CHROM. 3400

DETERMINATION OF NEUTRAL SUGARS IN GLYCOPROTEINS BY GAS–LIQUID CHROMATOGRAPHY*

W. F. LEHNHARDT AND RICHARD J. WINZLER
Department of Biochemistry, State University of New York at Buffalo, Buffalo, N.Y. 14214 (U.S.A.)
(Received January 18th, 1968)

SUMMARY

A procedure is described for the quantitative hydrolysis, derivatization, and analysis by gas–liquid chromatography of the neutral monosaccharides in glycoproteins. The new liquid phase combination employed gives resolution of eleven alditol acetates and shows a low level of baseline drift during temperature programming. Application of the analysis to a number of different glycoproteins is given.

In the course of studies on the structure of oligosaccharides from glycoproteins, a method was required which would quantitate on a submicrogram scale the individual neutral sugars occurring in protein hydrolysates. The method described below has satisfied this need. It involves hydrolysis of the protein sample with Dow 50 in the hydrogen form, reduction of the sugars to the corresponding alcohols, acetylation with acetic anhydride in pyridine, and gas chromatography of the acetylated alcohols.

Much attention has been given in recent years to the gas chromatographic analysis of carbohydrates, and extensive reviews have been published[1–4]. Of the various potential derivatives, we have chosen to use the O-acetyl glycitols, proposed by GUNNER *et al.*[5,6] and extended by other workers[7–11].

The procedure is quite similar to that recently described by KIM *et al.*[9]. However, there is sufficient difference in the methodology, in the column and operating conditions, and in the time required for analysis to merit presentation of this paper.

PROCEDURE

Apparatus and chromatographic conditions

A Microtek Model 220 gas chromatograph equipped with a temperature programmer and flame ionization detectors was employed. The injector port was modified by the use of a glass insert (8.4 cm × 1 cm O.D. × 0.25 cm I.D.) to reduce the dead volume of the vaporizing chamber. The U-shaped glass column 4 mm I.D. × 1.83 m containing by weight 0.75 % HiEFF-1BP, 0.25 % EGSS-X, and 0.1 % 144–B (phenyl-

* This work was done under the sponsorship of the Commission on Influenza of the Armed Forces Epidemiological Board and was supported by the U. S. Army Medical Research and Development Command, Department of the Army, under Research Contract No. MD-2803 and also by a grant from the U. S. Public Health Service No. CA08492-03.

diethanolamine) on 60/80 Gas-Chromosorb Q (Applied Science Laboratory, State College, Pennsylvania, Lot 690-42B).

The conditions for chromatography were:
Column temperature: initial 160°, final 210°.
Program rate: 1.3°/min.
Detector cell temperature: 325°.
Injector temperature: 225°.
Transfer temperature: 235°.
Hydrogen (to detector): 50 ml/min.
Air (to detector burner): 189 ml/min.
Air (to detector purge): 189 ml/min.
Nitrogen (carrier): 40 ml/min.
Chart speed: 30 in./h.

A centrifuged bio-dryer, VirTis Research Equipment, Model 10-310, was used for all concentrations at reduced pressure. The 6 × 50 mm Kimax culture tubes and 5 × 50 mm micro-polyethylene disposable centrifuge tubes were obtained from Arthur H. Thomas Company. Silicone blind hole rubber septa were used to seal hydrolysis tubes.

Reagents

Pyridine, acetic acid, acetic anhydride, methanol, hydrochloric acid, Dowex 1 X8 (HCO_3^-) 200/400 mesh, and Dowex 50 X2 (H^+) 200/400 were "Baker Analyzed" reagents. The pyridine was refluxed for 1 h with ninhydrin, distilled with protection from moisture, and stored over KOH until used. Sodium borohydride was obtained from Fischer Scientific Company. All parent carbohydrates were purchased from Calbiochem and were recrystallized until pure when tested by gas chromatography as described below. The corresponding alditols and alditol acetates were prepared as described by ABDEL-AKHER *et al.*[12] and recrystallized until pure by gas chromatography. Orosomucoid was prepared by the method of BEZKOROVAINY AND WINZLER[13]

Application to glycoproteins

Glycoprotein samples, 0.1 to 3 mg, containing about 0.1 μmole of neutral sugar, are dried in 6 × 50 mm culture tubes under reduced pressure in the bio-dryer. Samples are stored in a desiccator at this stage until they are hydrolyzed. The dried samples are dissolved in 50 μl of H_2O and 50 μl of a 40 % w/v suspension of Dowex 50 X2 (H^+) 200/400 mesh resin in 0.02 *N* HCl is added. The sample tubes are sealed with silicon rubber septa, mixed, and placed in a steam bath for 40 h.

The samples are removed from the hydrolysis bath, allowed to cool, and 50 μl of an internal standard solution containing 0.0277 to 0.2771 μmoles of arabitol, depending on the carbohydrate content, is injected through the septum into the sample. The sample is thoroughly mixed and centrifuged for 2 min at 2800 r.p.m. to sediment sample from the septum and walls of the tube.

The septa are removed, and 0.4 ml of H_2O is added to each tube with thorough mixing. The mixture is transferred with a disposable pipette to columns containing Dowex 1 X8 (HCO_3^-) resin, and allowed to elute into 12 ml siliconized conical centrifuge tubes. These columns are prepared in 6 in. disposable pipettes with a glass wool plug by the addition of 50 μl of a 20 % w/v suspension of Dowex 1 X8 (HCO_3^-)

200/400 mesh resin. A glass wool plug is employed to separate the Dowex 1 X8 (HCO_3^-) from the Dowex 50 X2 (H^+) used in hydrolysis.

The hydrolysis tubes are washed twice with 0.3 ml of water, the washings also being passed through the column. The elution of the column is continued with 1 ml of a 50 % v/v solution of methanol–water, and combined eluates are concentrated to dryness in the bio-dryer under reduced pressure.

The residues in the conical centrifuge tubes are dissolved in 100 μl of water and transferred to a 5 × 50 mm polyethylene disposable centrifuge tube using a 100 μl Lang-Levy pipette. The centrifuge tubes are washed with two 100 μl portions of water and the washings also transferred to the 6 × 50 mm tube. The samples are then concentrated to dryness in the bio-dryer under reduced pressure.

The samples are dissolved in 50 μl of water and treated for 1 h at room temperature with 50 μl of 0.22 *M* $NaBH_4$. Excess $NaBH_4$ is decomposed by the addition of 50 μl glacial acetic acid, and the samples are concentrated to dryness on the bio-dryer.

Borate is removed as the volatile trimethyl borate by the addition of three 100 μl portions of a 1:1000 v/v HCl–methanol solution with concentration to dryness in the bio-dryer under reduced pressure after each addition. The samples are usually held overnight at this stage in a desiccator to insure dryness.

The samples are acetylated for 15 min at 100° with 50 μl of pyridine and 50 μl of acetic anhydride. They are then removed, mixed, and replaced in the steam bath for an additional 15 min. The samples are then cooled and 1 to 10 μl aliquots injected directly into the gas chromatograph. Samples can be kept for at least three months at this stage without decomposition.

RESULTS

Separation of standard monosaccharides

The procedure gives well separated peaks for most of the common sugars from trioses to hexoses. This is shown in Fig. 1. Of the twelve compounds shown in this

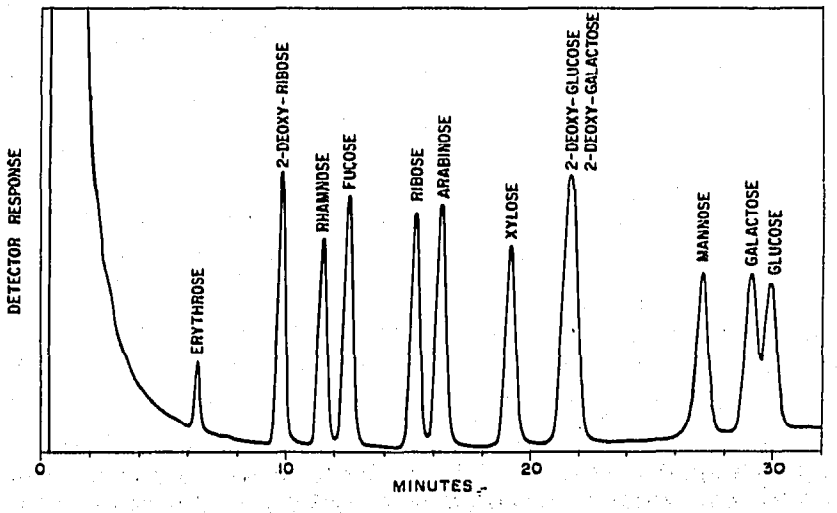

Fig. 1. Gas–liquid chromatography of the alditol acetates produced from a standard mixture of the parent carbohydrates. Liquid phase and column parameters are given in the text.

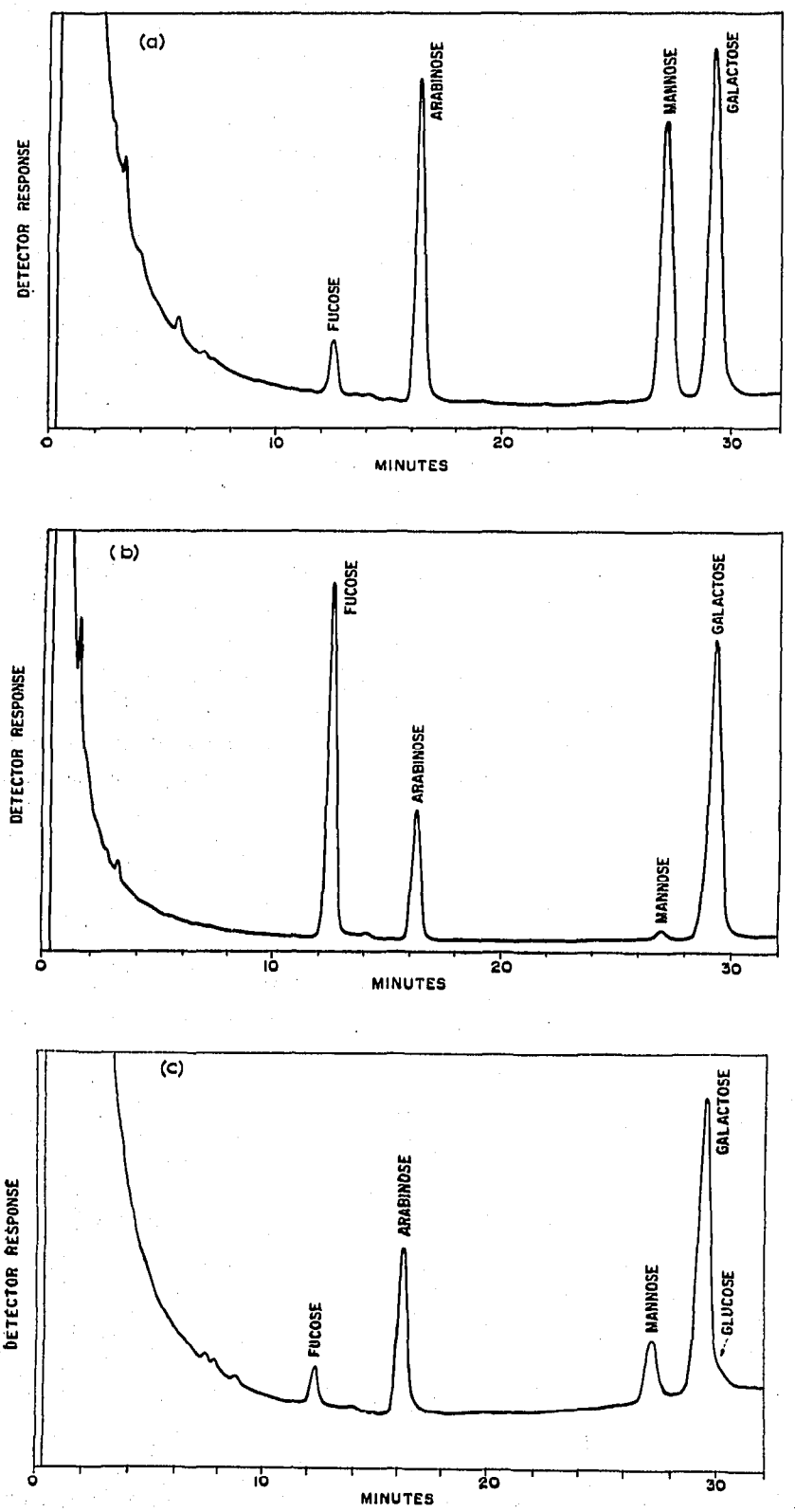

Fig. 2. Gas–liquid chromatography of the alditol acetates of the neutral sugars released after hydrolysis of various glycoproteins. Arabitol was added as the internal standard. (a) Orosomucoid; (b) canine submaxillary mucin; (c) a glycopeptide released by the action of trypsin from intact human erythrocytes. Liquid phase and column conditions are described in the text.

TABLE I

THE CARBOHYDRATE VALUES OBTAINED FROM VARIOUS GLYCOPROTEINS BY RESIN HYDROLYSIS, DERIVATIZATION, AND ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY OF THE ALDITOL ACETATES

Parent carbohydrate	*Orosomucoid (μMoles/mg)*	*Canine submaxillary mucin (μMoles/mg)*	*Glycopeptide released by trypsin from human erythrocytes (μmoles/mg)*
Fucose	0.0453	0.6250	0.0546
Mannose	0.3017	0.0148	0.1108
Galactose	0.3842	0.6983	0.5830
Glucose	—	—	trace

chromatogram the only complete overlap is that of 2-deoxyglucose with 2-deoxygalactose. Separation of glucose from galactose is not complete in this system. Overlap can naturally be expected between pairs of sugars which produce identical alcohols upon reduction, *e.g.* arabinose and lyxose.

The procedure has been applied to a number of glycoproteins. Fig. 2 shows chromatograms obtained with orosomucoid, canine submaxillary mucin, and a glycopeptide released by trypsin from intact human erythrocytes[14]. Table I gives the content of galactose, mannose, and fucose for each of these proteins calculated from the relation:

$$\mu\text{moles/mg} = \frac{(\text{area of } x)\quad(\mu\text{moles of arabitol})}{(\text{area of arabitol})\,(\text{response factor})\,(\text{mg of protein})}$$

Time required for hydrolysis

Orosomucoid, an α-1 acid glycoprotein containing the neutral sugars, fucose, mannose, and galactose, was used to determine the time required for hydrolysis. It was dried in an Abderhalden drying pistol for three days at 76° under reduced pressure, and a weighed amount dissolved in distilled water. Aliquots containing 0.9450 mg were placed in 6 × 50 mm culture tubes and concentrated to dryness on the bio-dryer.

The optimal time for hydrolysis of orosomucoid was determined using two different methods of hydrolysis. One method involved treatment with Dowex 50 X2 (H^+) resin in 0.01 *N* HCl as described under Methods, Fig. 3. The other procedure involved the solution of the dried protein in 6 × 50 mm culture tube in 50 μl of water followed by the addition of 50 μl of 2 *N* H_2SO_4. The tubes were sealed with silicone rubber septa and placed in a steam bath for the appropriate time. Fifty microliters of water containing 0.2761 μmoles of arabitol was injected into each sample and passed through an ion exchange column to deionize the sample. These columns consisted of dispopipettes containing a glass wool plug, 1 ml of a 20 % w/v suspension of Dowex 1 X8 (HCO_3^-) 200/400 mesh resin in H_2O, another glass wool plug, and 50 μl of a 40 % w/v suspension of Dowex 50 X2(H^+) 200/400 mesh resin in H_2O. The hydrolyzed samples were passed through the column and eluted and washed into centrifuge tubes as previously described. The rest of the procedure was that already described.

The results of this hydrolysis (Fig. 4) show that the release of fucose and galactose preceded the release of mannose in both hydrolytic procedures. The destructive

effect of the H_2SO_4 hydrolysis on the sugars is quite apparent, and the quantitative values never rise to the maximum obtainable by Dowex 50 hydrolysis.

Hydrolysis with Dowex 50 shows maximal values reached after about 30 h, with very little destruction with prolonged hydrolysis periods.

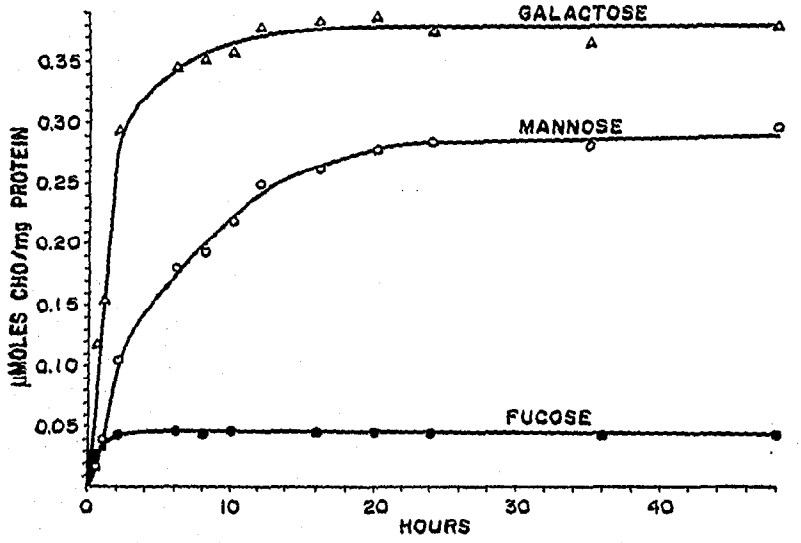

Fig. 3. Liberation of neutral monosaccharides from orosomucoid by hydrolysis with Dowex 50 X2 (H^+) resin in 0.01 *N* HCl and analysis by gas–liquid chromatography.

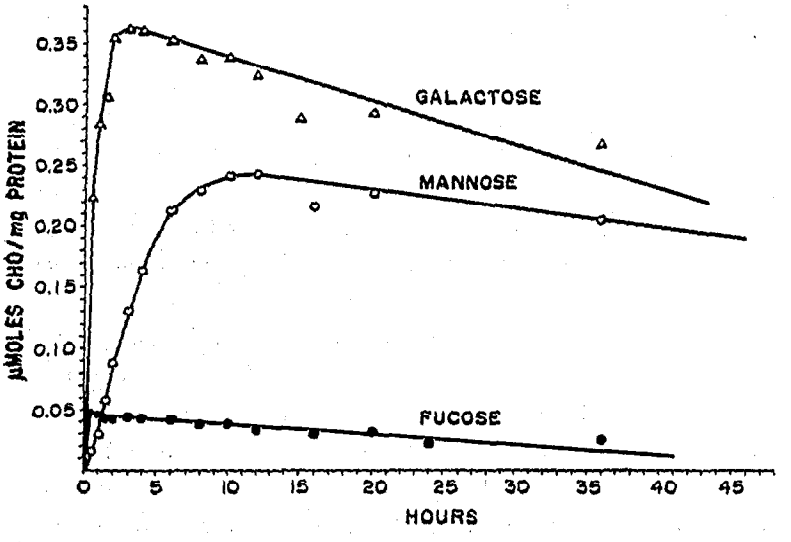

Fig. 4. Liberation of neutral monosaccharides from orosomucoid by hydrolysis with 1 *N* H_2SO_4 and analysis by gas–liquid chromatography.

Determination of response factor

The relative detector response for each monosaccharide was calculated with respect to arabitol. Samples of different carbohydrates were dried for 48 h at 76° under reduced pressure in an Abderhalden drying pistol. Each sample was weighed accurately in a range between 9.5 and 10 mg on a Cahn electrobalance and diluted to 5 ml with water. Twenty microliter aliquots of each sample were combined in a micro

polyethylene disposable tube and concentrated to dryness under reduced pressure. The samples were reduced with 0.11 M $NaBH_4$ and acetylated using the procedure already described.

The relative response factors were calculated using the following equation:

$$\text{Response factor} = \frac{(\text{area of } x)\ (\mu\text{moles arabitol})}{(\text{area of arabitol})\ (\mu\text{moles of } x)}$$

The results for several monosaccharides are shown in Table II. These response factors were used in the calculation of neutral sugars in unknown samples.

TABLE II

RELATIVE RESPONSE FACTORS AND RELATIVE RETENTIONS FOR THE ALDITOL ACETATES

The values were calculated using arabitol pentaacetate as the standard. The retention time for arabitol pentaacetate is about 16 min under the conditions described.

Parent carbohydrate	*Response factor*	*Relative retention*
2-Deoxyribose	0.841	0.593
Rhamnose	1.135	0.702
Fucose	1.089	0.765
Ribose	0.937	0.937
Arabinose	1.000	1.000
Xylose	0.965	1.178
2-Deoxyglucose	1.101	1.317
Mannose	1.182	1.665
Galactose	1.211	1.784
Glucose	1.197	1.839

Accuracy and reproducibility of method on standard sugars

Samples of L-fucose, D-mannose, and D-galactose were dried for 24 h at 76° under reduced pressure in an Abderhalden drying pistol. Three different mixed standard solutions were prepared, each containing 1.7 to 1.8 mg/10 ml of each of the above monosaccharides.

Aliquots of 300 μl were taken from each of the standards and placed in 6 × 50 mm glass culture tubes and concentrated to dryness in the bio-dryer.

Fifty microliters of H_2O and 50 μl of a 40 % (w/v) suspension of Dowex 50 X2 (H^+) resin in 0.02 N HCl was added to each tube; the tubes sealed with a silicone rubber septum. The samples were not hydrolyzed. The internal standard was added and the samples carried through the previously described procedure and the recovery of each sugar calculated for each experiment. The results indicate that the reproducibility of the method with known standards is 100 ± 3.8 %.

Reproducibility of method on a glycoprotein sample

Ten 300 μl aliquots of a solution of orosomucoid containing 3.273 mg/ml in 6 × 50 mm culture tubes were concentrated to dryness in the bio-dryer. The samples were hydrolyzed for 40 h in a steam bath using Dowex 50 X2 (H^+) resin and were carried through the standard procedure. The μmoles/mg of fucose, galactose, and

mannose were calculated for each sample. The results of these experiments indicate that the reproducibility of the method with a glycoprotein is within 3 % for each of the sugars.

Proportionality of response with amount of protein

In order to demonstrate that the neutral sugar values obtained is proportional to the glycoprotein added, 0.366, 0.733, and 1.466 mg of orosomucoid were subjected to the standard procedure. The relation of fucose, galactose, and mannose to the amount of orosomucoid added is shown in Fig. 5. These results show that the method gives linear relationship with different amounts of protein.

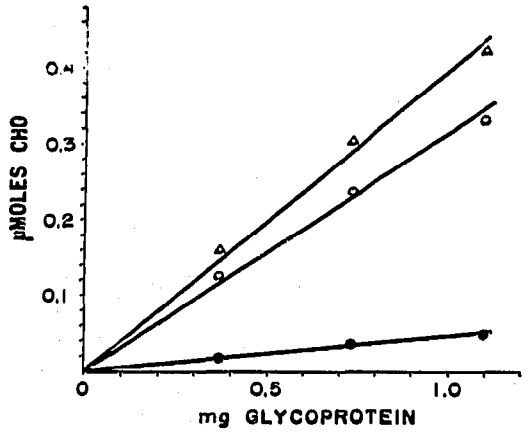

Fig. 5. Proportionality study on the glycoprotein orosomucoid showing the linear relationship between the amount of carbohydrates released and the amount of protein hydrolyzed (–●–) Fucose, (–○–) mannose and (–△–) galactose.

The method that has been described has been applied to a number of glycoproteins including albumin and aldolase which have no carbohydrate. These proteins yield no carbohydrate peaks in the gas chromatogram under the conditions that we have described.

A number of small unidentified peaks have been observed with several glycoproteins. One of these is between arabinose and xylose and another between 2-deoxyglucose and mannose. The nature of these peaks has not yet been established.

Studies similar to those of CROWELL *et al*[5] have been made varying the time, temperature, and concentration for the borohydride reduction steps. The conditions described in the procedure seem optimal for convenience and precision. Similarly, studies have been made of the conditions for acetylation in order to select the optimum for convenience and precision.

The present method is convenient, in that is employs a single column which does not bleed appreciably over the temperature range programmed. Freshly prepared columns give somewhat better resolution, but rapidly change to a steady performance. Columns have been used for more than 200 determinations without significant change in their characteristics.

The sensitivity of the method can be markedly increased by using balanced dual columns, but such sensitivity is not usually necessary.

The importance of the bio-dryer for the success of this procedure should be

emphasized, since with the small tubes used, other drying procedures are either too prolonged or lead to loss of material.

ACKNOWLEDGEMENT

The authors thank J. E. BLEECHER for his diligent efforts on the experimental work connected with this investigation.

REFERENCES

1 C. T. BISHOP, *Advan. Carbohydrate Chem.*, 19 (1964) 95.
2 C. T. BISHOP, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. X, Interscience, New York, 1962, p. 1–42.
3 H. W. KIRCHER, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. I, Academic Press, New York, 1962, p. 13–20.
4 W. W. WELLS, R. BENTLEY AND C. C. SWEELEY, in H. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964.
5 S. W. GUNNER, J. K. N. JONES AND M. B. PERRY, *Can. J. Chem.*, 39 (1961) 1892.
6 S. W. GUNNER, J. K. N. JONES AND M. B. PERRY, *Chem. Ind. (London)*, *Ser. A*, (1961) 255.
7 E. P. CROWELL AND B. B. BURNETT, *Anal. Chem.*, 39 (1967) 121.
8 J. A. HAUSE, J. A. HUBICKI AND G. G. HAZEN, *Anal. Chem.*, 34 (1962) 1567.
9 J. H. KIM, B. SHOME, T. H. LIAO AND J. G. PIERCE, *Anal. Biochem.*, 20 (1967) 258.
10 J. M. OADES, *J. Chromatog.*, 28 (1967) 246.
11 J. S. SAWARDEKER, J. H. SLONEKER AND A. JEANS, *Anal. Chem.*, 37 (1965) 1602.
12 J. ABDEL-AKHER, J. K. HAMILTON AND F. SMITH, *J. Am. Chem. Soc.*, 73 (1951) 4691.
13 A. BEZKOROVAINY AND R. J. WINZLER, *Biochim. Biophys. Acta*, 49 (1961) 559.
14 R. J. WINZLER, E. D. HARRIS, D. J. PEKAS, C. A. JOHNSON AND P. WEBER, *Biochemistry*, 6 (1967) 2159.