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The Determination of Glucosamine and Galactosamine in some Glycoproteins by Radioisotope Dilution

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1. The principle of radioisotope dilution was applied on a semi-micro scale to the determination of glucosamine and galactosamine in some glycoproteins, such as immunoglobulins, a urinary glycoprotein and blood-group-specific substances. 2. The glycoprotein was hydrolysed in the presence of [1-^{14}C]glucosamine or [1-^{14}C]-galactosamine or both. The amino sugars were made to react with naphthyl isothiocyanate and the products formed were isolated by the method of Scott (1962). The specific radioactivities determined from liquid-scintillation counting and the extinction at 240mμ or 222mμ were used to calculate the content of amino sugars in the protein analysed. 3. Where the values could be compared with those found by other workers, differences were in general not very great. The advantages of the method are that high concentrations of acid can be employed and undesirable side reactions, which may occur with the free sugars, do not affect the results. A potential source of error of the method is discussed.

Methods for the determination of hexosamine in glycoproteins depend on the complete release and recovery of amino sugars from glycoproteins after acid hydrolysis. The use of high acid concentrations and high temperatures during hydrolysis are desirable (Johansen, Marshall & Neuberger, 1960) to minimize the formation of acid-stable hexosaminidic linkages (Moggridge & Neuberger, 1938). Under severe hydrolytic conditions appreciable destruction of the hexosamines can occur (see Neuberger & Marshall, 1966). In the present work the principle of radioisotope dilution as used previously for non-amino sugars (François, Marshall & Neuberger, 1962; Graham & Neuberger, 1968) was applied to the determination of 2-aminohexoses in glycoproteins. In this procedure loss of amino sugars due to destruction during hydrolysis and to reaction with other substances such as uronic acids (Ogston, 1964; Jacobs & Muir, 1963) does not affect the accuracy of the method, provided that free and bound hexosamines are destroyed at the same rate.

The usual method for the determination of glucosamine and galactosamine depends on their separation on columns of sulphonic acid resin (Gardell, 1953) and their assay by one of the many modifications of the Elson–Morgan method (see Neuberger & Marshall, 1966). A method has been described in which the amino sugars are allowed to react with phenyl isothiocyanate and the derivatives formed separated by paper electrophoresis in sodium molybdate buffer and measured by their extinction at 240mμ (Scott, 1962). These compounds are most probably 4-hydroxy-3-phenyl-5-tetrahydroxybutylimidazolidinethiones (Scott, 1964). The method has been modified by Dr J. E. Scott (personal communication), who increased the sensitivity by replacing phenyl isothiocyanate by naphthyl isothiocyanate (Stockwell & Scott, 1967) and who improved the procedure for isolating the amino sugar derivatives.

MATERIALS AND METHODS

Materials. [1-^{14}C]Glucosamine hydrochloride was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. It was diluted with non-radioactive sugar in aqueous solution to give a specific radioactivity of about 0·2mc/g. The sugar was purified by chromatography on a column of Dowex 50 (X4; H^+ form; 200–400 mesh) with 0·33N-HCl as the solvent and crystallized from aqueous ethanol.

[1-^{14}C]Galactosamine hydrochloride was synthesized from D-lyxose as described by Kuhn, Leppelman & Fischer (1959), except that the H^{14}CN was generated in a closed system and allowed to diffuse into the stirred suspension of lyxosylamine in dry pyridine. D-Lyxose was prepared from calcium D-galactonate by the Ruff degradation or purchased from Sigma (London) Chemical Co., London, S.W. 6.

Methyl *N*-acetyl-β-D-glucosaminide (2-acetamido-2-deoxy-β-D-glucopyranoside) was isolated by chromatography on purified Celite 545 (Hall, 1962*a*) from a mixture of the anomers prepared by the method of Zilliken, Rose, Braun & György (1955). The anomeric mixture (0·4g.) was

dissolved in the lower phase (1·12ml.) of the solvent system ethyl acetate–propan-1-ol–water (4:1:2, by vol.), and the solution was mixed with Celite 545 (2·54g.; Johns–Manville Corp., New York, N.Y., U.S.A.). The moist solid was transferred to a column (2cm. wide) of Celite 545 (65g.) packed by the method of Hall (1962*b*). The column was developed with the upper phase of the solvent system and a portion (0·3ml.) of each fourth fraction (10ml.) was evaporated to dryness. The residue was dissolved in water (3ml.) and the extinction at 205mμ was measured. Fractions numbered 72–100 were pooled and evaporated to dryness and the residue was crystallized from propan-1-ol to give the α-anomer (yield 111mg.), m.p. 194–195° and $[\alpha]_D^{24}$ +128° (*c* 1·0 in water). Fractions numbered 130–175 were treated similarly to give the β-anomer (yield 173mg.). Recrystallization from ethanol gave material with m.p. 197–200° and $[\alpha]_D^{24}$ +45° (*c* 1·0 in water).

Naphthyl isothiocyanate was a product of Kodak Ltd., Kirkby, Lancs. Pyridine was refluxed over KOH and distilled. Dimethylaminododecane was a gift from Armour Hess Chemicals Ltd., Leeds.

Blood-group-specific substances from ovarian-cyst fluids were a gift from Professor W. T. J. Morgan, F.R.S. Rabbit γ-globulin was prepared by precipitation (five times) with Na_2SO_4 by the procedure of Kekwick (1940). Human urinary glycoprotein, prepared by the method of Tamm & Horsfall (1952), was given by Miss W. Smith of this Laboratory.

Measurement of radioactivity. Portions (0·5ml.) were either added to 6·5ml. of scintillator mixture (XDC; Bruno & Christian, 1961) and counted in a scintillation counter (Tritomat; Isotopes Development Ltd., Reading) or dried on aluminium planchets at 70° under reduced pressure and counted in a gas-flow Geiger–Müller counter (model D47; Nuclear–Chicago Corp.). Another method of scintillation counting used involved drying smaller portions (0·1ml.) on glass-fibre disks (Davies & Cocking, 1966) as described previously (Graham & Neuberger, 1968). For all methods the number of counts recorded increased linearly with the amount of radioactive sugar present up to at least 20μg. The presence of barium acetate decreased the efficiency of scintillation counting by about 3% and the efficiency of gas-flow counting by about 16%.

Hydrolysis of glycoproteins. Blood-group-substance samples were dried to constant weight over P_2O_5 *in vacuo* at 78°. Rabbit γ-globulin was dissolved in 0·01N-HCl and the concentration was determined from the extinction at 278mμ by taking $E_{1cm.}^{1\%}$ to be 13·5 (Crumpton & Wilkinson, 1963). Urinary glycoprotein prepared by the method of Tamm & Horsfall (1952) was dissolved in water and a portion was dried at 100° to constant weight to determine the concentration. A mixture of known amounts of glycoprotein and radioactive amino sugars in 6N-HCl (twice-distilled) was heated in sealed evacuated tubes at 100° for 6hr. The tube contents were concentrated to dryness *in vacuo* over NaOH and H_2SO_4.

Reaction with naphthyl isothiocyanate. The dried residue containing the hexosamine hydrochloride was dissolved in water (0·1ml.) and mixed with the reagent (0·2ml.) in a small stoppered centrifuge tube. The reagent consisted of 4% (w/v) naphthyl isothiocyanate in 5% (v/v) dimethylaminododecane in pyridine. The tube was heated at 56° for 15min. and cooled. The solution was extracted successively with about 2ml. each of 1% (v/v) dimethylaminododecane in benzene, benzene–*n*-heptane (1:1, v/v) and *n*-heptane. Samples of the aqueous phase were applied as lines to Whatman no. 4 or Whatman Benchcote paper (previously washed with water) and submitted to electrophoresis in 0·05M-sodium tungstate buffer adjusted to pH7·0 by the addition of freshly washed Dowex 50 (H^+ form). After electrophoresis for 1hr. with a potential gradient of about 45v/cm. the paper was dried and the spots were marked under an ultraviolet lamp with maximum emission at 257mμ. The spots were excised, cut into strips and shaken with 0·5% barium acetate (2·5ml.) for 20min. The paper was removed and the tubes were centrifuged. The extinction of the supernatant was measured at the extinction maximum of 222mμ and portions were assayed for radioactivity. In earlier experiments the extinction was measured at 240mμ, where there is a 'shoulder' on the extinction curve. The molar extinction coefficient at 222mμ (about 70000) is much greater than that at 240mμ (about 19000), but blank values are higher at the lower wavelength.

Determination of glucosamine by the Elson–Morgan reaction. The modification described by Kraan & Muir (1957) was used. The Cessi modification was scaled down to half of that described by Johansen *et al.* (1960) and the alkaline acetylacetone reagent was prepared by the method of Kraan & Muir (1957).

The hexosamine derivatives were not repurified to constant specific radioactivity. However, the possibility of interference by amino acids was investigated by carrying out the naphthyl isothiocyanate reaction on a mixture of 17 amino acids (0·125μmole of each; amino acid calibration mixture type I; Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). Mannose (0·54μmole) was also included so that the mixture would closely approximate to a glycoprotein hydrolysate. The reaction with naphthyl isothiocyanate, the extraction and electrophoresis were carried out under the same conditions. Under ultraviolet light two spots, which had moved 1·3cm. and 3·0cm., both towards the cathode, were revealed. When glucosamine was added to the amino acid mixture before the reaction a spot was seen that had moved 11·4cm. towards the anode. The galactosamine derivative migrated under the same conditions 6·3cm. towards the anode. These observations show that the napthyl isothiocyanate–amino acid reaction products that had not been extracted by organic solvents migrated towards the cathode and thus did not interfere with the determination of hexosamine.

RESULTS

To effect complete liberation of the amino sugars from their glycosidic linkages hydrolysis was carried out in 6N-hydrochloric acid for 6hr. at 100°. The hydrolysis mixture was heated *in vacuo* to avoid destruction by oxygen (Walborg & Ward, 1963). Johansen *et al.* (1960) found that hydrolysis of egg albumin or its glycopeptide in 5·7N-hydrochloric acid for 6hr. at 100° gave nearly the same amount of glucosamine as when standard conditions of hydrolysis (4N-hydrochloric acid for 6hr. at 100°) were employed, suggesting that glucosamine is fairly stable under these conditions. When D-[1-^{14}C]-glucosamine hydrochloride (0·453mg.) and methyl

Table 1. *Determination of known amounts of galactosamine*

[1-^{14}C]Galactosamine, diluted with various amounts of the non-radioactive sugar, was made to react with naphthyl isothiocyanate and the product was isolated by electrophoresis. Radioactivity was measured by scintillation counting on glass-fibre disks.

[1-^{14}C]Galactosamine hydrochloride (μg.)	Galactosamine hydrochloride (μg.)	Sp. radioactivity (counts/min./E_{222} unit)	Measured amount of galactosamine hydrochloride (μg.)	(%)
170·8	0	148·1	—	—
85·4	56·5	87·9	58·5	103
85·4	113·2	64·4	111·2	98
85·4	226·4	38·3	245·0	108

N-acetyl-β-D-glucosaminide (0·543mg.) were together subjected to these hydrolytic conditions, the amount of glucosamine recovered as measured by the Elson–Morgan method was 97% of theory. On the basis of the specific radioactivities (measured by counting at infinite thinness) before (85·7counts/min./μg.) and after (41·0counts/min./μg.) dilution, the recovery was 99%.

The naphthyl isothiocyanate derivatives of galactosamine and glucosamine were well separated by electrophoresis, moving distances of about 8cm. and 15cm. respectively during 1hr. in 0·05M-tungstate buffer at pH7 with an applied voltage of about 45v/cm. The assay of known amounts of galactosamine by using radioisotope dilution with the naphthyl isothiocyanate method gave reasonable results where the dilution of labelled sugar was not great (Table 1).

Glucosamine was determined in methyl *N*-acetyl-β-D-glucosaminide (0·185mg.) by hydrolysis in the presence of D-[1-^{14}C]glucosamine hydrochloride (0·165mg.) and comparison of the specific radioactivity (69·6counts/min./E_{222} unit) of the resulting glucosamine as the naphthyl isothiocyanate derivative with the specific radioactivity (140·2counts/min./E_{222} unit) of the same derivative prepared from the added D-[1-^{14}C]glucosamine hydrochloride. Radioactivity was measured by liquid-scintillation counting of the compounds on glass-fibre disks. From these results the glucosamine content of the glycoside was calculated to be 0·139mg., or 98·6% of theory.

Glucosamine content of rabbit immunoglobulin G. Rabbit immunoglobulin (15·2mg.) was hydrolysed in duplicate in the presence of D-[1-^{14}C]glucosamine hydrochloride (0·180mg.) and the dried hydrolysate was made to react with naphthyl isothiocyanate. As the products showed considerable trailing on electrophoresis the fluorescent areas on the paper were cut out and resubmitted to electrophoresis. The specific radioactivities of the eluted materials and of the added D-[1-^{14}C]glucosamine (186 and 379counts/min./E_{222} unit respectively, where radioactivity was measured at infinite thinness) gave values for the glucosamine content of 0·156 and 0·156mg., which correspond to 5·7 residues/immunoglobulin unit of mol.wt. 10^5, or 8moles/140000g. The hexosamine content assayed by the Cessi modification of the Elson–Morgan method after hydrolysis with 4N-hydrochloric acid for different times gave the following values: 6·3 and 5·9 moles/10^5g. after 4hr.; 5·5 and 5·9 moles/10^5g. after 6hr.; 5·7 and 5·8 moles/10^5g. after 8hr.

Hexosamine values previously reported are 5·9 moles/10^5g. (Fleischman, Porter & Press, 1963) and 7·0 moles/10^5g. (Utsumi & Karush, 1965); both values were obtained after hydrolysis in 4N-hydrochloric acid at 100° for 6hr.

Determination of glucosamine and galactosamine in blood-group-specific substances. Three samples of blood-group-specific substances (two B substances, preparation nos. 376 and 413, and an Le^a substance, preparation no. 350, Pusztai & Morgan, 1964) were hydrolysed in the presence of a mixture of ^{14}C-labelled glucosamine and galactosamine (Table 2). On combining the average glucosamine and galactosamine values for each substance, the total hexosamine contents are 31% for B 376, 29% for B 413 and 25% for Le^a 350. These values are similar to those found by Professor W. T. J. Morgan (personal communication), who applied the Elson–Morgan method after hydrolysis with 0·5N-hydrochloric acid at 100° for 18–20hr.: 33%, 28% and 27% respectively. The galactosamine values found for the B substances nos. 376 and 413 (Table 2) differ from the values calculated from the glucosamine/galactosamine ratios obtained by Professor Morgan (1·3 and 4·0 respectively) and are shown in Table 2.

Determination of glucosamine and galactosamine in urinary glycoprotein. Two samples of urinary glycoprotein (U 66 and U 67) were hydrolysed in the presence of a mixture of ^{14}C-labelled glucosamine and galactosamine (Table 3). After hydrolysis the amino sugars in the dried hydrolysate were adsorbed

Table 2. *Determination of glucosamine and galactosamine in blood-group-specific substances*

Hydrolysis was carried out in the presence of added ^{14}C-labelled galactosamine hydrochloride (93 μg.) and glucosamine hydrochloride (144 μg.) in duplicate experiments (i) and (ii). Naphthyl isothiocyanate derivatives of the amino sugars in the dried hydrolysate were separated by electrophoresis and eluted from the paper (see the text). Portions (0·5 ml.) were taken for scintillation counting and for extinction measurement at 240 mμ after suitable dilution.

Blood-group substance			Specific radioactivity of amino sugar (counts/min./E_{240} unit)		Measured hexosamine content	
Preparation	Amount (mg.)		Added	Recovered	(mg.)	(%)
B 376	0·816	D-Glucosamine				
		(i)	648	278	0·159	19·5 (18·7*)
		(ii)	628	269	0·160	19·6
		D-Galactosamine				
		(i)	801	375	0·0876	10·7 (14·3*)
		(ii)	3298	1474	0·0954	11·7
B 413	0·065	D-Glucosamine				
		(i)	648	309	0·131	21·7 (22·4*)
		(ii)	628	320	0·115	19·0
		D-Galactosamine				
		(i)	801	481	0·0513	8·5 (5·6*)
		(ii)	3298	2034	0·0479	7·9
Le^a 350	0·491	D-Glucosamine				
		(i)	648	384	0·082	16·8
		(ii)	628	582	0·077	15·7
		D-Galactosamine				
		(i)	801	514	0·0430	8·8
		(ii)	3298	2116	0·0431	8·8

* Values obtained by W. T. J. Morgan (personal communication). See the text for further details.

Table 3. *Determination of glucosamine and galactosamine in urinary glycoprotein*

The hydrolysis step, the reaction with naphthyl isothiocyanate and the electrophoretic separation were carried out as described in the text. The 'galactosamine' spot was resubmitted to electrophoresis. Elution of the fluorescent compounds from the electrophoretic strip was carried out with 0·5% $BaCl_2$ (1·5 ml.). Portions (0·1 ml.) were applied to glass-fibre disks for counting and the extinction of the solution was measured at 222 mμ after appropriate dilution.

Glycoprotein		Amount of added [1-^{14}C]hexosamine hydrochloride	Sp. radioactivity (counts/min./E_{222} unit)		Measured hexosamine content	
Preparation	Amount (mg.)	(mg.)	Added	Recovered	(μg.)	(%)
U 66	7·58	Glucosamine 0·656	140·2	57·5	784	10·4
		Galactosamine 0·0854	142·9	30·2	264	3·5
U 67	10·02	Glucosamine 0·656	140·2	50·0	984	9·8
		Galactosamine 0·0854	142·9	44·7	156	1·6

on and eluted (0·1 N-hydrochloric acid) from a small Dowex 50 (H^+ form) column (1 cm. × 5 cm.) before reaction with naphthyl isothiocyanate. As the amount of galactosamine in the glycoprotein is small relative to the glucosamine content, a considerable amount of amino sugar derivative had to be applied to the electrophoresis paper to obtain sufficient of the galactosamine derivative for measurement of specific radioactivity. As a result the glucosamine band trailed considerably owing to overloading of the paper. For this reason the galactosamine band was eluted and subjected to a further electrophoretic separation before measurement of specific radioactivity. Although the values for the glucosamine content of the glycoprotein are similar for the two preparations, the galactosamine contents differ considerably (Table 3). As the second preparation was dialysed for a much longer

period during its isolation (Miss W. Smith, personal communication), it is possible that a galactosamine-containing diffusible substance has been lost during dialysis. The hexosamine values found here are higher than those reported previously: 6·1% glucosamine and 1·1% galactosamine (Maxfield, 1966), and 10·3% total hexosamine (Rozenfel'd & Yusipova, 1968).

DISCUSSION

The use of radioisotope dilution for the determination of amino sugars in glycoproteins allows the use of drastic conditions of acid hydrolysis without having to correct for destruction, which is difficult to assess. High acid concentrations and high temperatures are desirable to diminish the possibility of formation of acid-stable hexosaminides. The method should also remain valid when substances are present that react with the hexosamines. For example, uronic acids appear to react with amino sugars (Jacobs & Muir, 1963), resulting in low results for hexosamine (Ogston, 1964).

The naphthyl isothiocyanate derivatives of the amino sugars are well separated on electrophoresis in tungstate buffer, they are easily detected in ultraviolet light and have the very high molar extinction coefficient at 222mμ of about 70000 (J. E. Scott, personal communication). At such a low wavelength many other substances also absorb light and care has to be taken that such substances are excluded or compensated for in the method. Amino acids and peptides in hydrolysates will also react with naphthyl isothiocyanate to form naphthylthiocarbamoyl derivatives. The extraction of the aqueous reaction mixture with 1% dimethylaminododecane in benzene removes those derivatives that are either acidic or neutral (J. E. Scott, personal communication), and the basic derivatives migrate towards the cathode.

From the trailing observed on electrophoresis of the naphthyl isothiocyanate derivatives of the hydrolysis products of rabbit immunoglobulin, it appeared that naphthyl isothiocyanate derivatives of other substances as well as the amino sugars may also remain in the aqueous phase during the extraction procedure. Re-electrophoresis of the excised glucosamine area gave a clean separation. Moreover, amino sugars in the urinary-glycoprotein hydrolysate had been partially separated from other hydrolysis products by ion-exchange chromatography before the coupling reaction. It may thus be expected that a preliminary ion-exchange separation of amino sugars before reaction with naphthyl isothiocyanate, followed by extraction of the reaction mixture with organic solvents and repeated electrophoresis of the hexosamine derivatives, will lead to a clean separation of the amino sugars from complex mixtures.

The radioisotope-dilution method for the determination of non-nitrogenous sugars (Graham & Neuberger, 1968) and of amino sugars may be an improvement on previous methods, but it still suffers from an important potential weakness. It is likely that protein-bound glucosamine, the reducing group of which is masked, is relatively stable to the destructive, as opposed to the hydrolytic, effect of acid. Destruction, associated with the production of non-chromogenic substances, is likely to occur mainly with the free amino sugar. If the added labelled free sugar is indeed destroyed to a significant extent before the protein-bound monosaccharide is liberated, the amount of sugar determined to be present in the protein will be in excess of the true value. This potential source of error will be minimized if the labelled monosaccharide is added not as the free sugar but as a glycoside that is hydrolysed by acid at a rate similar to that of the sugar residue in the protein. Methyl α-D-[^{14}C]-mannopyranoside has been used in this way already (Graham & Neuberger, 1968), and the corresponding amino sugar derivatives could be employed in a similar manner.

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