Carbohydrate Research, 96 (1981) 1–6
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

SYNTHESIS OF SEDOHEPTULOSE FROM NON-DIALYZABLE, ENDOGENOUS SUBSTRATES IN MAMMALIAN TISSUE EXTRACTS

PAUL P. HIPPS*, KAREN ACKERMANN, WILLIAM H. HOLLAND, AND WILLIAM R. SHERMAN
Departments of Psychiatry and Biological Chemistry, Washington University School of Medicine, St. Louis, MO 63110 (U.S.A.)

(Received March 3rd, 1980; accepted for publication in revised form, November 5th, 1980)

ABSTRACT

Sedoheptulose was observed to be formed at the rate of 12–120 nmol/g tissue/h in dialyzed rat and bovine tissue homogenates. The compound was identified by its gas-chromatographic retention time and by its mass spectrum. A standard of [^{14}C]-sedoheptulose was prepared from D-[^{14}C]fructose 6-phosphate and D-erythrose 4-phosphate for use in radioactive gas-chromatographic analysis in studies of possible precursors of the sedoheptulose. The reaction was stimulated by NAD^+. Evidence is presented to show that non-dialyzable sedoheptulose 7-phosphate may be the endogenous precursor. These results show that care must be exercised when using crude enzyme preparations and extremely sensitive analytical methods so that chromatographically unresolved products formed from non-dialyzable, endogenous substrates of low molecular weight do not introduce significant error in the analysis of the expected product.

INTRODUCTION

During a search for a *myo*-inositol epimerase in mammalian tissue extracts, we observed the enzyme-catalyzed synthesis of a substance having gas-chromatographic (g.l.c.) properties as the trimethylsilyl (Me_3Si) derivative similar to those of *scyllo*-inositol, the expected product. The mass spectrum of the unknown product was unusual in having a highly abundant ion at *m/z* 539, with only traces of ions at higher mass. The compound was ultimately identified as Me_3Si-sedoheptulose The identification and source of this sugar, which is not known to be a metabolic constituent of mammalian tissue, are discussed in this paper.

RESULTS AND DISCUSSION

The formation of an unknown incubation-product by tissue preparations. — When *myo*-inositol and NAD^+ were incubated with dialyzed, 15,000*g* supernatant solutions from rat tissue homogenates, we found a product having a retention time very close

*To whom inquiries should be directed.

0008-6215/81/0000–0000/$ 02.50,

to that of *scyllo*-inositol, when analyzed as the Me_3Si derivative. The retention time of Me_3Si-*scyllo*-inositol relative to Me_3Si-*myo*-inositol on columns of 3% of SE-30 at 160° was 0.80 and that of the unknown, 0.86. None of the substance was formed in the controls that did not contain cofactor (Table I). Similar results were obtained with incubations of dialyzed ammonium sulfate fractions, using *myo*-inositol as the substrate. Boiled controls were also inactive. The amount of the substance formed depended on the length of time of the incubation, suggesting initially that this was a product of *myo*-inositol metabolism

TABLE I

NAD$^+$-DEPENDENT SYNTHESIS OF SEDOHEPTULOSE IN RAT TISSUE[a]

Tissue	*Rate of sedoheptulose synthesis [nmol/h/g (wet wt.)][b]*
Brain	12 5
Testis	120
Kidney	68 8
Control[c]	<3

[a]Dialyzed 15,000*g* supernatant solution from homogenized tissue. [b]*myo*-Inositol (5 5mM) and 1.2mM NAD$^+$ were incubated with the tissue homogenates in 50mM Tris buffer, pH 8 5 at 25° in a total volume of 250 μL. [c]Controls were tissue homogenates incubated with *myo*-inositol without added NAD$^+$.

Identification of the incubation product as sedoheptulose (D-altro-*heptulose*). — Mass spectrometry showed that the unknown substance was not the Me_3Si derivative of any inositol. All of the Me_3Si-inositols give spectra consisting of the same ions, the abundances of which are characteristic of the inositol isomer[1]. None of these inositol-characteristic ions were present in the spectrum of the incubation product. The most striking aspect of the mass spectrum of the substance was the abundant ion *m/z* 539. When derivatized with *N,O*-bis(trimethylsilyl)acetamide-d_9, the *m/z* 539 ion shifted 45 a.m.u. to *m/z* 584, indicating the presence of 5 intact trimethylsilyl groups. This was supported by a high-resolution spectrum, which showed that *m/z* 539 had a composition of $C_{21}H_{51}O_6Si_5$. The absence of an ion 15 a.m.u. lower than *m/z* 539 is consistent with *m/z* 539 already having lost a neutral radical. Traces of an ion at *m/z* 642 suggested that this lost fragment could have been $\cdot CH_2OSiMe_3$. A series of losses of Me_3SiOH from *m/z* 539 (*m/z* 449, 359, and 269) were confirmed by Me_3Si-d_9 labeling and by the high-resolution spectrum. It became apparent that the spectrum was similar to those of Me_3Si-sorbose and Me_3Si-fructose[2], in which a major-abundance ion is formed by the loss of $\cdot CH_2OSiMe_3$ (M — 103) to form *m/z* 437 from the ketohexose. Partial spectra of Me_3Si-sedoheptulose and of the Me_3Si-unknown are given in Table II. After identification of the unknown as sedoheptulose, the spectrum of Me_3Si-sedoheptulose was reported by Okuda *et al.*[3].

TABLE II

THE PARTIAL 70-eV ELECTRON-IMPACT MASS SPECTRA OF AUTHENTIC Me_3Si-SEDOHEPTULOSE AND OF THE Me_3Si-UNKNOWN FROM INCUBATIONS OF TISSUE HOMOGENATES

	m/z											
	269	291	293	305	319	331	333	359	393	447	449	539
Standard[a]	4	10	14	8	39	10	9	49	3	7	12	100
Unknown[b]	4	7	7	9	20	11	8	61	6	5	12	100

[a]Me_3Si-Sedoheptulose prepared from sedoheptulose 7-phosphate and *E. coli* alkaline phosphatase. [b]Me_3Si-Sedoheptulose observed as formed in homogenates of rat testis.

The gas-chromatographic retention time of the unknown was found to be the same as that of authentic Me_3Si-sedoheptulose on two columns, confirming the structure of the 2-ketoheptose (retention time of the Me_3Si derivative of the unknown and sedoheptulose, relative to *myo*-inositol: on 1% of SE-30 at 160°, 0.86, 0.86; and on 3% of OV-17 at 160°, 0.84, 0.84). As described by Okuda *et al.*[3], a preceding shoulder on the main peak for sedoheptulose was observed, which they described as the pyranose form. In our study, the furanose form or second g.l.c. peak was preponderant.

The source of sedoheptulose. — *Post mortem* changes in tissue levels of various compounds have been known for some time. For example, changes in the tissue levels of di- and tri-phosphoinositides have been demonstrated by Dawson and Eichberg[4]. The degradation of liver glycogen and proteins results in the formation of 3-deoxy-D-*erythro*-hexosulose[5,6]. Degradation of one compound obviously results in the synthesis of another. However, the sedoheptulose observed in the tissue extracts described here arises not merely from the *post mortem* degradation of a tissue constituent, but is the result of enzymic synthesis from non-dialyzable, endogenous substrates. We have observed both phosphorylated and non-phosphorylated carbo-

TABLE III

EFFECT OF DIALYSIS ON CARBOHYDRATE CONCENTRATIONS FROM RAT TESTIS[a]

Compound	*Concentration [mmol/kg (wet wt.)] (× 10³)*		*% Remaining*
	Non-dialyzed	*Dialyzed*	
myo-Inositol	1330	13.4	1 0
scyllo-Inositol	10.2	0.162	1.6
Sedoheptulose	119	4 32	3.6
α,β-D-Glucose	149	6 26	4 2

[a]Rat testes (35 g) were homogenized in 50mM Tris buffer (35 mL), pH 7.5. After centrifugation, 43 mL of supernatant solution was dialyzed against 6 L of buffer that was changed 4 times in 24 h The volume recovered was 46 mL. Analysis was by selected-ion monitoring. Protein concentration before dialysis was 62 7 and after dialysis 42.8 mg/mL.

hydrates to be bound to cell-free tissue preparations in a form that is not dialyzable, but is susceptible to derivatization for gas chromatography. The data in Table III show that exhaustive dialysis leaves significant quantities of carbohydrates. The carbohydrates adsorbed to proteins are released by denaturation of the proteins following enzymic analysis and/or the addition of derivatizing reagents permitting their analysis. The sedoheptulose observed in these enzyme preparations may have been formed by the hydrolysis of sedoheptulose 7-phosphate. This sedoheptulose 7-phosphate might have been formed *via* the transketolase reactions using endogenous substrates, or it could have been already present in the dialyzed tissue-preparations.

After identification of the product as sedoheptulose, we prepared [^{14}C]sedoheptulose as a radioactive, gas-chromatographic standard. *myo*-[U-^{14}C]Inositol or *myo*[G-^{3}H]inosose-2 was incubated with a 30–70% ammonium sulfate fraction from bovine brain in order to determine if there was a precursor–product relationship between the cyclitols and the sedoheptulose. When the Me_3Si derivative of the incubation product and the Me_3Si-D-[^{14}C]sedoheptulose standard were analyzed by radioactive g.l.c., no tritiated or ^{14}C-labeled sedoheptulose was observed. These results showed that the synthesis of sedoheptulose was not the product of inositol–inosose metabolism.

Incubations without an added carbon source were partially dependent on added NAD^+, with a 4-fold stimulation observed in the presence of added cofactor. As shown in Table I, no sedoheptulose (<3 nmol/g) was observed in the tissues in the absence of added cofactors. Boiled-enzyme controls also showed no production of the ketoheptose. A bacterial source of the enzyme(s) was excluded by the presence of 0.02% sodium azide in the incubations. It was evident that sedoheptulose was formed in the rat tissue extracts at rates from 12–120 nmol/h/g from some endogenous source. The reaction was linear for ~2 h (Fig. 1).

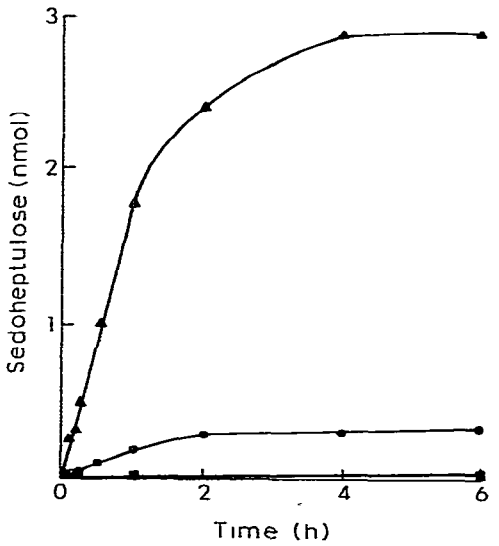

Fig 1. Effect of time on formation of sedoheptulose. Dialyzed rat-testis homogenate was incubated without NAD^+ (■) and with 1 2mM NAD^+ (●). Sedoheptulose 7-phosphate (5mM, pH 7.5) was incubated with the enzyme preparation in the presence of 1.2mM NAD^+ (▲).

An experiment in which sedoheptulose 7-phosphate was added to boiled and active enzyme preparations showed, by the formation of sedoheptulose in the active enzyme preparations, that there was sufficient phosphate monoesterase activity present to hydrolyze the substrate (Fig. 1). It is likely that this is the non-specific, non-Mg^{2+}-dependent phosphatase that is commonly observed in supernatant preparations from tissue: it provides a logical link to sedoheptulose 7-phosphate for the formation of the free sugar.

These observations again demonstrate the importance of care in excluding reactions from non-dialyzable, endogenous substrates of low molecular weight when using extremely sensitive analytical methods with relatively crude enzyme-preparations. The data described in this report show that the product from the endogenous substrate was chromatographically resolved from the expected product and readily detected. However, it is possible that endogenous substrates of low molecular weight may yield products that are not chromatographically resolved from the expected products, thus giving erroneous results.

EXPERIMENTAL

Enzyme preparation. — Rats were killed by decapitation. Tissues were removed immediately and kept on ice until used. Bovine brains were obtained fresh from the slaughterhouse and kept on ice until used. Tissues were homogenized in either water or 50mM Tris buffer, pH 7.5 containing 0.02% of sodium azide. After centrifugation at 15,000*g*, the supernatant solution was dialyzed overnight against the same buffer. The dialyzed, supernatant solutions were either used directly for assay or were made 30% saturated with solid ammonium sulfate. In the latter case, after stirring for 1 h, the precipitate was removed by centrifugation at 15,000*g* and discarded. The supernatant solution was then made 70% saturated with ammonium sulfate and stirred for 1 h. The precipitate was removed by centrifugation at 15,000*g*, dissolved in a minimum volume of buffer, dialyzed and used for analysis.

Enzyme analysis. — Assays were performed at 25° in 50mM Tris buffer, pH 8.5 containing 0.02% of sodium azide in a total volume of 250 μL. *myo*-Inositol or *myo*-inosose-2, when used as carbon source, were 5.5mM. NAD^+, when used, was either 0.62 or 1.2mM. After incubation, the reaction was stopped by placing the reaction vessel for 10 min in a boiling-water bath. Protein was precipitated by the addition of 25 μL of M zinc sulfate. After low-speed centrifugation, the supernatant solution was removed, lyophilized, and the Me_3Si derivative prepared by using 100 μL of 1:1 (v/v) pyridine–*N,O*-bis(trimethylsilyl)trifluoroacetamide containing 10% of chlorotrimethylsilane.

Analysis was by gas chromatography or by selected-ion monitoring with a gas chromatograph–mass spectrometer fitted with a 5-ft. glass column containing 1% of SE-30, or 3% of OV-17 on Gas Chrom Q. Mass spectra were obtained with an LKB 9000 g.l.c.–m.s. instrument. The ionization potential was 70 eV and the source and separator temperatures were 250°. Helium flow was 30 mL/min. Radioactive

g.l.c. was performed with a Nuclear Chicago Radioactivity Monitor, interfaced with a gas chromatograph.

Substrates. — Sedoheptulose was prepared by incubating sedoheptulose 7-phosphate (Sigma) with *Escherichia coli* alkaline phosphatase (Sigma) in the presence of 2mM magnesium chloride at pH 9.1. The Me_3Si derivative was prepared as already described.

[^{14}C]Sedoheptulose was prepared[7] by incubating D-[U-^{14}C]fructose 6-phosphate (50 μCi, 0.17 μmol, Amersham) and D-erythrose 4-phosphate (0.64 μmol, Sigma) in triethanolamine · HCl buffer (50mM), pH 7.6 containing 64mM NADH and 0.1 unit α-glycerophosphate dehydrogenase-triosephosphate isomerase with transaldolase (0.2 unit, Sigma), followed by incubation with *E. coli* phosphatase (0.1 unit, Sigma). The recovery was 2 μCi of [^{14}C]sedoheptulose.

myo-[U-^{14}C]Inositol (5 μCi) was purchased from New England Nuclear. *myo*-[G-^{3}H]Inososе-2 was prepared[7] by base-catalyzed enolization in 0.01 Ci of $^{3}H_2O$ (5 Ci/g, New England Nuclear).

ACKNOWLEDGMENT

This work was supported by Grant NS-05159 from the National Institutes of Health.

REFERENCES

1 W. R. SHERMAN, N. C. EILERS, AND S. L. GOODWIN, *Org. Mass Spectrom.*, 3 (1970) 829–840.
2 S. KARADY AND S. H. PINES, *Tetrahedron*, 26 (1970) 4527–4536.
3 T. OKUDA, S. SAITO, AND M. HAYASHI, *Carbohydr. Res.*, 68 (1979) 1–13.
4 R. M. C. DAWSON AND J. EICHBERG, *Biochem. J.*, 96 (1965) 634–643.
5 H. OTSUKA AND L. EGYÜD, *Biochim. Biophys. Acta*, 165 (1968) 172–173.
6 N. BAKER AND L. EGYÜD, *Biochim. Biophys. Acta*, 165 (1968) 293–296.
7 O. TCHOLA AND B. L. HORECKER, *Methods Enzymol.*, 4 (1966) 499–505.