The Rapid Determination of Small Amounts of Glucosamine and Galactosamine in Protein Preparations, with Special Reference to Skin Tissue

By C. H. PEARSON

Procter Department of Food and Leather Science, The University, Leeds 2

(Received 11 December 1962)

Determinations of the total hexosamine content of biological preparations, usually by modifications of the Elson & Morgan (1933) colorimetric method, are widely employed. It is frequently of greater interest, however, to know the separate amounts of glucosamine and galactosamine that are present. Various procedures for such determinations are now available (Gardell, 1958; Pogell & Koenig, 1958; Levvy & McAllan, 1959; Cessi & Serafini-Cessi, 1962; Scott, 1962), but the present paper is confined to ion-exchange chromatography.

Gardell (1953) separated and determined the hexosamines by displacement from a column of Dowex 50 resin with 0.3N-hydrochloric acid. With the recommended flow rate of only 1.5–2.0 ml./hr., and by collecting 0.5 ml. fractions, an analysis takes 48 hr. or more. Other workers (Rondle & Morgan, 1955; Crumpton, 1959; Holdsworth, 1961) have modified this method without claiming an improvement in the rate of separation.

An alternative procedure based on the method, with a Dowex 50 column (15 cm.), developed by Moore & Stein (1951) for the separation of basic amino acids, was put forward by Eastoe (1954). The hexosamines were eluted with 0.1M-citrate buffer, pH 6.75, at a flow rate of 4 ml./hr. Eastoe (1954) analysed the fractions by the ninhydrin colorimetric method and considered this to be more accurate than the Elson–Morgan technique used by Gardell (1953).

Moore & Stein's (1951) system for the separation of basic amino acids has since been modified (Moore, Spackman & Stein, 1958) and a carboxylic resin was preferred by Hannig (1959). The former workers now employ a column (15 cm.) of fractionated Amberlite 120 resin, together with a single buffer of comparatively high molarity, 0.35M, at pH 5.28. Glucosamine and galactosamine emerge as a single peak under these conditions (C. H. Pearson, unpublished work), but they were fairly well separated by Partridge & Elsdon (1961), who increased the length of the column to 30 cm. With a resin of small particle size a high flow rate (20 ml./hr.) was permissible, but unfortunately, when hydroxylysine was present, as in collagen hydrolysates, it overlapped galactosamine. Partridge & Elsdon

(1961) suggested that a longer column should be used under these circumstances but gave no further details.

Preparations of fibrous proteins such as collagen often contain only small amounts (0.01–0.1%) of hexosamines. They therefore offer specific difficulties in hexosamine analyses not encountered with the mucopolysaccharides and similar compounds (originating mainly from the interfibrillary ground substance) that have been isolated from skin tissue (Stacey & Barker, 1962) after various deproteinizing operations. Few publications, however, have dealt with this problem where the hexosamine:protein ratio is very low.

The acid-hydrolysis stage of the analysis requires careful investigation for any given type of sample. Before the optimum conditions of hydrolysis can be established, however, it is necessary to ensure that the free hexosamines present in the hydrolysates can be determined with reasonable precision. The object of the present work was to develop rapid and convenient ion-exchange methods that would give reliable values for hexosamines, when applied to hydrolysates of fibrous proteins.

MATERIALS AND METHODS

Pure compounds. Glucosamine hydrochloride and galactosamine hydrochloride were used for the preparation of standard curves and for recovery tests. The glucosamine (British Drug Houses Ltd.) was at least 99% pure and the galactosamine, a synthetic sample, was a generous gift from Professor R. Kuhn of Heidelberg who stated that it had the correct specific rotation and was 100% uniform when chromatographed by the Fischer & Nebel (1955) method.

Protein preparations for analysis. Four preparations derived from ox skin were employed. Most of the hair and the epidermis was removed from the skin which was then split into five layers with an industrial splitting machine (Turner Machinery Ltd., Leeds). Pieces of the top layer (corium minor) were repeatedly extracted with 0.1M-sodium phosphate buffer, pH 9.0, to remove most of the interfibrillary material, before dehydration with acetone. Hydrolysates of this preparation I were used in testing all the ion-exchange procedures. The other layers were first macerated in a Wiley mill (Apex Construction Ltd., Soho Square, London, W. 1) and then given the same treatment as for preparation I. Analyses are reported only for pre-

parations IV (fourth layer) and V (fifth or bottom layer). The final preparation (III P) was precipitated by adding ethanol to a phosphate buffer extract of the centre (third) layer of the skin. Collagen was the main component of preparations I, IV and V, whereas preparation III P consisted mainly of non-collagenous proteins.

Hydrolysis conditions. Samples (200–500 mg., depending on the amount of hexosamine present) of protein were hydrolysed in spring-clipped glass-stoppered tubes with 10–15 ml. of 4N-HCl for 4, 8 or 18 hr. at 100°. The hydrolysates were filtered and made up to volume (25 or 50 ml.). Samples (5 or 10 ml.) were evaporated at room temperature in a vacuum desiccator over P_2O_5 and NaOH, and kept at 4°.

Ion-exchange resins. Dowex 50 (X 8; 200–400 mesh) was prepared as described by Moore & Stein (1951). Amberlite CG-120 (type II) was fractionated hydraulically (Hamilton, 1958) and given the same treatment as the Dowex 50 except that it was stored in the moist H^+ form. The average particle sizes of the fractions of Amberlite were estimated approximately under the projection microscope with a stage micrometer. A slurry of the carboxylic resin Amberlite CG-50 (type II) (Na^+ form; 200 mesh) was decanted several times to remove the finer particles.

Chromatographic columns. Six types of columns were prepared: method A, Amberlite CG-120 (50 μ particles) (37 cm. $\times$ 0.6 cm.); method B, Dowex 50 (16 cm. $\times$ 1.0 cm.); method C, Amberlite CG-120 (40 μ particles) (30 cm. $\times$ 0.6 cm.); method D, Amberlite CG-120 (50 μ particles) (50 cm. $\times$ 0.6 cm.); method E, Amberlite CG-50 (50 cm. $\times$ 0.6 cm.); method S (small column for total hexosamine determinations, Amberlite CG-120 (40 μ particles) (4 cm. $\times$ 0.8 cm.).

Each column was equilibrated with the solution that was to be used for elutions: A, 0.3N-HCl; B, C and S, 0.1M-citrate buffer, pH 5.0 (Moore & Stein, 1951); D, 0.35M-citrate buffer, pH 5.26 (Moore *et al.* 1958); E, 0.2M-citrate buffer, pH 7.0 (Hannig, 1959). Columns A, B, C, D were maintained at 25–30° and E at 40°. Column S was run at room temperature (18–25°). A flow rate of either 3–4 ml./hr. (overnight, method A-1) or 20–40 ml./hr. (method A-2) was employed for the HCl procedure. Columns B and C were run at 4–5 ml./hr., and columns D and E at about 15 ml./hr. Approx. 20 ml. of effluent was collected from column S in 15–20 min. The higher flow rates were obtained by forcing the solutions through the columns under pressure of nitrogen.

Chromatographic procedure. For application to the column the dried hydrolysate residue was dissolved in 1–2 ml. of 0.3N-HCl or the appropriate buffer solution. A separating funnel containing the acid or buffer was connected to the column and approx. 1 ml. fractions were collected, a drop-counting instrument being used for high flow rates. Tubes were weighed so that the fraction volumes were accurately known in the portion of the chromatogram which contained the hexosamines. Columns B and C were regenerated as described by Moore & Stein (1951). In method A at least 50 ml. of 0.3N-HCl was collected after the emergence of the galactosamine peak; no further treatment was given before re-use except that the top 1–2 cm. of resin, which darkened, was occasionally replaced by fresh.

Elson-Morgan colorimetric method. The acetylacetone and Ehrlich reagents were prepared as described by Boas (1953) except that a 4N-sodium carbonate-sodium bicarbonate buffer, pH 10.0, based on the 2N solution used by Neuhaus

& Letzring (1957), was substituted for 1N-sodium carbonate solution in the acetylacetone reagent.

Procedure. Fractions, glucosamine standards and reagent blanks were analysed simultaneously in the rimless test tubes (6 in. $\times$ $\frac{5}{8}$ in.) used in the fraction collector. During heating they were covered with aluminium caps. The volumes of fractions containing hexosamine did not usually differ by more than 5%. Somewhat greater variations were found to be permissible, however, even with the HCl system, as long as the 4M-sodium bicarbonate buffer was used.

Distilled water (1 ml.) was pipetted into each fraction and a volume, equal to the average fraction volume, of 0.3N-HCl or the appropriate buffer was added to 1 ml. of standard (15, 30 and 45 μ g. in triplicate). A portion (1 ml.) of a freshly prepared solution of the acetylacetone reagent was added to each tube. Rapid but accurate pipetting was essential at this stage of the procedure because of the instability of hexosamines in alkaline solution. The tubes were heated at 89–92° for 45 min. (Boas, 1953) and quickly cooled. Ethanol (2.5 ml.) was added followed by 1 ml. of the Ehrlich reagent. After the solutions had been shaken several times to remove CO_2 , the extinctions at 530 $m\mu$ were determined in cells of 1 cm. light-path in a Unicam SP. 500 spectrophotometer, about 15 min. after the addition of the Ehrlich reagent.

Stability of standard solutions. Dilute aqueous solutions of glucosamine (and galactosamine) deteriorated fairly rapidly. The standards were therefore freshly prepared each day by diluting a stock solution (200–300 μ g./ml.). The latter was stable for 7 days at 4°.

Ninhydrin colorimetric method. The original reagent of Moore & Stein (1948) was used with column methods B and C, and the modified reagent (Moore & Stein, 1954) for methods D and E.

Total hexosamine determinations. The dry hydrolysate residue was transferred to column S with 1–2 ml. of 0.1M-citrate buffer, pH 5.0, and a graduated tube was placed beneath it. The solution was forced into the resin by nitrogen pressure and a further quantity of the buffer was added to the column. The first 7.0 ml. of effluent was discarded and the next 10.0 ml. was collected. Portions (2.0 ml.) were analysed by the Elson-Morgan method. The next few millilitres of effluent were collected for determinations of blank values. Before the column was used again 2N-NaOH was passed through followed by the 0.1M-citrate buffer, pH 5.0, until equilibrium was re-established.

RESULTS

Elson-Morgan colorimetric method. When 0.3N-hydrochloric acid was present it was necessary to use a concentrated buffer solution in the acetylacetone reagent. For fraction volumes up to 1.4 ml. the 4N-sodium carbonate buffer, pH 10.0, gave optimum pH conditions. The colour yields also seemed to depend on ionic strength, for a 2N-buffer, pH 10.0, gave lower and less reproducible extinction values, even when hydrochloric acid was not present in the fractions.

Relative colour yields were determined with 1 ml. of aqueous standards (30 μ g.) of glucosamine

and galactosamine with the addition of 1 ml. of the 0.3N-hydrochloric acid or buffer used in the column-chromatography methods. The following values were obtained for the percentage colour yield of galactosamine relative to glucosamine: A (0.3N-hydrochloric acid), 91; B and C (0.1M-citrate buffer, pH 5.0), 87; D (0.35M-citrate buffer, pH 5.26), 88. These results were used in calculating the galactosamine content of samples analysed by the different column methods and in recovery tests. When changes were made in the composition of the acetylacetone reagent and in the heating conditions, the relative colour yields were altered, as found by Strange (1960). Some of the discrepancies between the results obtained by previous workers can therefore be accounted for by variations in the experimental conditions, although it is probable that their galactosamine preparations were not always pure.

Analyses of preparation I. Tables 1 and 2 show the results obtained from the ion-exchange columns and Figs. 1-3 some of the corresponding elution curves. Of the long-column methods only method C failed to show any separation when the fractions were analysed by the Elson-Morgan technique. The hexosamine peaks were, however, only partially resolved on the carboxylic resin (method E). The separation in method D was superior to that found by Partridge & Elsdon (1961) on a shorter column.

A recovery test was carried out on method A by separating a mixture of pure hexosamines, giving recoveries of 106% of glucosamine and 99% of galactosamine. When the pure compounds were added to a hydrolysate of preparation I the corresponding recoveries were 97 and 95%. In view of these results and the consistently good separations that were achieved for all hydrolysates, the mean results from method A (Table 1) were used in assessing the accuracy of hexosamine values obtained from the other columns.

Method B was reliable when applied to 18 hr. hydrolysates of preparation I and there were no significant differences between the results obtained by the ninhydrin and Elson-Morgan analyses. Good separations of the hexosamines were unusual, however, for 4 hr. hydrolysates, and even when they occurred the quantitative results from ninhydrin analyses were much too high for glucosamine (Table 2; and peak X in Fig. 2a). Tryptophan is the only amino acid known to emerge with the hexosamines when a weakly acidic buffer is used for elution (Eastoe, 1954). As Hamilton & Anderson (1954) claimed that this amino acid was found in ion-exchange chromatograms of gelatin hydrolysates (6N-hydrochloric acid for 20 hr.), preparation I was analysed by the method of Spies & Chambers (1948). After hydrolysis for 4 hr. in 4N-hydrochloric acid no tryptophan was detected and therefore it cannot be present in peak X in Fig. 2 (a),

Table 1. *Analyses of protein preparation I from ox skin by using methods A-1, A-2 and S*

Samples (200 mg.) of the preparation were hydrolysed in 10 ml. of 4N-HCl, except for hydrolysates 8 and 9 (300 mg.) and 10 (100 mg.). Experimental details are given in the text. The Elson-Morgan colorimetric procedure was used in all cases and the galactosamine values have been corrected for the percentage colour yield relative to glucosamine under the appropriate conditions.

Hydrolysate no.	Hydrolysis for 4 hr.										Hydrolysis for 18 hr.									
	1	2	2	3	4	5	6	6	7*	7*	8	3	4	5	8	9	10			
Column method	A-1	A-1	S	A-1	A-2	A-2	A-2	A-2	A-2	A-2	A-1	S	S	S	S	S	S			
Glucosamine (%)	0.26	0.22	—	0.24	0.21	0.24	0.28	0.19	0.22	0.22	0.17	—	—	—	—	—	—			
Mean values	0.24			0.23			0.23			0.23			0.23			0.23				
Galactosamine (%)	0.10	0.08	—	0.10	0.07	0.08	0.11	0.06	0.07	0.09	0.07	—	—	—	—	—	—			
Mean values	0.09			0.08			0.08			0.08			0.08			0.08				
Total hexosamine (%)	0.36	0.30	0.37†	0.34	0.28	0.32	0.39	0.25	0.29	0.31	0.24	0.32†	0.35†	0.32†	0.26	0.31	0.37			
Mean values	0.33			0.31			0.31			0.31			0.33			0.28				

* Duplicate analyses on a single hydrolysate residue.

† Average of two determinations.

Table 2. Analyses of protein preparation I from ox skin by using methods B, C, D and E

The weights of samples hydrolysed are as given in Table 1. Experimental details are given in the text. Analysis was by the Elson-Morgan (E-M) or ninhydrin (N) method and the galactosamine values have been appropriately corrected, where necessary, for relative colour yields. Some analyses were carried out on alternate fractions from the column and are marked*.

Hydrolsate no. ...	Hydrolysis for 4 hr.			Hydrolysis for 18 hr.									
	11	11	11	12	12	12	8	9	4	4	9	9	8
Column method ...	B	B	B	B	B	B	B	C	C	C	D	D	E
Colorimetric method ...	E-M*	N*	N	E-M	N*	E-M*	E-M	E-M	E-M	E-M	E-M	N	E-M†
Glucosamine (%)	0.27	0.73	0.83	0.22	0.24	0.29	0.17	—	—	—	0.20	0.24	0.2
Galactosamine (%)	0.13	0.08	0.08	0.06	0.07	0.12	0.09	—	—	—	0.06	—	0.1
Total hexosamine (%)	0.40	0.81	0.91	0.28	0.31	0.41	0.26	0.28	0.26	0.30	0.26	—	0.3

† Approximate results only, owing to variations in fraction volumes (also galactosamine uncorrected for relative colour yield).

Fig. 1. Elution curves, obtained by using method A, of hydrolysates of protein preparations from ox skin. Experimental details are given in the text. (a) Preparation I, hydrolysed for 18 hr. (flow rate 20–40 ml./hr.). (b) Preparation V, hydrolysed for 8 hr. (flow rate 3–4 ml./hr.). Analyses were by the Elson-Morgan method. Gl, Glucosamine; Ga, galactosamine.

Fig. 3. Elution curves, obtained by using (a) method D and (b) method E, of hydrolysates of protein preparations from ox skin. Experimental details are given in the text. (a) Preparation I, hydrolysed for 18 hr. (b) Preparation I, hydrolysed for 18 hr. Analyses were by the Elson-Morgan method. Gl, Glucosamine; Ga, galactosamine.

Fig. 2. Elution curves, obtained by using method B (16 cm. $\times$ 1.0 cm. column), of hydrolysates of protein preparations from ox skin. Experimental details are given in the text. (a) Preparation I, hydrolysed for 4 hr. (b) Preparation I, hydrolysed for 18 hr. Analyses of alternate fractions were by the ninhydrin method (upper curves) and by the Elson-Morgan method (lower curves) respectively. Gl, Glucosamine; Ga, galactosamine; X, see the text; Y, hydroxylysine.

nor can it have interfered with any of the ninhydrin analyses for hexosamines on preparation I. Peak X in Fig. 2 (a) probably contains a peptide that is decomposed when the hydrolysis period is extended.

Reproducibility of method A. In this procedure the positions of the hexosamine peaks showed little variation. The blank tube (or tubes) between the peaks was found at 40 ± 5 ml. in six columns. The variation for successive runs on any given column was only about 2 ml.

The agreement between hexosamine values obtained from two columns was investigated by analysing samples from single hydrolysates (8 hr.) of preparations IV, V and III P, with the following results: (i) glucosamine (%): IV, 0.07, 0.07; V, 0.07, 0.08; III P, 0.73, 0.73; (ii) galactosamine (%): IV, 0.000, 0.00(4); V, 0.05, 0.05; III P, 0.18, 0.18.

These results showed that significant variations in hexosamine values do not occur when different columns are used (see also results for hydrolysate no. 7 of preparation I in Table 2). A small, but clearly defined, peak was found for galactosamine in one analysis of preparation IV. The peak contained approx. 4 μ g. of hexosamine, corresponding to 0.004 % on the protein weight.

A large discrepancy was found between duplicate determinations on hydrolysate no. 6 of preparation I (Table 1) but this was probably due to losses of hexosamine which may occur during evaporation of hydrolysates (even at room temperature). Nevertheless, in general the agreement between determinations on replicate hydrolysates prepared on different occasions was encouraging (Table 1). A definite effect was found, however, when even small changes were made in the ratio of the volume of hydrolysing acid to sample weight. Hydrolysate no. 8 (Table 1), for which 300 mg. of preparation I was taken, gave a significantly lower result for glucosamine by both methods A and B than the mean value obtained from hydrolysate nos. 3-7 (200 mg. hydrolysed). A comparative test with the small column with three different ratios of acid to sample (hydrolysate nos. 3-5; 8 and 9; 10) confirmed this conclusion so far as total hexosamine determinations were concerned.

DISCUSSION

Determination of total hexosamine. Direct determinations of total hexosamine on hydrolysates of preparation I, with various modifications of the Elson-Morgan method, gave results (0.45-0.60 %) that were spuriously high compared with values from column chromatography. This was to be expected for this type of sample, particularly in view of the work of Moss (1955). The small ion-exchange column(s) used for more accurate determinations of total hexosamine is a scaled-down version of method B. It gave more consistent recoveries of added glucosamine (99-100 % for at least 300 μ g. of added hexosamine) than the procedure of Boas (1953). A number of columns were operated simultaneously and less than half an hour was required for the purification of the hydrolysates. Results were obtained for preparation I that agreed quite well with the longer-column analyses.

Determination of the individual hexosamines. For the analysis of fractions from a column the ninhydrin method often provides a convenient alternative to the Elson-Morgan technique, although there is no advantage in sensitivity or accuracy compared with the modification of the latter which was developed in the present work. Ninhydrin may be used with method B, in which an overnight elution with 0.1 M-citrate buffer, pH 5.0, gives an excellent separation of the hexosamines and hydroxylysine. Castellani, Castellani-Bisi & Frigerio (1959) found that hydroxylysine and galactosamine overlapped when 0.1 M-citrate buffer, pH 6.7, was employed. The variability of some previous hydroxylysine values for gelatins and collagens may arise from the inclusion of hexosamine in the amino acid peak.

The present results do not support the criticism

of Watson (1958) that the 0.1 M-buffer procedure gives low and variable values for hexosamines. It was, however, found to be important not to use a short hydrolysis time for method B.

Use of ninhydrin with high-flow-rate procedures. Modern developments in amino acid analysis towards high-flow-rate procedures, which can be adapted for automatic column chromatography, need special consideration in relation to hexosamine analyses. The Elson-Morgan method would be more difficult to make automatic than the ninhydrin procedure. Unfortunately, however, the technique of using a single buffer of relatively high molarity with the sulphonic acid (Moore *et al.* 1958; Kersten & Zürn, 1959) or carboxylic (Hannig, 1959) Amberlite resins appears to be inappropriate for hexosamine analyses when hydroxylysine is present, unless the Elson-Morgan method is used. A good separation of the hexosamines has been obtained, however, by an automatic gradient-elution technique [G. D. Winter (Technicon Chromatography Corp.), personal communication]. It should also be possible to utilize the hydrochloric acid system for automatic analyses, as the ninhydrin technique may be used with method A (C. H. Pearson, unpublished work).

Rapid analyses with the Elson-Morgan method. A large number of analyses can be undertaken with method A without automation, as separations can be accomplished overnight or in less than 3 hr. The accurate prediction of the positions of the hexosamine peaks assists in their identification, even if only one peak is present, and greatly decreases the number of fractions that have to be analysed. A lengthy re-equilibration period is avoided if an alkali treatment is not given between runs. However, because amino acids are only slowly displaced by 0.3 N-hydrochloric acid, they will accumulate in the system from one analysis to the next. This accounts for the fact that the efficiency of a column deteriorates after some time, but frequent renewal of columns should not be necessary.

Method A has given reproducible results when the galactosamine peak contained only 10-20 μ g. of hexosamine (e.g. preparation IV above), but for the most accurate determinations 30-100 μ g. of each compound should be present. Higher column loadings that require an extension of the standard curve beyond about 45 μ g. are less satisfactory.

Unidentified peaks in methods A and B. A number of small unidentified peaks were observed in several experiments with Elson-Morgan analyses. Other investigators have also obtained additional Elson-Morgan-positive peaks under various conditions of ion-exchange-column chromatography (Partridge & Davis, 1955, 1958; Leach, 1960). The early peaks in Fig. 2 (a) (method B) were attributed to amino acid-neutral sugar interactions, which are fre-

quently the major cause of interference in direct Elson-Morgan determinations (Kent & Whitehouse, 1955). An irregular base-line after galactosamine occurred in analyses of preparation V by method A (Fig. 1b). Neutral sugars will not be present in this section of the chromatogram and from the curves given by Crumpton (1959), who used a similar column system, the ill-defined peaks would appear to be due to hexosamines. There is, however, the possibility of artifact formation, particularly during the preceding hydrolysis stage.

SUMMARY

1. Several methods for the determination of glucosamine and galactosamine by ion-exchange-column chromatography have been compared.

2. Hydrolysates of protein preparations from ox skin were used to investigate five different ion-exchange systems combined with the Elson-Morgan colorimetric method. The hexosamines were well separated by elution from a column of Dowex 50 with 0.1M-citrate buffer, pH 5.0 (method B). Good results were also obtained when Amberlite CG-120 was used with either 0.35M-citrate buffer, pH 5.26 (method D), or 0.3N-hydrochloric acid (method A).

3. The colour reaction with ninhydrin was applied as an alternative method for the analysis of the fractions in method B. Galactosamine was not separated from hydroxylysine in method D and therefore ninhydrin could not be employed.

4. The time required for the original hydrochloric acid procedure of Gardell (1953) was decreased. With a resin of small particle size, glucosamine and galactosamine were separated in 3 hr. (method A). This method was studied further and it was concluded that with Elson-Morgan analyses reliable results could be obtained for small amounts of the hexosamines even when large quantities of amino acids were present.

Valuable advice on techniques of column chromatography was given by S. Blackburn, J. E. Eastoe and A. A. Leach. I also acknowledge helpful discussions with Professor A. G. Ward and Dr R. Reed.

REFERENCES

- Boas, N. F. (1953). *J. biol. Chem.* **204**, 533.
 Castellani, A. A., Castellani-Bisi, C. & Frigerio, E. R. (1959). *Arch. Biochem. Biophys.* **80**, 57.
 Cessi, C. & Serafini-Cessi, F. (1962). *Biochem. J.* **82**, 43P.
 Crumpton, M. J. (1959). *Biochem. J.* **72**, 479.
 Eastoe, J. E. (1954). *Nature, Lond.*, **173**, 540.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Fischer, F. G. & Nebel, H. J. (1955). *Hoppe-Seyl. Z.* **302**, 10.
 Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
 Gardell, S. (1958). *Meth. biochem. Anal.* **6**, 289.
 Hamilton, P. B. (1958). *Analyt. Chem.* **30**, 914.
 Hamilton, P. B. & Anderson, R. A. (1954). *J. biol. Chem.* **211**, 95.
 Hannig, K. (1959). *Clin. chim. Acta*, **4**, 51.
 Holdsworth, E. G. (1961). *Biochim. biophys. Acta*, **51**, 295.
 Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Amino Sugars*, 1st ed., p. 181. London: Butterworths.
 Kersten, H. & Zürn, L. (1959). *Nature, Lond.*, **184**, 1490.
 Leach, A. A. (1960). *J. appl. Chem.* **10**, 367.
 Levvy, G. A. & McAllan, A. (1959). *Biochem. J.* **73**, 127.
 Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
 Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
 Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
 Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
 Moss, J. A. (1955). *Biochem. J.* **61**, 151.
 Neuhaus, O. W. & Letzring, M. (1957). *Analyt. Chem.* **29**, 1230.
 Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.
 Partridge, S. M. & Davis, H. F. (1958). *Biochem. J.* **68**, 298.
 Partridge, S. M. & Elsdon, D. F. (1961). *Biochem. J.* **80**, 34P.
 Pogell, B. M. & Koenig, D. F. (1958). *Nature, Lond.*, **182**, 127.
 Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **59**, xiii.
 Scott, J. E. (1962). *Biochem. J.* **82**, 43P.
 Spies, J. R. & Chambers, D. C. (1948). *Analyt. Chem.* **20**, 30.
 Stacey, M. & Barker, S. A. (1962). *Carbohydrates of Living Tissues*, 1st ed., pp. 37, 82. London: D. Van Nostrand.
 Strange, R. E. (1960). *Nature, Lond.*, **187**, 38.
 Watson, M. R. (1958). *Biochem. J.* **68**, 416.