Carbohydrate Research, 103 (1982) 181–185
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Simple procedures for the addition of a ^3H label to cellulose oligo- and polysaccharides and for esterification of the products with [^{14}C]nicotinic acid

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(Received July 6, 1981; accepted for publication, September 15th, 1981)

The metabolism and excretion of nicotinic acid have been extensively investigated in rats and humans[1–3], but the nicotinic acid in cereals, which is naturally esterified to a (1→4)-β-D-glucan, is nutritionally unavailable to these animals[4–7] for reasons that are obscure. Further, if the macromolecules containing the bound nicotinic acid are administered orally[8] or intraperitoneally[9] to nicotinic acid-deficient rats, the normal urinary products of nicotinic acid metabolism (N^1-methylnicotinamide and 1-methyl-2-pyridone-5-carboxamide) are not present. Instead, the metabolite trigonelline (N^1-methylnicotinic acid betaine) is detected in the urine[8,9]. It has been postulated[3] that, in such animals, an increased permeability of the intestinal mucosa allows the uptake of the macromolecular complex and that the nicotinoyl residues are then enzymically N^1-methylated, so that trigonelline is produced on hydrolysis. To test this theory, radiolabelled, non-digestible polysaccharides esterified with radiolabelled nicotinic acid were required, so that the metabolic fate of each component could be followed.

Techniques for obtaining alditols from polysaccharide hydrolysates by reduction with NaB^3H_4 have been described[10], but very few labelled polysaccharides are available from commercial sources and there is no report of the labelling of cellulose with ^3H. Nicotinic esters of ethylene glycol, inositol, and glycerol have been described[11], and also those[12] of D-glucose and other monosaccharides. We now report on the use of borotritide to label *O*-methylcellulose and oligo- and polysaccharides derived from cellulose, and on the esterification of the products with [^{14}C]nicotinic acid.

The procedure is simple and rapid, and products were stable for several months at −20° in the freeze-dried form. The oligosaccharides obtained by partial hydrolysis of cellulose were reduced with borotritide and fractionated on Bio-Gel P-2. The

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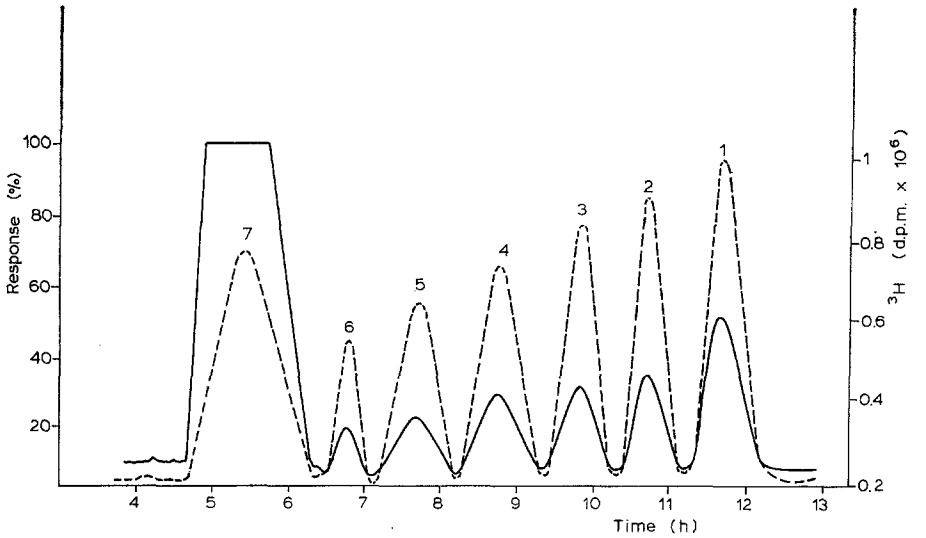

Fig. 1. Chromatogram of NaB^3H_4-reduced cellulose oligosaccharides on a column (0.9 × 150 cm) of Bio-Gel P-2, eluted at 7 ml/h with deaerated, deionised water: ——, refractive index; – – – –, ^3H content.

TABLE I

EXTENT OF ^3H-LABELLING OF REDUCED CELLULOSE OLIGO- AND POLY-SACCHARIDES

Parent carbohydrate	*^3H (μCi/mg)*
Cellobiose	99.10
Cellulose oligosaccharides[a]	
Peak 3	64.12
Peak 4	46.30
Peak 5	40.65
Peak 6	31.07
Peak 7	17.82
O-Methylcellulose	0.41

[a]Peaks 3–7 in Fig. 1.

elution profile (Fig. 1) exhibited 7 peaks, corresponding to d.p. 1–6 plus the material in the void volume. The elution profile for ^3H coincided with that obtained by using the refractive index. The extent of labelling is shown in Table I; as the molecular weight increases, the specific activity decreases, consistent with labelling of the original reducing end-groups only.

When these ^3H-labelled saccharides were administered intraperitoneally to animals, the ^3H subsequently detected in the urine was still attached to carbohydrate of molecular weight similar to that administered. The ^3H-labelled oligosaccharides

TABLE II

THE DEGREE OF ESTERIFICATION OF NaB^3H_4-REDUCED CELLULOSE OLIGO- AND POLY-SACCHARIDES WITH [^{14}C]NICOTINIC ACID

Parent carbohydrate	*D.e.*[a]	$^{14}C/^3H$
Cellobiose	1.50	1.3
Cellulose oligosaccharides[b]		
Peak 3	1.22	2.8
Peak 4	1.00	4.0
Peak 5	0.95	6.1
Peak 6	0.65	9.1
Peak 7	0.24	43.5
O-Methylcellulose	0.15	85.6

[a]Degree of esterification. [b]Peaks 3–7 in Fig. 1.

were also administered orally to rats. Chromatography of the resulting urine on Bio-Gel P-2 showed that the degradation products of the oligosaccharides in the first 5 h accounted for only 7% of the ^{3}H-labelled carbohydrates excreted during this period[9]. Maximum urinary excretion of the ^{3}H-labelled oligosaccharides was also detected during this period. The results given above and elsewhere[9] suggest that the ^{3}H-labelled oligosaccharides are suitable for determination of the permeability of the small intestine.

Various procedures for the esterification of monosaccharides with nicotinic acid have been reported[13]. The method found suitable for our requirements involved conversion[11] of [^{14}C]nicotinic acid into nicotinoyl chloride hydrochloride, and its reaction[12] with the ^{3}H-labelled carbohydrate in methyl sulphoxide–pyridine. The products were purified by chromatography on Bio-Gel P-2; the ^{3}H and ^{14}C were eluted in the same position. That esterification had occurred was established with the product from [^{3}H]-*O*-methylcellulose. Saponification followed by chromatography on Bio-Gel P-2 led to elution of ^{3}H in the void volume of the column, and ^{14}C in the position corresponding to nicotinic acid.

The degree of esterification by [^{14}C]nicotinic acid can be assessed by the $^{14}C/^3H$ ratio, which is highest for *O*-methylcellulose and lowest for cellobiose (Table II), indicating that the extent of esterification is related to the size of the saccharide. A degree of esterification of 5 for hexoses and 4 for pentoses was obtained by Forlano *et al.*[12].

EXPERIMENTAL

[^{14}*C*]*Nicotinoyl chloride hydrochloride.* — Nicotinic acid (500 mg, containing 250 μCi of [*carboxyl*-^{14}C]nicotinic acid) was stirred with redistilled thionyl chloride (20 ml) for 5 min and then heated in a sealed tube at 80° for 2 h. The excess of

thionyl chloride was evaporated from the cooled mixture by using filtered air. Dry benzene (20 ml) was added and the mixture concentrated to dryness at 50° under a stream of filtered air.

Chromatography on Bio-Gel P-2. — A column (0.9 × 150 cm) at 60° was eluted with deaerated, deionised water at 7 ml/h. The eluate was monitored with a Waters Associates refractometer 401 monitor, as described by John *et al.*[13]. The sample load was 1 ml. Fractions (1 ml) were collected, and a portion of each was assayed for radioactivity in a Tri-Carb Counter 2650 using a toluene-based scintillation fluid [PPO (7.5 g), POPOP (0.45 g), toluene (1500 ml), and Triton X-100 (750 ml)]. Fractions that corresponded to the peak of the material required and contained radioactivity were combined and freeze-dried.

Borotritide reductions. — The cellulose oligo- or poly-saccharide (25–500 mg) was added to NH_4OH (4 ml) followed by 25–50 mCi of NaB^3H_4 (500–600 mCi/mmol) at room temperature. After 2–6 h, the unreacted borotritide was decomposed with glacial acetic acid, the mixture was concentrated, and methanol (5 × 1 ml) was distilled from the residue at 30° in a stream of filtered air. The products were purified by chromatography on Bio-Gel P-2 as described above.

Cellulose (1 g) was treated[9] with 70% sulphuric acid (100 ml) for 24 h at 5°. The partial hydrolysate, at 5°, was diluted to 10 ml with water, neutralised with barium carbonate, centrifuged, and freeze-dried, to yield a mixture of oligosaccharides that was reduced, as described above, for 4 h. A solution of the product in water (1 ml) was subjected to chromatography on Bio-Gel P-2. The elution profile is shown in Fig. 1.

The *O*-methylcellulose reduced (for 6 h) was a Sigma product, and had a mol. wt. of $\sim 10^5$ and a d.s. of 1.6.

[^{14}C]Nicotinoylation of ^3H-labelled carbohydrates. — The following general method was used. [^{14}C]Nicotinoyl chloride hydrochloride (50 μCi) in methyl sulphoxide (1 ml) was added to methyl sulphoxide (3 ml) containing 10–20 mg of the ^{3}H-labelled carbohydrate followed by pyridine (5 ml). The mixture was shaken at 0° for 24 h and then at 16° for 24 h. The solvent was evaporated by using filtered air at 60°. Water (3 × 3 ml) was evaporated at 50° from the residue, a solution of which in water (2 ml) was then chromatographed on Bio-Gel P-2. Fractions (1 ml) were analysed for ^{3}H and ^{14}C content as described above. The fractions associated with the peak containing the radioactivity were combined and freeze-dried.

[^{3}H]-*O*-Methylcellulose gave one peak, eluted in the void volume. A portion (10 mg) of the product was treated with 2M NaOH (5 ml) at 100° for 15 min. The hydrolysate was neutralised with acetic acid, concentrated to 1 ml by using filtered air at 40°, and chromatographed on Bio-Gel P-2. The elution profile for ^{3}H showed only one peak, which was eluted in the void volume. The carbohydrate content was assayed by the anthrone procedure[14]. The ^{14}C-radioactivity was eluted in one peak corresponding to the position of nicotinic acid, and the identity was confirmed by h.p.l.c. [Waters Associates Model 6000A pumping system, a Model U6k loop injector, and a Model 440 u.v. detector (254 nm)]. A Partisil 10 SCX column (50

cm × 4.5 mm i.d.) was used together with a pre-column of reverse-phase C_{18} material (Vydac-201, 33–44 μm, Waters Associates). Elution at 215 ml/min was effected with 15mM potassium citrate (pH adjusted to 2 with sulphuric acid) containing 5% of methanol. The material analysed was extracted with methanol (500 μl), the extract was centrifuged for 15 min at 300*g*, and a 5-μl portion was used for h.p.l.c.

The product of esterification of [^{3}H]cellobiitol (30 mg) gave one peak of ^{3}H- and ^{14}C-radioactivity on Bio-Gel P-2.

Esterification of each of the ^{3}H-labelled oligosaccharides (isolated by chromatography on Bio-Gel P-2) gave one broad peak in chromatography on Bio-Gel P-2, the elution position of which varied according to the chain length of the oligosaccharide.

REFERENCES

1 E. KODICEK, *Biochem. J.*, 34 (1940) 712–723.
2 E. KODICEK, *Biochem. J.*, 34 (1940) 723–735.
3 K. K. REDDI AND E. KODICEK, *Biochem. J.*, 53 (1953) 286–294.
4 J. B. MASON, N. GIBSON, AND E. KODICEK, *Br. J. Nutr.*, 30 (1973) 297–311.
5 J. B. MASON AND E. KODICEK, *J. Am. Assoc. Cereal Chem.*, 50 (1973) 637–646.
6 D. K. CHAUDHURI AND E. KODICEK, *Biochem. J.*, 47 (1950) xxxiv.
7 J. B. MASON AND E. KODICEK, *Biochem. J.*, 120 (1970) 509–514.
8 J. B. MASON AND E. KODICEK, *Biochem. J.*, 120 (1970) 515–523.
9 J. S. SANDHU AND D. R. FRASER, *Biochem. J.*, 200 (1981) 495–500.
10 P. PREHM AND A. SCHEID, *J. Chromatogr.*, 166 (1978) 461–467.
11 C. O. BADGETT AND C. F. WOODWARD, *J. Am. Chem. Soc.*, 69 (1947) 2907–2912.
12 E. A. FORLANO, J. O. DEFERRARI, AND R. A. CADENAS, *Carbohydr. Res.*, 21 (1972) 484–486.
13 M. JOHN, G. TRENEL, AND H. DELLWEG, *J. Chromatogr.*, 42 (1969) 476–484.
14 H. G. ALBAUM AND W. W. UMBREIT, *J. Biol. Chem.*, 167 (1947) 369–376.