QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF FUCOSE AS TRITIATED FUCITOL*

MAKOTO MURAKAMI** AND RICHARD J. WINZLER
Department of Biochemistry, State University of New York at Buffalo, Buffalo, N.Y. (U.S.A.)
(Received December 14th, 1966)

The reduction of the carbonyl group of reducing sugars to the corresponding sugar alcohols with sodium borohydride has been advantageously used in carbohydrate chemistry. It seemed possible that this reaction might be adapted to the determination of reducing sugar in ultramicro quantity by employing tritium-labeled sodium borohydride to introduce tritium into the corresponding alcohol. We have applied this principle to the estimation of L-fucose, a sugar of biochemical and immunological interest for which present analytical methods are relatively insensitive and unprecise. Fucose and hexoses, with ribose added as an internal standard, were reduced with tritium-labeled sodium borohydride, and the labeled reduction products were separated by thin-layer chromatography. The amount of radioactivity fixed in the sugar alcohols was proportional to the molar amounts of the sugars used. The method has been applied to the estimation of fucose content of orosomucoid. The procedure as described requires 0.3–1.6 μg of the sugar, but much greater sensitivities are possible.

MATERIALS AND METHODS

Sugar preparations (L-fucose, D-ribose, D-galactose, D-mannose, L-fucitol, D-ribitol, D-galactitol and D-mannitol) were analyzed reagents obtained from Mann Research Laboratories.

To 10 mC of tritium-labeled sodium borohydride (purchased from New England Nuclear Corporation) was added enough recrystallized non-radioactive sodium borohydride to bring the total to 1 mmole. This was dissolved in 10 ml of 0.01 *N* NaOH. One ml aliquots containing 1 mC of tritium, 0.1 mmole of sodium borohydride and 0.01 mmole of NaOH were lyophilized in test tubes, and stored until used.

Shortly before use, 1 ml of water was added to a test tube containing the radioactive sodium borohydride. 10 μl of this solution was added to a mixture of fucose (0.02–0.1 μmole) and ribose (0.02–0.1 μmole) in 20 μl of water. After incubation overnight at room temperature, one drop of glacial acetic acid was added to destroy the excess borohydride, and the mixture was lyophilized.

The sugar alcohols were separated by thin layer chromatography on Kieselguhr. Twenty grams of Kieselguhr containing $CaSO_4$ as a binder was mixed with 40 ml of

* This investigation was supported by grants from the National Institutes of Health (CA-AM 08492) and from the Armed Forces Epidemiological Board (DA49-193-MD-2803).
** Present address: Rikagaku Kenkyusho, The Institute of Physical and Chemical Research, Komagome, Bunkyo-ku, Tokyo, Japan.

0.1 *M* phosphate buffer (pH 5.7) in a mortar. A layer 0.25 mm thick was applied with an applicator to 20 × 20 cm plates. The plates were dried at room temperature for 15 min and were then kept in an oven at 105° for 2 h and stored in a desiccator until used.

The solvent system utilized for separation of the sugar alcohols was butanol–pyridine–water (6:4:3 by volume)[1].

The lyophilized samples were dissolved in 10 μl of water, and 1 μl was spotted on the plate. When the solvent had ascended to the top edge of the plate (about 3.5 h), the plates were dried overnight at room temperature. For the qualitative localization of sugars and sugar alcohols, sodium meta-periodate and benzidine[2] were used as detection reagents.

The location of radioactivity in the chromatograms was determined by cutting the dried, unstained Kieselguhr into forty, 5 mm sections. Each section was scraped off the plate and transferred into liquid scintillation counting vials. One-half ml of water was added, followed by 10 ml of the scintillation medium[3]. The samples were counted for 10 min on a model 70008 Nuclear-Chicago liquid scintillation counter.

Estimation of the fucose content of orosomucoid

Human orosomucoid (1–2 mg) prepared as described by BEZKOROVAINY AND WINZLER[4], was hydrolyzed with Dowex 50W-X8 resin (200–400 mesh, H^+ form) at 100° for 24 h[5]. The resin and hydrolyzate were separated by filtration and washing through a sintered glass filter. The filtrates were transferred to a 50 ml Erlenmeyer flask, and the hydrolyzate neutralized with Dowex 2-X8 (200–400 mesh, bicarbonate form)[6]. The resin was removed by filtration and rinsed with three 1 ml aliquots of water. The combined filtrate and washings were lyophilized. The residue was transferred quantitatively to a small test tube (1 × 7.5 cm) using 0.5 ml of water, relyophilized, and the lyophilized residue dissolved in 10 μl of water. Ten μl of ribose (0.1 μmole) was added as an internal standard followed by 10 μl of the tritium-labeled borohydride (1 μmole, 10 μC). The remainder of the procedure was as already described.

RESULTS

Fig. 1 shows the chromatographic separation of ribose, galactose, mannose and fucose and for these same compounds reduced with sodium borohydride as described in the text; *i.e.*, ribitol, galactitol, mannitol and fucitol. Fucitol and ribitol are well separated from each other and from the hexitols, but galactitol is not separated from mannitol in this chromatographic system. The same results were found when the quantitative distribution of radioactivity was studied following reduction of the sugars with tritium-labeled sodium borohydride. As is shown in Fig. 2, three radioactive peaks are found when fucose and ribose are reduced with tritium-labeled borohydride and chromatographed. Two have the R_F values of ribitol and fucitol, while another unidentified component moves with the solvent front.

Various amounts of ribose (0.02–0.1 μmole) were reduced with tritium-labeled sodium borohydride in separate tubes, and one-tenth of the reduced sugar alcohol in each tube was applied to the chromatographic plates. The sum of radioactivity in the ribitol peak plotted against the amount of ribose added gives the linear relationship

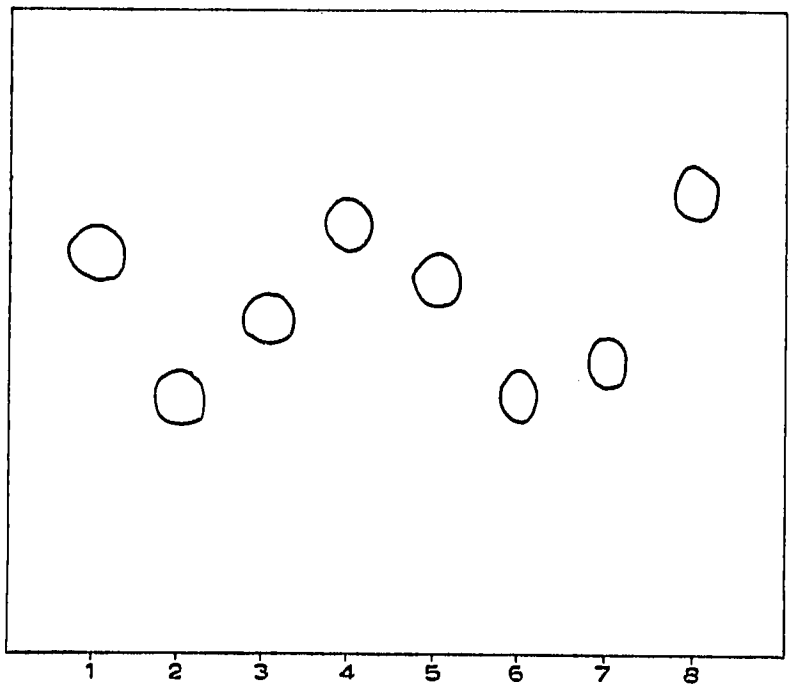

Fig. 1. Thin-layer chromatogram of the sugars and sugar alcohols. The circled spots correspond to: 1 = ribose (R_F 0.57); 2 = galactose (R_F 0.32); 3 = mannose (R_F 0.46); 4 = fucose (R_F 0.62); 5 = ribitol (R_F 0.53); 6 = galactitol (R_F 0.32); 7 = mannitol (R_F 0.38); 8 = fucitol (R_F 0.68).

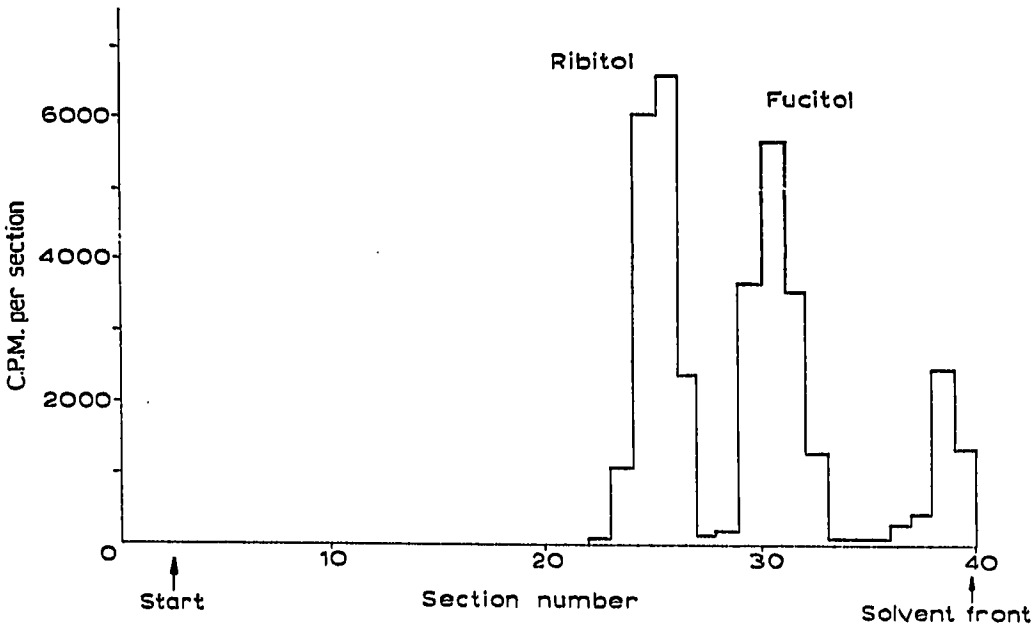

Fig. 2. Separation of radioactive fucitol and ribitol by thin-layer chromatography. In this experiment a mixture of fucose and ribose (0.1 μmole each) was reduced by the tritium-labeled sodium borohydride (1 μmole, 10 μC) and one-tenth of the amount was applied to the chromatographic plate. The plate was cut into 0.5 cm sections and the radioactivity in each section determined.

shown in Fig. 3. Similar linearity was found with fucose. For practical utilization of this procedure for fucose determination, it was felt that addition of a fixed quantity of ribose to a sample prior to reduction would serve as an internal standard. Fig. 4 shows the ratio of counts fixed in the fucitol component to those in ribitol at various amounts of fucose (0.002–0.01 μmole) and a fixed amount of ribose (0.01 μmole).

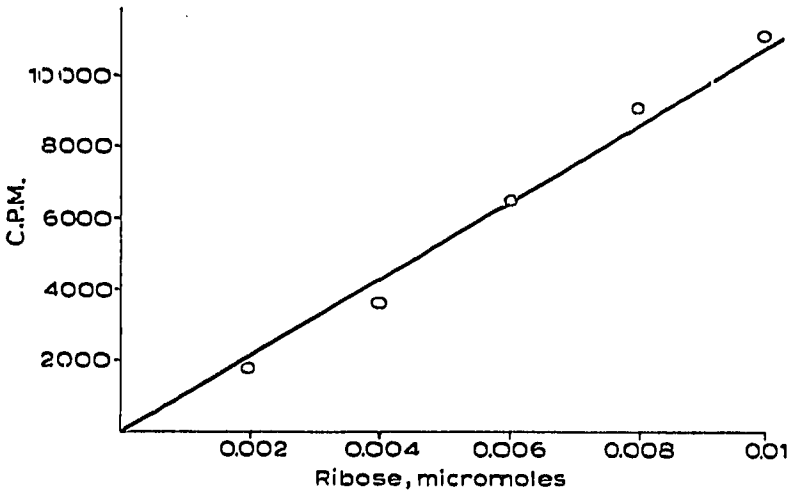

Fig. 3. Linear relationship between increasing concentrations of ribose and the counts per minute fixed in ribitol.

As is shown in Fig. 4 the counts fixed in fucitol are slightly lower than those of ribitol reduced from an equimolar amount of ribose. In 25 experiments the counts fixed in fucitol were 88.7 $\pm$ 4.2 % of those fixed in an equimolar amount of ribose. This difference persisted with samples of fucose and ribose recrystallized several times and dried to constant weight. The reason for this difference is not apparent.

Routinely a period of 16 h has been used for the reduction with borohydride. That this is an adequate time is evident from Fig. 5 which shows that the reduction of fucose and ribose is completed within 1 h under the conditions employed. It is evident that fucose is reduced somewhat more slowly than ribose but that both are fully reduced in 1 h.

The procedure outlined has been applied to the determination of the fucose content of human orosomucoid. Results given in Fig. 6 show that the radioactivity in the fucitol and ribitol peaks are separated from the large unresolved radioactive peak, consisting of galactitol and mannitol. The fucose content of the glycoprotein was calculated from the relationship:

$$\text{fucose } (\mu\text{mole}) = \text{ribose } (\mu\text{mole}) \times \frac{\text{c.p.m. in fucitol}}{\text{c.p.m. in ribitol}} \times \frac{100}{88.7}$$

$$\text{fucose } (\%) = \frac{\text{fucose } (\mu\text{mole}) \times 164 \times 100}{\text{orosomucoid } (\mu\text{g})}$$

The average fucose content of human orosomucoid in 5 determinations by the present method was 0.66 % $\pm$ 0.06 S.D. Previous data on the fucose content of

orosomucoid determined by the method of DISCHE AND SHETTLES[7] have varied from 0.7 to 1.5 %[4,8–13].

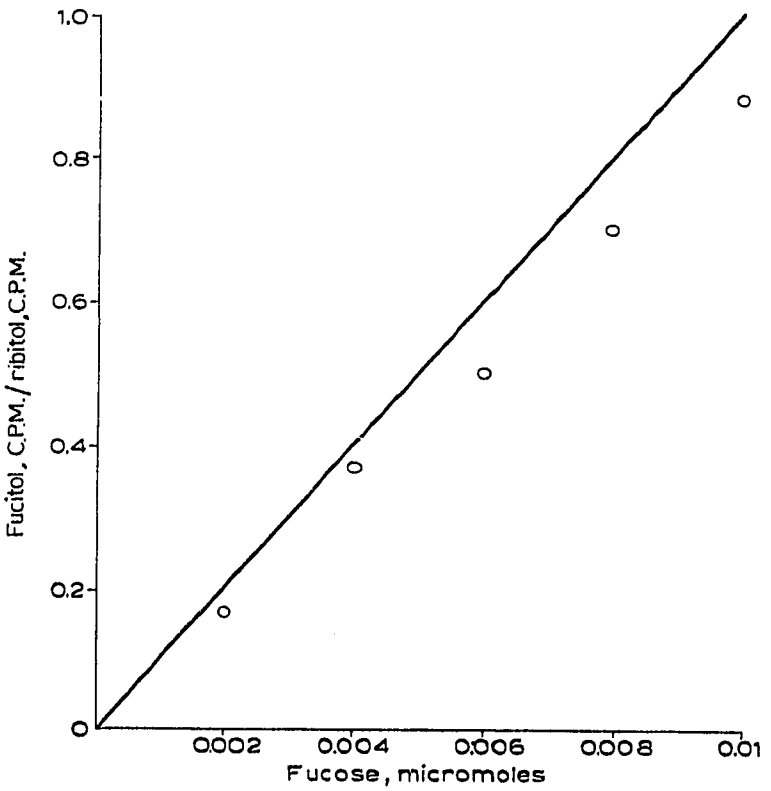

Fig. 4. Ribose as an "internal standard" for fucose determination. A constant amount of ribose (0.1 μmole) was mixed with varying amounts of fucose (0.02–0.1 μmole) and the mixture reduced. One-tenth of the amount was chromatographed and counted as described in the text. The solid line results from the assumption that fucose and ribose fix the same number of counts per mole. The points are experimental.

DISCUSSION

The procedure described here is potentially adaptable to give much greater sensitivities by utilizing borohydride of higher specific activity, and the chromatography of a larger proportion of the sample. The procedure is also adaptable to any sugar which can be chromatographically separated after reduction to an alcohol. Another application of the procedure is its adaptation as an exceedingly sensitive method for determination of reducing end groups in oligosaccharides and polysaccharides.

It would be expected that the same amount of tritium should be fixed into the sugar alcohols prepared from equimolar amounts of sugars. The present results, however, showed that counts in fucitol were always lower than those in equimolar amounts of ribitol. Possible causes of this discrepancy include weighing errors, presence of impurities or moisture, incomplete reduction of fucose by the tritiated sodium borohydride, a non-reductive exchange between tritium of the sodium borohydride and hydrogen atoms of the sugar alcohol which was greater with ribose, and differences

between quenching rates associated with the liquid scintillation counting of the radioactive sugar alcohols. Each of these possibilities was investigated and was eliminated. The ribose and fucose samples used in the present experiments were prepared several times in separate weighings with similar results.

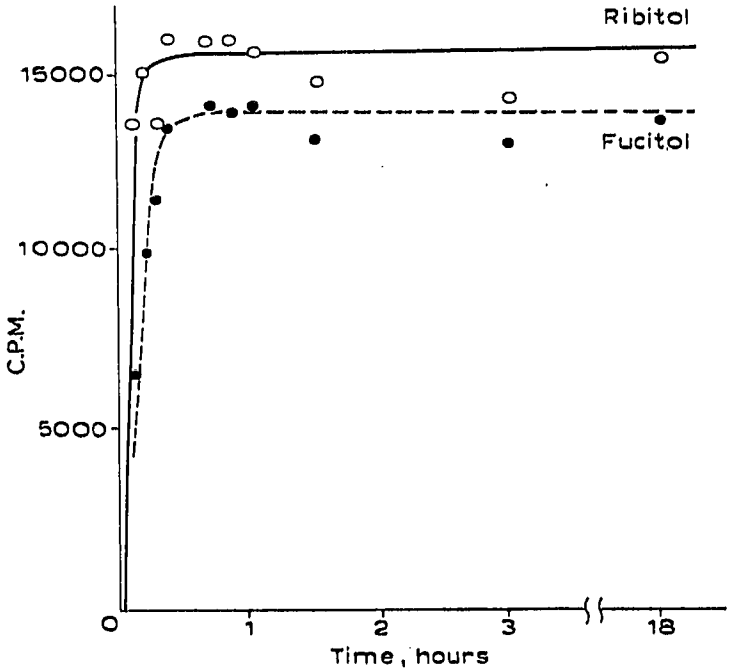

Fig. 5. Effect of time on reduction of fucose and ribose (0.01 μmole of each).

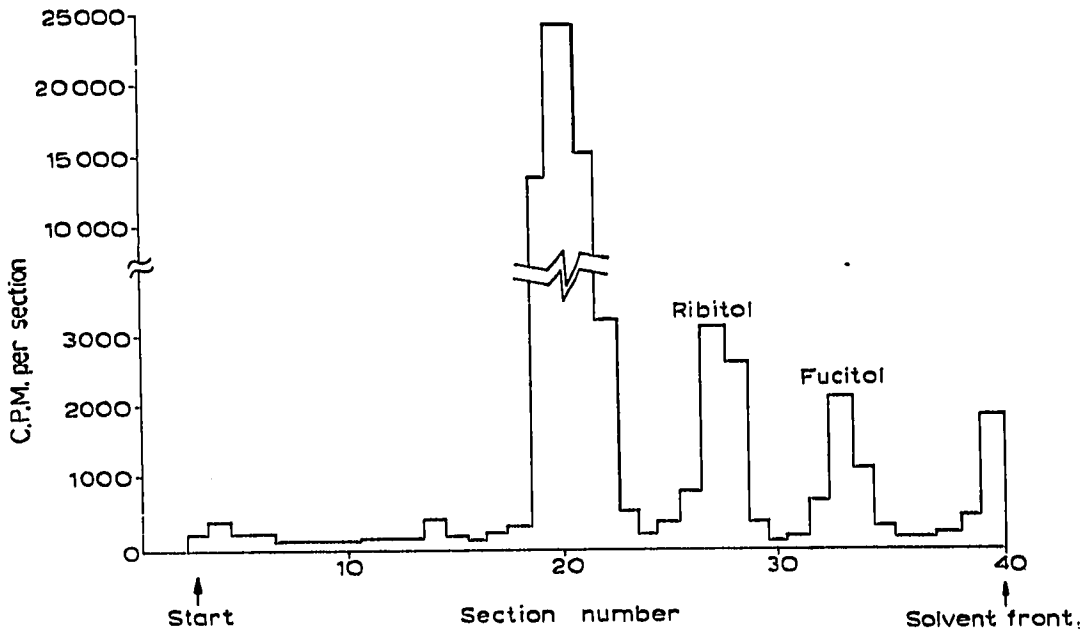

Fig. 6. Separation of radioactive neutral sugar alcohols in reduced hydrolysate of 1.6 mg of orosomucoid. Ribose (0.1 μmole) was added as an internal standard prior to reduction. One-twentieth of the reduction product was chromatographed and counted.

Ribose and fucose were recrystallized from hot ethanol and the crystallized sugars were dried *in vacuo* over calcium chloride at room temperature to a constant weight. The experiments using the recrystallized preparations gave the same results as those obtained by using the commercial preparations of the highest quality. Using gas–liquid chromatography of the trimethyl silyl derivatives no carbohydrate impurity could be detected in the recrystallized fucose and ribose.

Ribose and fucose were reduced with non-radioactive sodium borohydride under precisely the conditions described. Tritium-labeled sodium borohydride was then added to the reduced sugar mixture. No radioactivity was fixed in the sugar alcohols. It thus can be concluded that no exchange reaction occurred between tritium of the sodium borohydride and hydrogen of the sugar alcohols. It should be emphasized that reduction of the sugars with sodium borohydride was quantitative, since no radioactivity was found in the sugar alcohols previously reduced by the addition of non-radioactive sodium borohydride. Counting efficiencies of the samples determined by the channel ratio method[14,15] showed no differences between ribitol and fucitol.

The cause of the lower incorporation of tritium into fucitol is thus not clear. It is possible that there are steric differences which favor fucose reduction by the non-radioactive borohydride. Another possibility is that fucose is less stable than ribose leading to some destruction during reduction with borohydride.

The relationships are sufficiently constant, however, that the procedure can be applied to the determination of very small amounts of fucose.

SUMMARY

A procedure has been described which provides a highly sensitive method for fucose estimation. Fucose with ribose added as an internal standard, was reduced with tritium-labeled sodium borohydride, and the resulting radioactive sugar alcohols, fucitol and ribitol, were separated by thin-layer chromatography on Kieselguhr. Tritium was fixed into the sugar alcohols in proportion to amounts of sugars added. This method was applied to estimation of fucose content of human orosomucoid. The procedure as described requires about 0.3–1.6 μg of fucose, and is capable of much greater sensitivity.

REFERENCES

1 D. HAMERMAN, K. W. BARTZ AND A. REIFE, *Anal. Chem.*, 27 (1955) 1524.
2 D. WALDI, *J. Chromatog.*, 18 (1965) 417.
3 J. P. DAVIDSON AND P. FEIGELSON, *Intern. J. Appl. Radiation Isotopes*, 2 (1957) 1.
4 A. BEZKOROVAINY AND R. J. WINZLER, *Biochim. Biophys. Acta*, 49 (1961) 559.
5 C. MUND AND H. VENNER, *Z. Physiol. Chem.*, 338 (1964) 145.
6 E. F. WALBORG, L. CHRISTENSSON AND S. GARDELL, *Anal. Biochem.*, 13 (1965) 177.
7 Z. DISCHE AND L. B. SHETTLES, *J. Biol. Chem.*, 175 (1948) 595.
8 L. ODIN, *Acta Chem. Scand.*, 9 (1955) 862.
9 I. YAMASHINA, *Acta Chem. Scand.*, 10 (1956) 1666.
10 S. KAMIYAMA AND K. SCHMID, *Biochim. Biophys. Acta*, 58 (1962) 80.
11 E. H. EYLAR AND R. W. JEANLOZ, *J. Biol. Chem.*, 237 (1962) 622.
12 N. HEIMBURGER, K. HEIDE, H. HAUPT AND H. E. SCHULTZE, *Clin. Chim. Acta*, 10 (1964) 293.
13 P. WEBER, I. BORNSTEIN AND R. J. WINZLER, *Anal. Biochem.*, 14 (1966) 100.
14 G. A. BRUNO AND J. E. CHRISTIAN, *Anal. Chem.*, 33 (1961) 650.
15 E. T. BUSH, *Anal. Chem.*, 35 (1963) 1024.