gardell, 1958). Cessi & Serafini-Cessi (1962), by a distillation method, have used borate to release selectively the 2-methylpyrrole (the most important Elson–Morgan chromogen), due to D-galactosamine, from the mixture of the acetylacetone condensation products with D-galactosamine and D-glucosamine. Slein (1952) estimated galactosamine directly. Using yeast hexokinase, he showed that glucosamine but not galactosamine was phosphorylated in the presence of ATP. The phosphorylation of glucosamine under these conditions is well authenticated (Brown, 1951; Grant & Long, 1952). Glucosamine 6-phosphate was then removed by precipitation with barium hydroxide–zinc sulphate, leaving galactosamine to be determined in the supernatant. Slein suggested that the method would be useful when only small amounts of galactosamine were present in a mixture with glucosamine. The present paper extends Slein's (1952) observations and improves the method of separating galactosamine from other products in the reaction mixture. The method is applied to *Aspergillus niger*-cell-wall hydrolysates.

MATERIALS

D-Glucosamine hydrochloride (W. Kerfoot and Sons, Burnley, Lancs.) was recrystallized from 3N-HCl, water and finally by a methanol–acetone–water procedure (Roseman & Ludowieg, 1954) to give $[\alpha]_D^{25}+71{\cdot}8°$ (*c* 2·0 in water). D-Galactosamine hydrochloride (L. Light and Co. Ltd., Colnbrook, Bucks.) had $[\alpha]_D^{25}+96{\cdot}0°$ (*c* 2·4 in water); another sample (California Corp. for Biochemical Research), recrystallized once from methanol–acetone–water, had $[\alpha]_D^{25}+96{\cdot}4°$ (*c* 3·2 in water). All samples were chromatographically pure with respect to other amino sugars, non-nitrogenous sugars and amino acids. Dowex 50 (minus 400 mesh, 200–325 wet-mesh range; X 5) (California Corp. for Biochemical Research) was used in the ion-exchange columns. ATP was the disodium salt (98% pure) (Sigma Chemical Co.); solutions were adjusted to pH 6·5–7·0 with NaOH before use.

Yeast hexokinase was Sigma grade 3 (150 000 Kunitz–MacDonald units/g.). It had no phosphatase activity; the use of such a grade of enzyme is highly important in the assay described in this paper.

CO_2-free NaOH (0·01N) was prepared by 'method B' of Vogel (1951). All other chemicals were of analytical reagent quality.

Phosphate buffer (0·25M) was made by dissolving 39 g. of $NaH_2PO_4,2H_2O$ in ion-free water, adjusting the pH to 7·62 with 2N-NaOH and making up to 1 l. Acetate buffers (0·1 and 1·0M) were made by adjusting solutions, containing 0·1 and 1·0 mole of NaOH respectively in 900 ml. of ion-free water, to pH 5·2 with acetic acid and then making up to 1 l. Tris buffer was the buffer at pH 7·6 and *I* 0·2 described by Datta & Grzybowski (1961).

METHODS

Assay methods. Total hexosamine was estimated by the method of Boas (1953), with glucosamine hydrochloride as standard.

Titrimetric experiments were done in the Radiometer titrator (Type TTT/C) with a Titrograph assembly (Type

SBR2c) (both supplied by Radiometer, 72 Emdrupvej, Copenhagen, NV).

Phosphate was estimated by the method of Chen, Toribara & Warner (1956).

Hydrolysis of cell walls. Cell walls of *A. niger* were prepared by the method outlined by Crook & Johnston (1962), with a Mickle tissue disintegrator and a Braun cell homogenizer (Braun, Apparatebau, Melsungen, W. Germany). Samples (25 mg.) were heated in 4 ml. of 4N-HCl for 24 hr. at 100° to give optimum release of hexosamine. Under these conditions, glucosamine was destroyed to the extent of 12·5%. Solutions were filtered, HCl was removed *in vacuo*, and the residue (containing about 3 mg. of hexosamines), in 3 ml. of 0·2N-HCl, was loaded on a column of Dowex 50 (H^+ form) (Boas, 1953), washed with 10 ml. of water and eluted with 10 ml. of 2N-HCl. A sample (1 ml.) of the resulting solution on suitable dilution was used for the determination of total hexosamine; the remainder, after complete removal of HCl, was dissolved in 5 ml. of water (12–14 μmoles of hexosamine present) and used for the assay of galactosamine.

Growth of Aspergillus niger. A modified Czapek–Dox medium (Oxoid CM95, Oxo Ltd., London) was used. Spores from a malt extract–yeast extract slope were transferred to the medium (90 ml.), shaken for 24–30 hr. (204 strokes/min.) and inocula (10 ml.) taken from this culture were transferred to 190 ml. portions of medium. Cultures were then usually shaken for 54 hr. Growth was at 30°. Variations from these conditions are mentioned in Table 4.

Table 1. *Consumption of alkali during phosphorylation of hexosamines*

The reaction mixtures contained in addition to the amino sugars (as hydrochlorides): 30 μmoles of $MgCl_2$, 10 μmoles of ATP and about 6 μmoles of NaOH to bring the pH initially to 7·62; 50 units of yeast hexokinase were used to start the reaction; the pH was maintained constant by the addition of 0·01N-NaOH by the Radiometer titrator. Reaction mixtures (final vol. 3·1–3·7 ml.) were bubbled with CO_2-free nitrogen. The temperature of the reaction vessel was 25°.

Expt. no.	Hexosamine added (μmoles)		NaOH used (μmoles)
	Galactosamine	Glucosamine	
1	—	2·20	1·70
2	2·25	—	0
3	0·14	3·30	2·75
4	—	3·30	2·75

RESULTS

Phosphorylation experiments. Slein's (1952) method depends on the non-phosphorylation of galactosamine and this was first checked. In the phosphorylation of glucosamine by ATP in the presence of yeast hexokinase, 1 proton/molecule is released and this can be followed titrimetrically. Typical results obtained with glucosamine and galactosamine are shown in Table 1. It is evident that galactosamine is not phosphorylated. However, the uptake of alkali with glucosamine is only about 80% of that expected. This is probably due to an increase in the pK of the amino group (by about 0·5–1·0 pK unit) on phosphorylation of the molecule (the pK of the amino group of glucosamine is probably about 7·8; Dr S. P. Datta, personal communication). Thus, at pH 7·6. some of the released protons from the reaction protonate the amino group and are not titratable. The results shown in Table 2 provide further evidence for the non-phosphorylation of galactosamine and reveal that glucosamine is completely phosphorylated.

Factors affecting the separation and estimation of galactosamine. Preliminary experiments showed that the precipitation procedure with barium hydroxide–zinc sulphate (Slein, 1952) leaves small amounts of glucosamine (presumably as glucosamine 6-phosphate) in the supernatant. For example, in one experiment phosphorylating mixtures were set up containing, respectively, glucosamine hydrochloride (720 μg.) plus galactosamine hydrochloride (29 μg.), glucosamine hydrochloride (720 μg.), and galactosamine hydrochloride (29 μg.). After precipitation with barium hydroxide–zinc sulphate and removal of a sample (3 ml. from 5·7 ml.) for the Elson–Morgan determination, the corresponding extinctions observed were (average of duplicates): 0·088, 0·024, and 0·064. Allowing for the low extinctions—a consequence of carrying out the Elson–Morgan reaction in the presence of tris buffer—it is clear that the separate galactosamine and glucosamine determinations are additive for the mixture. Thus the glucosamine was not completely removed and remained at a level comparable with that of

Table 2. *Disappearance of acid-labile phosphate during phosphorylation of hexosamines*

The incubation mixtures contained in addition to the amino sugar (as hydrochloride): 10 μmoles of $MgCl_2$, 2·5 μmoles of ATP, 25 units of yeast hexokinase and 51·4 μmoles of tris buffer, pH 7·6, in a final vol. of 0·9 ml. Incubation was at 30° for 1 hr. The reaction was stopped by adding 1·1 ml. of 10% (w/v) trichloroacetic acid. After centrifuging, 1 ml. of the supernatant was added to 1 ml. of 2N-HCl, heated for 7 min. at 100°, cooled, diluted to 10 ml. and a sample (0·5 ml.) taken for the determination of phosphate.

Hexosamine added (μmoles)		Acid-labile phosphate present (μmoles)		
Glucosamine	Galactosamine	Initially	Finally	Utilized
1·93	—	4·91	2·91	2·00
—	1·73	4·91	4·91	0

galactosamine. These results were confirmed by using a similar set of reaction mixtures obtained after phosphorylation in titrimetric experiments. Brown (1951) also found the procedure to remove 'substantially all' of the phosphorylated glucosamine, but presumably not all. In the present experiments shaking reaction mixtures with Dowex 1 (CO_3^{2-} form) was ineffective in removing glucosamine 6-phosphate, whereas passage through a small column of Dowex 2 (Cl^- form) gave recovery in too big a volume. Finally it was found possible to separate galactosamine from mixtures and to recover it in a small volume (5 ml.) by passing reaction mixtures through a column of Dowex 50 buffered to pH 5·2, subsequently eluting the galactosamine with 2N-hydrochloric acid. The method is thus a modification of that of Boas (1953). It was important to equilibrate columns with a strong buffer (I 1·0) followed by a weak buffer (I 0·1), otherwise contraction of the resin in strong buffer prevented complete equilibration. Further, because of contraction and expansion of the resin, elution with 2N-hydrochloric acid from small columns used in the Boas (1953) method did not achieve quantitative recovery if hexosamines had been loaded in water. For the determination of total hexosamine this was avoided by loading the hexosamines in 0·2N-hydrochloric acid. Galactosamine could also be recovered quantitatively if loaded in solutions of I 0·1–0·2; reaction mixtures here had I about 0·2. Buffer should be absent from the final Elson–Morgan reaction, otherwise depression of colour and poor reproducibility ensue. Finally, columns should contain fairly similar amounts of resin so that the amount of sodium chloride eluted from the columns with 2N-hydrochloric acid is about the same, thus avoiding a variable depression of colour formation in the Elson–Morgan reaction (cf. Boas, 1953).

The method finally devised for the separation of galactosamine is outlined below. Ten small columns of Dowex 50 (X5) were prepared as described by Boas (1953) by quickly pipetting 5 ml. of a 1:1 (w/v) suspension of resin in water into glass tubes, (1 cm. × 25 cm.) tapering to 1–2 mm. at the tip. These were buffered at pH 5·2 by passing 10 ml. of M-acetate buffer followed by 10 ml. of 0·1M-acetate buffer through the columns. This routine was critical for equilibration. Incubation mixtures for the determination of galactosamine typically had the composition outlined under Table 4 for *A. niger* cell walls. Incubation mixtures were set up in duplicate or triplicate for both the unknown and for the galactosamine standards (25 μg.), and arranged so that final estimates were made on 25–60 μg. of galactosamine hydrochloride. A water blank was also included. Enzyme was added to all mixtures to eliminate any subsequent chromogenic interference from it. Incubation was at 30° for 1 hr. to ensure complete phosphorylation of the amounts of glucosamine in Tables 3 and 4. After incubation, 0·025 ml. of chlorophenol red solution was added to each mixture followed by 0·1 ml. of M-acetate buffer. Mixtures were titrated to pH 5·2 with 0·1N-hydrochloric acid against the same amount of indicator in 0·1M-acetate buffer. The same volume of acid was always required for each mixture. A portion (1–5 ml.) of the resulting solution or of a dilution of the same, containing 100–300 μg. of galactosamine (the amount chosen to allow 25–60 μg. to be taken for the Elson–Morgan determination), was then placed on a buffered ion-exchange column. In any one series of tubes (*a*) dilution if necessary was to the same volume and (*b*) the volume put on the column was the same. The reaction mixture was allowed to pass through the column, then 5 ml. of 0·1M-acetate buffer, pH 5·2, was added to complete the removal of the indicator, ATP, ADP, PO_4^{3-} ions, glucosamine 6-phosphate and enzyme (isoelectric point 4·6–4·8). Finally acetate was removed by passing 5 ml. of ion-free water through the column, and the galactosamine was eluted with 2N-hydrochloric acid into a 5 ml. flask (elution is complete in this volume) and estimated as described by Boas (1953). For analysis the same volume of eluate must be taken from each flask in any one series. In calculating the percentage of total hexosamine which was galactosamine, allowance was made for the fact that the colour yield of galactosamine was only 89 % of that of glucosamine in the Boas assay (cf. Crumpton, 1959).

Determination of galactosamine in experimental mixtures. Mixtures of the two hexosamines were set up as shown in Table 3 and determinations of galactosamine made as outlined above. Recoveries were calculated from a single concentration of galactosamine hydrochloride since Beer's law holds in the method of assay used (Boas, 1953) up to about 50 μg. of amino sugar. This was checked and the upper limit was found to be about 100 μg., although determinations were usually made on less than 60 μg. of galactosamine hydrochloride. Because of the large amount of sodium chloride eluted from the buffered columns, the colour yield for a given weight of amino sugar was approximately two-thirds of that in the original method of Boas (1953).

The results show that a clear-cut separation of galactosamine from glucosamine was achieved, and are further evidence for the complete phosphorylation of glucosamine and the non-phosphorylation of galactosamine. The mean recovery was 99·7 % (S.D. ± 1·7 %) for 14 results.

Application of the method to the determination of galactosamine in Aspergillus niger *cell walls.* Seven preparations of the cell walls of *A. niger* were

Table 3. *Recovery of galactosamine from experimental mixtures with glucosamine*

Incubation mixtures contained in addition to the amino sugars (as hydrochlorides): 30 μmoles of $MgCl_2$, 5–8 μmoles of ATP, 150 μmoles of NaH_2PO_4, pH 7·6, and 50 units of yeast hexokinase in a final vol. of 2·9 ml. Incubation was at 30° for 1 hr. Galactosamine was then estimated as described in the text. Each value recorded under 'Hexosamine recovered' refers to a separate incubation mixture.

Hexosamine added to incubation mixture (μg.)			Hexosamine recovered (μg.)	Percentage recovery based on galactosamine added
Glucosamine		Galactosamine		
718	+	0	0, 0	0, 0
718	+	29	29, 29	100, 100
604	+	49	50, 48, 49	102, 98, 100
754	+	77	77, 83, 77	100, 108, 100
604	+	102	102, 104, 100	100, 102, 98
747	+	144	142, 145	99, 101
0		102	100, 101	98, 99

Table 4. *Hexosamine content of preparations of* Aspergillus niger *cell walls*

Hydrolysates were prepared and analysed as described in the text. Incubation mixtures for galactosamine estimation contained: 30 μmoles of $MgCl_2$, 6–10 μmoles of ATP, 150 μmoles of NaH_2PO_4, pH 7·6, 3·5 μmoles of total hexosamine (containing in these experiments up to 0·55 μmole of galactosamine) and 50 units of yeast hexokinase in a final vol. of 2·9 ml. Incubation was at 30° for 1 hr. 'Total hexosamine' is the value for galactosamine and glucosamine calculated as free amino sugar, not corrected for destruction. 'Hexosamine' is expressed as free, not anhydro, amino sugar. Preparations 1 and 2 were slow-growing, having been started from slopes, 8 and 7 weeks old respectively. By serial transfer, all other cultures were started from material which had been brought to a maximal rate of growth.

Prep. no.	Total hexosamine (μg./mg. of cell wall)	Galactosamine (% of total hexosamine)	Hexosamine (% of cell wall)		Growth conditions	
			Glucosamine	Galactosamine	Duration of shaking (hr.)	Temp.
1	89	17·7	7·3	1·60	52	30°
2	97	12·1	8·6	1·17	47	30
3	116	6·3	10·9	0·70	54	30
4	122	5·2	11·5	0·64	54	30
5	128	6·2	12·0	0·79	54	30
6	120	6·9	11·2	0·83	54	30
7	95	4·0	9·1	0·38	54	33

made—nos. 3, 4, 5 and 6 in the Mickle tissue disintegrator and nos. 1, 2 and 7 in the Braun cell homogenizer. Analyses were then made as shown in Table 4. The values are not corrected for destruction during hydrolysis. Results were reproducible; thus from one hydrolysate of preparation 1, values of 17·3 and 17·5 % were obtained, and from another 17·5, 18·0 and 18·0 %.

DISCUSSION

The method outlined fulfils a need for an accurate way of determining galactosamine directly when present in admixture with a large amount of glucosamine. Thus the method is capable of quantitatively separating and of accurately determining galactosamine when it represents as little as 4 % of the total hexosamine. Presumably galactosamine present in proportions much higher than those studied here can be determined by the method. It is not known how other amino sugars would react in the incubation mixtures. The basis of the method, introduced by Slein (1952), has the merit that no chemical modification of the galactosamine, involving possible loss, is necessary during the determination.

The results for the hexosamine content of the cell wall of *A. niger* are variable. However, from records of cultural conditions, the results of Table 4 have been listed approximately in the order of physiological age of the cultures and support the idea of a progressive depletion, or dilution with other sugars, of the cell-wall galactosamine as the age of the culture increases. The results of Distler & Roseman (1960) on the acid-washed mycelium of *Aspergillus parasiticus* show the galactosamine content to fall, and glucosamine to rise and fall, as growth proceeds. The present results show a similar trend.

SUMMARY

1. The observations of Slein (1952) on the non-phosphorylation of galactosamine by ATP and yeast hexokinase have been confirmed.

2. A method is presented for separating galactosamine from glucosamine before assaying it.

3. The method is applied to the analysis of hydrolysates of *Aspergillus niger* cell wall.

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Studies on Carbohydrate-Metabolizing Enzymes

9. THE ACTION OF ISOAMYLASE ON AMYLOSE*

BY O. KJØLBERG AND D. J. MANNERS
Department of Chemistry, University of Edinburgh

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The incomplete degradation of amylose by β-amylase was first shown by Peat, Pirt & Whelan (1952*a*, *b*), who reported β-amylolysis limits of only 68–70% for various samples of amylose. These observations have been confirmed by several workers, e.g. Hopkins & Bird (1953), Neufeld & Hassid (1955), Cowie, Fleming, Greenwood & Manners (1957). Peat *et al.* (1952*a*, *b*) also showed that impure preparations of β-amylase caused complete degradation of amylose owing to the concurrent action of β-amylase and a second carbohydrase, 'Z-enzyme'. At this time (1952–3) Z-enzyme was believed to exert a debranching action on amylose (Peat & Whelan, 1953), and, since Z-enzyme preparations showed β-glucosidase activity, it was suggested that the barriers to β-amylase were β-glucosidic linkages (Peat, Thomas & Whelan, 1952*c*). However, later work (Cunningham, Manners, Wright & Fleming, 1960; Banks, Greenwood & Jones, 1960) has shown that Z-enzyme activity is caused by a trace of α-amylase, and involves the random hydrolysis of a small number of α-(1→4)-glucosidic linkages. The barriers to β-amylase in amylose are therefore bypassed by Z-enzyme, and not selectively removed as was originally suggested.

An additional complication in this work was the finding by Gilbert (1958), later confirmed by others (Banks, Greenwood & Thomson, 1959; Liddle, Manners & Wright, 1961), that oxygen treatment of amylose introduces barriers to enzymic degradation. It is therefore necessary to prepare amylose under anaerobic conditions to avoid inadvertent modification of the substrate, as almost certainly occurred in the earlier studies.

The nature of the barriers to β-amylase has been the subject of several investigations. Possibilities include (*a*) an anomalous linkage [i.e. other than the α-(1→4)-glucosidic type] in the middle of an amylose chain, (*b*) a branch point, or (*c*) the presence of ester phosphate groups, although treatment of amylose with phosphatase preparations does not increase the β-amylolysis limit (Peat *et al.* 1952*c*; Banks & Greenwood, 1961). We have therefore re-examined the possibility that the anomalous linkages represent α-(1→6)-glucosidic inter-chain linkages of the type present to the extent of 4–5% in amylopectin.

The presence of anomalous linkages in amylose is inferred from the specificity requirements of

* Part 8: Cunningham, Manners & Wright (1962).