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The Estimation of Galactose, Mannose and Fucose in Glycoproteins by Radioisotope Dilution

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1. The principle of radioisotope dilution, as used previously for the estimation of mannose in egg albumin, was applied on a semi-micro scale to the estimation of fucose, mannose and galactose in some glycoproteins. The sugars were separated by partition chromatography on columns of Celite 545. 2. The release of mannose from egg albumin in 2N-hydrochloric acid at 100° after various times was determined by the radioisotope-dilution method and found to have a half-time of 7min. 3. The destruction of mannose in 2N-hydrochloric acid after 3hr. at 100° was found to be small if air was excluded. The destruction was slightly increased by the presence of lysozyme containing tryptophan in an amount equimolar with the mannose. The same amount of free tryptophan caused considerable loss of mannose. 4. Analytical values are reported for the non-amino sugar contents of egg albumin, rabbit γ-globulin and some samples of blood-group-specific substances. The values found were similar to the most reliable estimates published previously.

Although much work has been done on the estimation of neutral sugars in glycoproteins there is still no method that completely overcomes all the difficulties involved. To estimate a glycosidically bound sugar it must first be released from its linkage. The acidic conditions required for complete hydrolysis of a glycosidic linkage bring about some destruction of the released sugar (see Neuberger, Marshall & Gottschalk, 1966). It is difficult to correct for this destruction as the kinetics of release of a sugar, if it is bound in several different positions within a particular glycoprotein, are complex. In addition, some amino acids of the protein moiety are likely to enhance sugar degradation (François, Marshall & Neuberger, 1962). It is also likely that, where *N*-acetylhexosamine residues are attached to non-amino sugars, the latter may not be completely released owing to the formation of relatively acid-stable hexosaminidic linkages (Moggridge & Neuberger, 1938; Gottschalk & Ada, 1956).

Colour reactions carried out on the intact glycoprotein suffer from the disadvantage that the colour produced is not specific for one sugar. At least one amino acid, tryptophan (see Neuberger & Marshall, 1966), causes interference and a true blank value is rarely, if ever, obtainable.

A radioisotope-dilution method, developed for the estimation of mannose in egg albumin, was designed to overcome the problem of correcting for destruction of released sugar (François *et al.* 1962). Radioactive mannose was added to egg albumin, the mixture was hydrolysed and the mannose was isolated as the phenylhydrazone. The change in specific radioactivity of the mannose before and after hydrolysis of the egg albumin gave a measure of the mannose content. The aim of the present work was to extend this method to the estimation of small amounts of several neutral sugars in glycoproteins. Although we do not claim to have solved the problem of sugar analysis in glycoproteins, further extension of this approach should lead to a better estimate of the sugar content of glycoproteins than those obtained previously.

MATERIALS

Rabbit γ-globulin was prepared by precipitation with Na_2SO_4 according to Kekwick (1940). Blood-group-specific substances from ovarian-cyst fluids were a gift from Professor W. T. J. Morgan, F.R.S. Egg albumin, crystallized five times from $(NH_4)_2SO_4$, was prepared as described by Warner (1954). Lysozyme was a crystalline product from hen's-egg white.

L-[1-^{14}C]Fucose was obtained from Calbiochem, Los Angeles, Calif., U.S.A. D-[1-^{14}C]Mannose, D-[U-^{14}C]-mannose and D-[1-^{14}C]galactose were products of The Radiochemical Centre, Amersham, Bucks. Methyl α-D-mannopyranoside [m.p. 193–194°, $[\alpha]_D$+77° (c 1 in water)] and methyl α-D-[^{14}C]mannopyranoside (m.p. 193–194·5°, sp. radioactivity about 32μc/g.) were synthesized as described by Mowery (1963).

METHODS

Hydrolysis of glycoproteins and glycosides

The concentration of rabbit γ-globulin was determined by measurement of the extinction at 280 mμ of a solution in 0·01 N-HCl, with $E^{1\%}_{1cm.}$ taken as 13·5 (Crumpton & Wilkinson, 1963). A solution of the glycoprotein was mixed with [^{14}C]mannose and [^{14}C]galactose (in amounts similar to those present in the glycoprotein) in a ground-glass-stoppered tube. The tube was heated in a boiling-water bath until the solution temperature was at least 95°, whereupon sufficient redistilled HCl (about 6 N and of known concentration) was added to make a final concentration of 2 N- (or 1 N-) HCl. The tube was flushed with N_2, the stopper was taped on and heating was continued for a further 2 hr.

Blood-group-specific substances. A sample of glycoprotein was weighed out and dried at 78° *in vacuo* over P_2O_5 (Pusztai & Morgan, 1964) to constant weight. The glycoprotein was dissolved in water and made up to a known volume. For hydrolysis a portion of the solution in a Pyrex test tube was mixed with ^{14}C-labelled monosaccharide(s) (purified by chromatography) and sufficient water and redistilled HCl (or H_2SO_4, where stated) were added to give the required acid concentrations in a total volume of 10 ml. The tube was constricted, flushed with N_2, sealed and heated in boiling water.

Egg albumin. Egg albumin was dissolved in water, the solution was filtered through sintered glass (no. 3 porosity) and a portion was taken for estimation of dry wt. (at 78° *in vacuo* over P_2O_5). Hydrolysis was carried out in evacuated and sealed Pyrex test tubes unless otherwise stated. For experiments on the destruction of mannose and on the hydrolysis of glycosides, the solutions were heated in 2 N-HCl in evacuated and sealed Pyrex test tubes.

Removal of acid after hydrolysis. On removal from the boiling-water bath the hydrolysis tube was cooled and opened. Acid was removed by addition of freshly washed Dowex 1 (X4; HCO_3^- form; 100–200 mesh) until the solution was neutral. The slurry was poured into a column that was coupled to a small column of Dowex 50 (X4; H^+ form; 100–200 mesh). The columns were washed with water and the effluent and washings were evaporated to dryness on a rotary evaporator with a bath temperature of 45°. In early experiments with rabbit γ-globulin Amberlite IR-4B (OH^- form) was used as the anion-exchange resin, but the sugar recovery was variable. The acetate form of Dowex 1 was used as the anion-exchange resin in some later experiments, as it can be used in a column and as the sugars are maintained in an acidic environment. Quantitative recovery of sugars was obtained from both the HCO_3^- and the acetate forms of Dowex 1.

^{14}C-*labelled monosaccharides*

Radioactive sugar was diluted with non-radioactive sugar in aqueous solution to give a specific radioactivity of about 100 μc/g. The solution was concentrated to a syrup, and upon addition of methanol and propan-2-ol the sugars slowly crystallized at 4° during several days. The crystalline material was dried at 78° *in vacuo* over P_2O_5 for 1–2 hr.

When the ^{14}C-labelled sugars had been stored for many months they were repurified by chromatography on a Celite 545 column (see below) before they were used for isotope-dilution experiments. For example, D-[1-^{14}C]galactose (117 mg.) was dissolved in the aqueous phase (0·66 ml.) of the ethyl acetate–propan-1-ol–water (4:1:2, by vol.) solvent, the solution was mixed with Celite 545 (1·5 g.) and the mixture was applied to a column of Celite 545 (12 g.). The column was developed with the ethyl acetate phase and portions of each fourth fraction (10 ml.) were counted at infinite thinness. The fractions nos. 30–54 were pooled and evaporated to dryness. The residue was crystallized and dried. The ^{14}C-labelled sugars used showed after chromatography a change in specific radioactivity of less than 3%.

Estimation of reducing sugars. The hexoses isolated by column chromatography were estimated either by the 2-aminobiphenyl method (Timell, Glaudemans & Currie, 1956) or by the ferricyanide method (Park & Johnson, 1949). In the ferricyanide method the concentration of H_2SO_4 in the ferrous ammonium sulphate reagent was increased from 0·05 N to 0·1 N to avoid turbidity, which was occasionally observed. Fucose was assayed by a modification (Gibbons, 1955) of the method of Dische & Shettles (1948).

Column chromatography. The neutral sugars were separated as described by Hall (1962*a*,*b*) on columns of Celite 545 (Johns-Manville Corp., New York, N.Y., U.S.A.) by using the two-phase solvent system ethyl acetate–propan-1-ol–water (4:1:2, by vol.) (Hall, 1962*b*). Ethyl acetate was purified by shaking with 5% Na_2CO_3 followed by saturated $CaCl_2$, and it was dried over anhydrous K_2CO_3 and distilled. Propan-1-ol was distilled from solid $SnCl_2$.

Resolution of mixtures of sugars (up to 5 mg.). Celite 545 (10 g.), mixed with the lower phase (4·4 ml.) of the solvent, was tamped in small portions into a precision-bore glass column (1 cm. diam.) having a water jacket maintained at a constant temperature close to that of the laboratory. The sugar sample was dissolved in the lower phase (0·1 ml.) and mixed with Celite 545 (200 mg.) and the mixture was tamped on to the top of the column. The upper phase of the solvent was run from a reservoir through thin Teflon tubing on to the top of the column. Fractions (10 ml.) were collected either by drop counting or by an organic solvent siphon. Columns were freshly packed for each run. Radioactive sugars were detected by transferring a sample (about 0·5 ml.) from each fraction on to metal planchets, which were dried at 100° and counted at infinite thinness. The fractions comprising a sugar peak were combined and the solvent was removed in a rotary evaporator with a water bath temperature at 40°. The residue was dissolved in water and portions of the solution were taken for radioactive and colorimetric assay.

Paper chromatography. For the isolation of [^{14}C]mannose by paper chromatography the dried material was dissolved in boiled water to give a mannose concentration of about 2%. A portion (0·02 ml.) of the solution was applied as a line (2 in. long) to Whatman no. 541 paper that had been washed successively with 1% (v/v) acetic acid, water and ethanol. The paper was developed overnight with the upper phase of ethyl acetate–propan-1-ol–water (4:1:2, by vol.). After the paper was dried the areas corresponding to mannose, as revealed on guide strips sprayed with aniline hydrogen phthalate (Partridge, 1949), were cut out and eluted at 4° with 5 ml. of aq. 25% ethanol. Portions (0·5 ml.) of the eluate in triplicate were assayed by the *o*-aminobiphenyl method and put on planchets for counting at infinite thinness.

Thin-layer chromatography. Thin-layer chromatography of [^{14}C]mannose on $NaBH_4$-reduced cellulose was carried out essentially as described by Wolfrom, de Lederkremer & Schwab (1966) except that $NaBH_4$-reduced Whatman CC41 cellulose was used and spread as a slurry (32g. in 80ml. of water) with an applicator having a clearance of 0·4mm. For chromatography, [^{14}C]mannose was dissolved in sufficient aq. 30% ethanol to make a concentration of about 1·2% and 5μl. of the solution was applied along a 2cm. line on the cellulose layer. Each thin-layer plate (20cm. × 20cm.) could accommodate two unknown samples, standard [^{14}C]mannose and a blank as well as two marker spots. After four developments with ethyl acetate–pyridine–water (12:5:4, by vol.) the plates were developed with ethyl acetate and dried with a hair-drier. The areas of cellulose, corresponding to the marker spots of mannose revealed by alkaline $AgNO_3$ reagent, were scraped off with a razor blade and shaken with 0·8ml. of freshly boiled water for 15min. The suspension was centrifuged and portions (0·1ml.) of the supernatant were assayed for ^{14}C by scintillation counting, and for mannose by the ferricyanide method (Park & Johnson, 1949).

Performic acid oxidation

Performic acid was prepared according to Moore (1963). Sufficient H_2O_2 was used to give about a fourfold excess over the calculated amount that would be required to oxidize the susceptible amino acids present. The dried glycoprotein was dissolved in performic acid (2ml.) and left at 0° for 4hr. Ice-cold water (about 30ml.) was added and the mixture was freeze-dried.

To remove any salt present before oxidation, rabbit γ-globulin (55mg.) was dissolved in water (1ml.) and precipitated with acetone (3ml.). The precipitate was washed with acetone–methanol (1:1, v/v) and dried *in vacuo*. During this procedure the loss of material absorbing at 280mμ amounted to 0·3% of the glycoprotein taken.

Measurement of radioactivity

Counting at infinite thinness. Portions (0·5ml.) in triplicate or quadruplicate of an aqueous solution of the ^{14}C-labelled sugar were carefully placed in the centre of aluminium planchets (3·2cm. diam.), which were then heated at about 70° under reduced pressure (water pump) until dry. The planchets were assayed for radioactivity in a gas-flow Geiger–Müller counter (Nuclear–Chicago Corp. model D47) operating in the plateau region and fitted with a Micromil window and an automatic sample-changing device. The gas used was 1·5% butane in helium. The number of counts recorded for each planchet was greater than 10^4. The efficiency of counting ^{14}C-labelled sugars was about 25% and the background count was usually about 20counts/min.

For amounts of up to 100μg. of radioactive sugar a linear relationship was found between the amount of ^{14}C-labelled sugar and the number of counts obtained. Dilution of ^{14}C-labelled sugar with up to twice the amount of non-radioactive sugar did not affect the measured radioactivity within the experimental error of about ±2%. This method of counting was used for most of the work.

Liquid-scintillation counting. The samples of [1-^{14}C]-mannose that were obtained from thin-layer chromatograms were assayed for radioactivity by liquid-scintillation counting. Portions (0·1ml.) in triplicate of each sample were dried on Whatman GF/A 2·1cm. glass-fibre disks (Davies & Cocking, 1966), which were counted in 1·0ml. of scintillation fluid [0·4% 2,5-diphenyloxazole and 0·02% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] in a scintillation counter (Nuclear–Chicago Corp. model 6860, Mk I^{TM}) in which the attenuator setting was adjusted to give a mximum counting rate for [^{14}C]mannose in this system.

The efficiency of counting was found to be about 90% and to be constant for volumes of scintillator between 0·5 and 1·0ml. The background count of vials containing 1ml. of scintillator was usually 14counts/min., which increased by about 1count/min. when a glass-fibre disk was placed in the scintillator. Another scintillator, 0·4% (w/v) 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen (Ciba Ltd., Basle, Switzerland) in toluene, gave similar results.

The measured specific radioactivity of [1-^{14}C]mannose did not vary by more than ±0·6% when amounts from 5μg. to 55μg. of radioactive sugar were applied to the glass-fibre disk. When [1-^{14}C]mannose (9·1μg.) was diluted with 10·4μg., 20·8μg. and 41·6μg. of non-radioactive mannose, decreases in the measured radioactivity of 0·2%, 0·6% and 1% respectively were observed. The determination by isotope dilution and thin-layer chromatography of a known amount of pure mannose gave estimates of 99·0% and 99·7% of the actual value.

RESULTS

Hall (1962*b*) has described the separation of L-rhamnose, L-fucose, D-mannose and D-glucose by partition chromatography on Celite columns. The method was scaled down and found to give good resolution of mixtures of L-fucose, D-mannose and D-galactose, the common neutral sugar components of glycoproteins (Fig. 1).

The isotope-dilution method for the estimation of sugars in glycoproteins was designed to avoid low values of sugar content caused by sugar destruction under the conditions of acid hydrolysis required for the complete cleavage of glycosidic bonds. However, it is possible that the isotope-dilution method may give an estimate of the sugar content higher than the true value, if the ^{14}C-labelled sugar that is added to the glycoprotein is destroyed to a significant extent before the protein-bound sugar has been completely released. This possibility was discussed by François *et al.* (1962) and on the data then available it was considered that the error would be small. To assess the factors involved with greater accuracy the destruction of mannose under the conditions of acid hydrolysis and in the presence of some relevant compounds was determined (Table 1). The destruction of mannose, when heated in 2N-hydrochloric acid at 100° for 3hr. in an evacuated sealed tube, was about 4%, which is less than would be expected from the figure of 23% reported by François *et al.* (1962) for a 5hr. heating period. The difference is likely to be due to the absence of

oxygen in the present experiment. These authors also found that under the same conditions the presence of 1 M-cysteine increased the destruction of mannose to 42%. As both tryptophan and the sulphur-containing amino acids are destroyed on heating in acid to a greater extent if carbohydrate is present (Martin & Synge, 1945) it was of interest to investigate also the stability of mannose in the presence of tryptophan and of a tryptophan-containing protein (Table 1). The greater destructive effect of tryptophan when free, rather than when it is bound in lysozyme, may be due to different reactivities in the free and bound states or may reflect competitive reactions of tryptophan with other amino acids as well as with mannose (see Martin & Synge, 1945). Addition of ribose greatly diminished the destructive effect of tryptophan on mannose.

The liberation of mannose with time from egg albumin in 2 N-hydrochloric acid at 100° was determined by radioisotope dilution (Fig. 2). Half the mannose in egg albumin was released in about

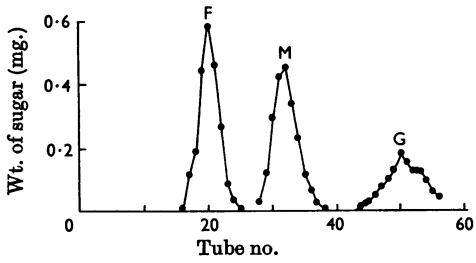

Fig. 1. Separation of L-fucose (F), D-mannose (M) and D-galactose (G) on a Celite 545 column with ethyl acetate–propan-1-ol–water (4:1:2, by vol.) as the solvent system. The aqueous phase of the solvent system, containing L-fucose (2·4 mg.), D-mannose (2·0 mg.) and D-galactose (1·1 mg.), was mixed with Celite 545 (0·4 g.) and chromatographed on a column of Celite 545 (10 g.) as described in the text. A portion (1·0 ml.) of each fraction (10 ml.) was dried and assayed by the *o*-aminobiphenyl method (Timell *et al.* 1956).

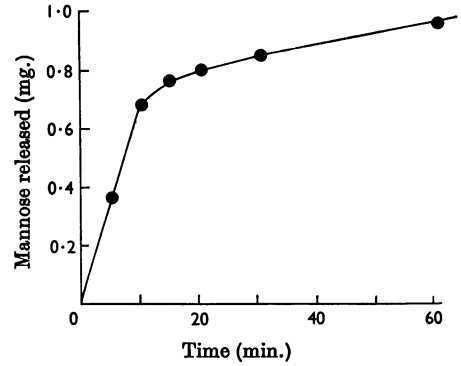

Fig. 2. Release of mannose from egg albumin in 2 N-HCl at 100°. Egg albumin (494 mg.) dissolved in water (20 ml.) was heat-denatured (pH 7·5, 85°, 8 min.) and treated with pepsin (5·5 mg.) at pH 2·5 (formate buffer, 0·5 M) and 37° for 24 hr. The solution was made to 50 ml. and portions (5 ml.) were mixed with [U-^{14}C]mannose (463 μg.) and sufficient 6 N-HCl to make a concentration of 2 N. The solutions were heated for various times in stoppered tubes under N_2. Free mannose was isolated by chromatography on Celite columns and assayed for specific radioactivity, which was used to estimate the amount of mannose released.

Table 1. *Recovery of mannose after heating in acid in the presence of various substances*

Mannose (483 μg., 2·68 μmoles) was heated with the compounds listed in 2 N-HCl (3 ml.) for 190 min. at 100° in test tubes sealed under vacuum. Tube no. 5 was not heated. To each solution was added [1-^{14}C]mannose (478 μg., 2·65 μmoles) and the mannose was isolated by paper chromatography and assayed for radioactivity at infinite thinness and colorimetrically by the *o*-aminobiphenyl method (Timell *et al.* 1956). The specific radioactivity of the [1-^{14}C]mannose was 77·3 counts/min./μg. before and 78·1 counts/min./μg. after paper chromatography. From tube no. 5 the value of the specific radioactivity of the [1-^{14}C]mannose added was calculated as 78·2 counts/min./μg., which was the value used to estimate the mannose recovered, i.e. mannose recovered = 478 (78·2/sp.radioactivity − 1).

Tube no.	Substance added	Sp. radioactivity (counts/min./μg.)	Mannose recovered (μg.)	Mannose recovered (%)
1	None	39·6	466	96·5
2	Tryptophan (575 μg., 2·83 μmoles)	42·1	410	84·9
3	Tryptophan (575 μg., 2·83 μmoles) + D-ribose (797 μg., 5·3 μmoles)	40·3	450	93·1
4	Lysozyme (7 mg.)*	40·1	454	94·0
5	None	38·9	483	100

* Contains tryptophan (2·94 μmoles) and cysteine (1·96 μmoles).

Table 2. *Estimation of mannose in egg albumin*

The compounds were mixed as indicated below and heated in 5ml. of 2N-HCl at 100° for 185min. in sealed evacuated tubes. Mannose was isolated by thin-layer chromatography and assayed for specific radioactivity (see the Methods section). The mannose content of egg albumin was assumed to be 2·0%.

Mannose (μg.)	Methyl α-D-mannopyranoside (μg.)	Egg albumin (mg.)	Estimated mannose content (μg.)	Estimated mannose content % of theoretical content
408*	558	—	537	103·7
			508	98·1
408*	—	22·85	458	100
			458	100
403	556*	—	403	100
—	556*	22·85	430	94
			435	95

* Labelled with ^{14}C.

7min. and virtually complete liberation of the mannose occurred in 1hr. François *et al.* (1962) estimated a half-time of 10min. for the release of mannose based on the data of Neuberger (1938) and Nuenke & Cunningham (1961) for the liberation of reducing groups from egg albumin in 1·5N-hydrochloric acid. They also found that 97% of the mannose of egg albumin was split off after 1hr. in 2N-hydrochloric acid. Therefore in 2N-hydrochloric acid in the absence of air, the ratio of the rate of liberation of mannose from egg albumin to the rate of destruction of free mannose will be very much greater than the minimum value of 12 suggested by François *et al.* (1962).

Differential destruction during acid hydrolysis of added labelled sugar, or of the sugar arising from the glycoprotein, might be avoided if the radioactive sugar was added in the form of a glycoside having a rate of cleavage in acid similar to that of the glycoprotein sugar. For this purpose methyl α-D-[^{14}C]-mannopyranoside was synthesized. It has a half-time of hydrolysis in 2N-hydrochloric acid at 100° of 3min. (calculated from the data of Overend, Rees & Sequeira, 1962) compared with 7min. for the release of mannose from egg albumin under the same conditions. The addition of radioactive mannoside was compared with that of [^{14}C]mannose in the estimation of the mannose content of egg albumin (Table 2). The value found with the radioactive methyl mannoside was about 5% lower than that with free radioactive mannose, and this difference may be just within the error of the method. It does not appear therefore that use of the radioactive glycoside in this case is of particular advantage.

In an earlier experiment a mixture of [U-^{14}C]-mannose (1·53mg., sp. radioactivity 54·9counts/min./μg.), D-galactose (1·24mg.) and egg albumin (90·6mg.) was heated in 2N-hydrochloric acid (25ml.) at 100° for 2hr. in a stoppered tube. The mannose, isolated from the hydrolysate by Celite chromatography, had a specific radioactivity of 25·1counts/min./μg., which gave a value for the mannose content of egg albumin of 2·01%. François *et al.* (1962) found the mannose content of egg albumin to be 2·0±0·06% by using an isotope-dilution method and 2·0±0·1% by using the orcinol–sulphuric acid colorimetric method.

The amounts of mannose and galactose in rabbit γ-globulin were also estimated by the isotope-dilution method (Table 3). One sample of this glycoprotein was oxidized with performic acid before hydrolysis to oxidize tryptophan and the sulphur-containing amino acid residues, which are the amino acids most likely to cause destruction of sugars during hydrolysis. Prior performic acid oxidation of egg albumin, fibrinogen and fibrin has been used to prevent interference by tryptophan in the anthrone–sulphuric acid reaction for the estimation of hexose (Hörmann & Gollwitzer, 1962, 1963). Since the hexose values found after oxidation agreed with those obtained by reading the anthrone colour at a wavelength where tryptophan did not interfere, it is probable that no sugar destruction occurred during oxidation. Similarly Spiro (1963) reported that performic acid oxidation of fetuin at 0° did not result in any loss of carbohydrate. We found that the colour given by methyl α-D-glucopyranoside and αα′-trehalose in the orcinol–sulphuric acid reaction was not affected by prior performic acid oxidation at 0° for 4·5hr.

Performic acid oxidation of rabbit γ-globulin did not affect the values that we obtained for the galactose and mannose contents within the errors of

the method employed. The average values of the galactose and mannose contents of rabbit γ-globulin are then 2·9 and 5·8 moles respectively/mole of rabbit γ-globulin (assuming the mol.wt. to be 140000). Therefore the combined content of mannose and galactose residues is $1·0 \pm 0·1\%$ in a ratio of 2:1.

Previous values reported for the combined mannose and galactose residue content of rabbit γ-globulin, as estimated by the colorimetric orcinol–sulphuric acid method, are 0·67% (Nolan & Smith, 1962) and 1·11% (Fleischman, Porter & Press, 1963), which includes a 'trace' of fucose. Both groups of authors found a mannose/galactose ratio of 2:1 by paper chromatography. A value of 1·03% non-amino hexose was obtained by Utsumi & Karush (1965) after the hydrolysis of rabbit γ-globulin in 0·5N-hydrochloric acid at 100° for 16hr.

The fucose and galactose contents of three samples of blood-group-specific substances were also determined by radioisotope dilution (Tables 4 and 5). Since fucose is readily released from blood-group substances (see, e.g., Watkins, 1966), conditions of hydrolysis were employed suitable for the estimation of fucose alone. The fucose value for B413 substance was no greater when more severe hydrolytic conditions were employed. If

Table 3. *Estimation of mannose and galactose in rabbit γ-globulin*

Rabbit γ-globulin was hydrolysed in the presence of ^{14}C-labelled mannose and galactose at 100° for 2hr. in a total volume of 5ml. for the first two analyses and 25ml. for the last two analyses listed below. The hydrolysate was deionized and the neutral sugars were separated by chromatography on a column of Celite 545 (see the text). The isolated monosaccharides were estimated by the *o*-aminobiphenyl method except for the oxidized sample, where the ferricyanide method was employed.

Concn. of HCl (N)	Amount of γ-globulin taken (mg.)	^{14}C-labelled sugars added (μg.)	Sp. radioactivity (counts/min./μg.)		Sugar found	
			Initial	Final	(μg.)	(moles/10^5g.)
1·0	57·9	Mannose (451)	54·2	27·1	451	4·33
		Galactose (281)	51·3	27·3	247	2·37
2·0	22·5	Mannose (212)	53·2	30·2	161	3·98
2·0	65·9	Mannose (1015)	54·7	37·2	477	4·02
		Galactose (491)	49·7	34·7	212	1·79
2·0	55·0*	Mannose (382)	56·0	27·5	396	4·30
		Galactose (237)	51·6	29·3	180	1·96

* Protein oxidized with performic acid.

Table 4. *Estimation of* L-*fucose in blood-group-specific substances by radioisotope dilution*

Hydrolysis was carried out in an atmosphere of N_2 in sealed tubes at 100°. Fucose was isolated by Celite chromatography and estimated by the method of Gibbons (1955). Radioactivity was measured at infinite thinness.

Blood-group substance	Conditions of hydrolysis	Amount of glycoprotein taken (mg.)	Amount of [1-^{14}C]fucose added (mg.)	Sp. radioactivity of L-fucose (counts/min./μg.)		Fucose content		
				Added	Recovered	(mg.)	(%)	Colorimetric value* (%)
B376	0·1N-H_2SO_4, 60min.	1·527	0·222	55·3	24·2	0·285	18·7	17
	N-HCl, 10min.	1·527	0·222	55·3	24·1	0·287	18·8	
B413	0·1N-H_2SO_4, 60min.	1·148	0·241	43·4	25·2	0·174	15·2	16
	N-HCl, 70min.	1·209	0·222	50·9	28·6	0·173	14·3	
	2N-HCl, 3hr.	1·209	0·222†	47·0	26·9	0·166	13·7	
Le^a350	0·2N-H_2SO_4, 60min.	3·59	0·178	42·6	20·9	0·185	5·2	8·4
	2N-HCl, 3hr.	3·924	0·177†	47·0	20·5	0·229	5·8	

* Values found by W. T. J. Morgan (personal communication) by the method of Gibbons (1955) on the intact glycoprotein.
† Radioactive galactose was also added and determined (see Table 5).

Table 5. *Estimation of galactose in samples of blood-group-specific substances by radioisotope dilution*

Hydrolysis was carried out in 10ml. of 2N-HCl under N_2 in a sealed tube for 3hr. at 100°. The galactose was isolated by Celite chromatography and determined by the ferricyanide method (Park & Johnson, 1949). Samples were counted at infinite thinness.

Blood-group substance	Loss on drying* (%)	Amount of glycoprotein taken (mg.)	Amount of [1-^{14}C]galactose added (mg.)	Sp. radioactivity of galactose (counts/min./μg.)		Galactose content	
				Added	Recovered	(mg.)	(%)
B376	11·2	2·624	0·942†	47·3	27·7	0·665	25·4
	11·9	3·054	0·89	53·1	28·7	0·754	24·7
B413	16·0	6·77	1·78†	48·7	23·8	1·86	27·5
	15·7	2·296	0·445	47·9	18·8	0·693	30·2
		1·209	0·338‡	66·6	30·0	0·412	34·1
Le^{a}350	15·9	3·45	1·035	51·0	27·5	0·884	25·6
	16·2	3·59	1·035	48·9	25·6	0·984	26·2
		3·924	0·676‡	66·6	25·2	1·111	28·3

* Dried over P_2O_5 at 78° *in vacuo*.

† [1-^{14}C]Galactose was not purified by chromatography.

‡ Radioactive L-fucose was also added and determined (see Table 4).

the difference in the fucose values for Le^{a}350 substance is significant complete release of fucose may not have been achieved under the milder conditions of hydrolysis. For a maximum recovery of fucose from serum glycoproteins it was necessary to hydrolyse with 0·6N-sulphuric acid at 100° for 60min. (Gyorky & Houck, 1965). The radioisotope-dilution results for fucose in the B-specific substances were similar to those obtained colorimetrically. A considerable difference between the two methods was observed for the fucose value of Le^{a}350 substance. The galactose contents of these particular blood-group B-substance samples has not been estimated by other methods. Previously reported values for the galactose content of blood-group B-specific substances as determined colorimetrically range from 25 to 38% (see Watkins, 1966). Quantitative paper chromatography of the hydrolysis products (0·5N-hydrochloric acid, 100°, 16hr.) from Le^{a}350 substance gave a value of 28·8% galactose (Pusztai & Morgan, 1961), which is not incompatible with the values reported here, considering the wide variation obtained for a particular estimation. The reproducibility of the radioisotope-dilution method is likely to be considerably improved if the radioactivity is measured by liquid-scintillation counting, which was found to be much more reliable than the method employed in the present experiments.

DISCUSSION

This paper is largely concerned with the major problem in sugar analysis which arises from the destruction of sugars during the course of acid hydrolysis. Experiments were carried out on the effect of hot acid on the recovery of mannose, which is a common constituent of glycoproteins, to assess some of the factors that are likely to enhance the destruction of sugars. Mannose alone in the absence of air was found to be fairly stable in 2N-hydrochloric acid at 100°. In comparison with previous results, where air was not excluded (François *et al.* 1962), it would appear that the presence of oxygen caused a significant increase in destruction of mannose, as has been shown for the amino hexoses (Walborg & Ward, 1963). For the amino acids, tryptophan and cysteine, which are rendered less stable in acid by the presence of carbohydrate, free tryptophan in equimolar amount with the sugar present was found to enhance sugar destruction considerably. However, the destructive effect of protein-bound tryptophan was found to be much less, demonstrating that free tryptophan is not a valid control for the tryptophan bound in a glycoprotein. In an earlier paper (François *et al.* 1962) it was shown that the presence of M-cysteine almost doubled the rate of mannose destruction in acid. As air was not excluded in that experiment and as cysteine was in a vast excess of the mannose present, it is not likely that cysteine, in the absence of air, bound in a glycoprotein in relatively small amount would have a marked influence on sugar destruction.

The destructive effect of tryptophan and cysteine in a glycoprotein may be overcome by preliminary oxidation with performic acid, or by addition of a sufficient quantity of a suitable reactive sugar which does not occur in the glycoprotein. Similarly, addition of the radioactive sugar in the form of a

glycoside would greatly diminish any preferential destruction of added labelled sugar. These methods would be useful in cases where the tryptophan content of a glycoprotein is high relative to the sugar content. In the many glycoproteins that have a relatively high sugar content and little tryptophan, the radioisotope-dilution method is unlikely to give estimates that are significantly different from the true value.

From the limited data presented it appears that the results obtained by radioisotope dilution are similar in general to the values found by colorimetric methods, i.e. the thioglycollic acid–sulphuric acid method, which in glycoproteins appears to be specific for fucose (Gibbons, 1955), and the orcinol–sulphuric acid method, which seems adequate when only one non-amino sugar is present (see Johansen, Marshall & Neuberger, 1960). However, detailed comparisons are difficult because of the variation in the values obtained by the radioisotope-dilution method. Errors in this method arise from two measurements of radioactivity and two colorimetric estimations. Determination of radioactivity by liquid-scintillation counting, rather than by the method used for most of this work, is likely to give more precise results.

The advantage of using isotope dilution for the estimation of neutral sugars in glycoproteins is that relatively severe conditions of hydrolysis may be used, thus removing the need to establish optimum conditions of hydrolysis and decreasing the possibility of trapping sugars in acid-stable hexosaminidic linkages. Also loss of sugar during the isolation procedure does not affect the final result. The method should be particularly useful where the destruction of free sugar during hydrolysis is likely to be high.

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