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PREPARATIVE-SCALE SEPARATION BY ANION-EXCHANGE CHROMATOGRAPHY OF SIX PER-C-DEUTERATED INOSITOL EPIMERS PRODUCED DURING C-^{1}H–C-^{2}H EXCHANGE REACTIONS WITH RANEY NICKEL IN DEUTERIUM OXIDE

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ABSTRACT

After a ^{1}H–^{2}H exchange reaction of *myo*-inositol with deuterated Raney nickel in deuterium oxide, six epimers (*scyllo-*, *chiro-*, *neo-*, *allo-*, *muco-*, and *epi*-inositol), in addition to *myo*-inositol, were identified as their per-*O*-(trimethylsilyl) derivatives by gas–liquid chromatography on a fused-silica capillary column of SE-30, and also by gas–liquid chromatography–mass spectrometry. Preparative-scale separation of six of these deuterium-labeled inositols (98–99% deuterated) was achieved by anion-exchange chromatography, after partial purification of the epimerization products by recrystallization. That is, anion-exchange chromatography using Dowex 1 resin completely resolved *myo-*, *scyllo-*, *allo-* and *muco*-inositol as their borate complexes. Although *neo*-inositol was only partially separated from *chiro*-inositol, these two inositols were completely resolved from the four other epimers.

INTRODUCTION

Hydrogen atoms bonded to carbon atoms having a free hydroxyl group, in various carbohydrates, undergo ready ^{1}H–^{2}H exchange in deuterium oxide in the presence of Raney nickel catalyst[1]. Although the replacement of ^{1}H by ^{2}H takes place primarily with retention of configuration, inversion is also frequently observed[2–5]. During the course of the ^{1}H–^{2}H exchange reaction of *myo*-inositol, there were detected *scyllo-*, *chiro-*, and *neo*-inositol (as isomerization products) by ^{13}C-n.m.r. analysis[6]. By gas chromatography and mass spectrometry, we have observed the presence of at least six inositol isomers produced from *myo*-inositol during the ^{1}H–^{2}H exchange reaction with deuterated Raney nickel.

Because of the complexity of the product mixture, repeated recrystallization results in low yield, as well as being time-consuming. No adequate paper chromatography system has been developed[7], and, although paper electrophoresis provides better separations[8], the method is unsuitable for use on a preparative scale. Column chromatography has an advantage in the preparative-scale purifica-

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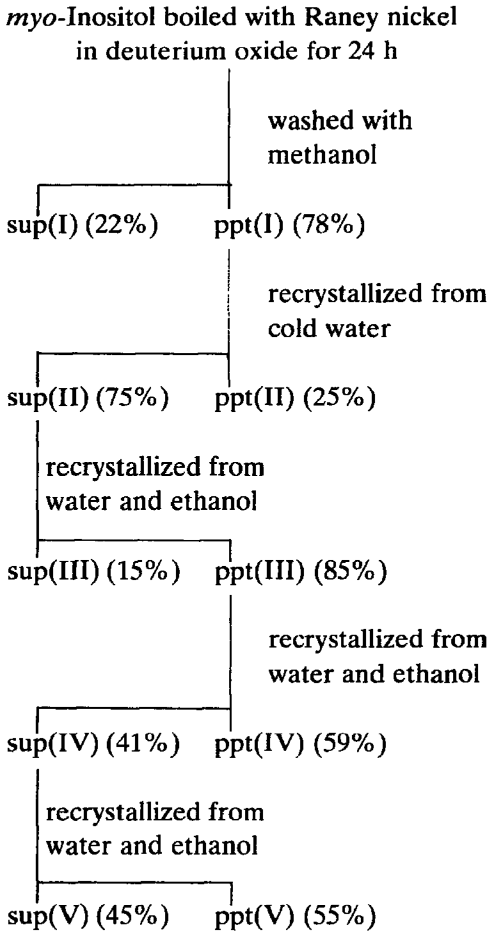
tion, but only mixtures of two inositol epimers have been resolved by this method[9-11]. In the present study, preparative-scale purification of the six inositol epimers has been achieved by elution from a Dowex 1 column with a gradient–stepwise-gradient sequence of boric acid solutions.

EXPERIMENTAL

Inositols. — *myo*-Inositol was purchased from Sigma Chemical Co. (St. Louis, MO). Other standard inositols were generous gifts of Dr. L. Anderson (University of Wisconsin-Madison).

Preparation of Raney nickel. — Raney nickel (obtained from Sigma Chemical Co., St. Louis, MO, for the time-course experiments and from Aldrich Chemical Co., Milwaukee, WI, for the deuterium-labeling experiments) was washed with water until the pH of the wash liquid was neutral. Deuterated Raney nickel was prepared by washing 5 times with deuterium oxide. The volume to be used was the packed volume after centrifugation at 600*g* for 5 min.



Scheme 1. Recrystallization protocol for deuterium-labeled inositols; sup = supernatant, ppt =

Deuterium-labeling of myo-*inositol.* — *myo*-Inositol (5 g) was three times dissolved in deuterium oxide (50 mL) and dried. It was then dissolved in deuterium oxide (150 mL) mixed with deuterated Raney nickel (25 mL), boiled under reflux (for 24 h in the first experiment, and 12 h in the second experiment), and the suspension cooled, filtered through Whatman No. 1 filter-paper supported on a medium-porosity sintered-glass frit, and the solid washed 3 times with hot deuterium oxide. The filtrate and washings were combined and evaporated to dryness. In the first experiment, the residue was washed 5 times with methanol and the remaining solid crystallized (see Scheme 1). In the second experiment, recrystallizations only from water and ethanol were performed.

The extent of deuterium exchange was measured by 400-MHz ^{1}H-n.m.r. spectroscopy of the inositols as hexaacetates, in C^2HCl_3, using a Bruker WH-400 spectrometer, and by g.l.c.–m.s. of the per-*O*-(trimethylsilyl)ated inositols, using *m/z* 217, 305, and 507 as characteristic ions.

Epimerization study. — *myo*-Inositol (1 g) was boiled under reflux with water (30 mL) and Raney nickel (5 mL) for 3, 5, 8, 24, and 48 h, the suspension filtered, and the solid washed 3 times with hot water. The filtrate and washings were combined and lyophilized.

*Per-*O*-(trimethylsilyl)ation of inositols.* — Each sample of lyophilized inositol (<1 mg) was treated with 0.1 mL of Trisil Z (Pierce Chemical Co., Rockford, IL) in a small vial (4 mL capacity), sealed with a Teflon disc secured with a screw cap, and heated overnight or longer at 60–65°. The thorough drying before reaction was found important, and both higher temperature and longer reaction-time, compared with conditions for neutral sugars[12], were required for full derivatization. The per(trimethylsilyl)ated *scyllo*-inositol was rather insoluble at room temperature, and thus samples were injected directly from the mixture resulting from the overnight treatment at 60–65°.

G.l.c. analysis. — The per(trimethylsilyl)ated inositols were separated, and detected, by using a Varian Model 1740 gas–liquid chromatograph with a Model 477 integrator fitted with a glass column (1 m × 2 mm) packed with SE-33 (3%, coated on Gas Chrom Q, 80–100 mesh), operated isothermally at 170°, with injector and detector temperature of 280°, and nitrogen as the carrier gas; or by using a Perkin–Elmer, Sigma 3B gas chromatograph equipped with a Spectra-Physics SP 4100 computing integrator fitted with a fused silica capillary column (15 m × 0.25 mm) of SE-30, operated at an initial temperature of 150° for 1 min, followed by a programmed temperature increase, at 6° per min, to 230°, with an injector and detector temperature at 300°, and helium as the carrier gas. Flame-ionization detectors were used with both chromatographs. Compositions presented are the relative ratios of peak areas printed out by the integrator.

G.l.c.–m.s. analysis. — The g.l.c.–m.s. analyses were carried out by using an automated Finnigan 1020 g.l.c.–mass spectrometer equipped with a fused-silica capillary column (30 m × 0.25 mm) of SE-54 and operated in the electron-ionization mode (70 eV) with an ion-source temperature of 95°. Samples were injected

TABLE I

INOSITOL COMPOSITIONS OF THE FRACTIONS OBTAINED FROM *myo*-INOSITOL SOLUTIONS BOILED WITH RANEY NICKEL IN DEUTERIUM OXIDE FOR 24 HOURS

Inositols	%							
	Sup(I)	*Ppt(I)*	*Ppt(II)*	*Sup(III)*	*Ppt(III)*	*Ppt(IV)*	*Sup(V)*	*Ppt(V)*
allo-[a]	7	2	0	10	0	0	1	0
neo- and/or *muco*-	1	5	3	0	3	3	0	2
chiro-	17	16	1	42	13	4	61	29
scyllo-	0	29	82	2	16	22	4	7
myo-	1	44	14	27	68	71	33	62
Unknown I	27	4	0	19	0	0	1	0
Other unknowns	48	0	0	0	0	0	0	0

[a] This peak contained at least one unknown component (unknown II in Fig. 2).

with a split ratio of 100:1. Other g.l.c. conditions were as described for the capillary column of SE-30.

Dowex 1 anion-exchange chromatography. — The lyophilized reaction-product (100 mg) from treatment of *myo*-inositol with Raney nickel in water for 5 h at 100° was dissolved in 0.01M boric acid (2 mL) and loaded under N_2 pressure onto a column (0.9 × 30 cm) of Dowex 1-X8 (borate) resin (200–400 mesh). The column was washed successively with water (100 mL), the first linear gradient of boric acid (50 mL of 0.05M boric acid + 50 mL of 0.1M boric acid), 200 mL of 0.1M boric acid, the second linear boric acid gradient (100 mL of 0.1M boric acid + 100 mL of 0.2M boric acid), and 200 mL of 0.3M boric acid, delivered from a peristaltic pump at 30 mL per h. Fractions (5 mL each, except for the 0.1M boric acid washing which was collected as 2.5-mL fractions) were collected and their inositol content determined according to Isaaks *et al.*[13], using *myo*-inositol as a standard.

A longer column (0.9 cm × 60 cm) was used for the separation of the larger quantities of deuterium-labeled inositols. Approximately 600 mg of a partially purified mixture (see Scheme 1 and Table I) was dissolved in 4 mL of 0.01M boric acid, and applied to the column under N_2 pressure. The column was successively washed, at 30 mL per h, with 100 mL of water, the first gradient solution (200 mL of water + 200 mL of 0.1M boric acid, or 250 mL of water + 250 mL of 0.1M boric acid), 200 mL of 0.1M boric acid, the second linear gradient (100 mL of 0.1M boric acid + 100 mL of 0.3M boric acid) and 200 mL of 0.3M boric acid. Fractions (5 mL each) were collected, and the inositol content determined[13], using nonlabeled *myo*-inositol as a standard.

The boric acid in each fraction was removed by repeated evaporation with methanol. The boric acid-free inositols were lyophilized, per(trimethylsilyl)ated, and identified by g.l.c. and m.s.[14].

The columns were used repeatedly after washing with water.

RESULTS

Deuterium-labeling of myo-*inositol in deuterium oxide with Raney nickel.* — Analysis of the crude product of deuterium exchange with *myo*-inositol by g.l.c. on a packed column of SE-33 showed the presence of many components, five of which had retention times corresponding to *allo-*, *neo-* + *muco-*, *chiro-*, *scyllo-*, and *myo*-inositol, respectively. Washing of the crude product with methanol removed almost all non-inositol compounds, leaving a white residue that contained the six inositols and a small proportion of an unknown substance (see Table I). Some *chiro-* and *allo*-inositol were removed by the washing. Only 44% of the white residue was *myo*-inositol. Recrystallization from cold water (see Scheme 1) removed mostly *scyllo*-inositol. Two further recrystallizations from water and ethanol purified *myo*-inositol by up to 71%, with a recovery of 80% from the starting, methanol-insoluble precipitate (see Table I).

In a second experiment, more *scyllo-* and *neo*-inositols were removed by a first recrystallization from water and ethanol. After four further recrystallizations, the purification of *myo*-inositol was increased to 88%, but with only 50% recovery from the original filtrate.

The extent of deuterium exchange of *myo*-inositol at 12 h was 98.6% (measured by n.m.r.) or 99% (measured by g.l.c.–m.s.).

Epimerization of myo-*inositol during treatment with Raney nickel in water.* — Problems associated with epimerization during the exchange reaction were investigated by conducting the reaction in water. Reaction times progressively increased from 3 to 48 h led to the production of increasingly dark brown filtrates and a lowering of product dry-weights. One gram of *myo*-inositol yielded 780, 770, 670, 430, and 180 mg of product mixture after 3, 5, 8, 24, and 48 h reaction time, respectively. The peak heights of at least six unknown compounds, whose per-trimethylsilyl derivatives all had shorter retention-times on SE-33 than those of the inositols,

TABLE II

COMPOSITIONS OF INOSITOL EPIMERS PRESENT IN THE *myo*-INOSITOL SOLUTIONS BOILED WITH RANEY NICKEL IN WATER[a]

Inositols	*Boiling time (hours)*		
	3	*5*	*8*
	Inositol (%)		
allo-[b]	6	8	11
neo- and/or *muco-*	9	8	6
chiro-	20	21	23
scyllo-	23	23	24
myo-	42	40	36

[a] Other unknown peaks are not included. [b] This peak contained at least one unknown component (unknown II in Fig. 2).

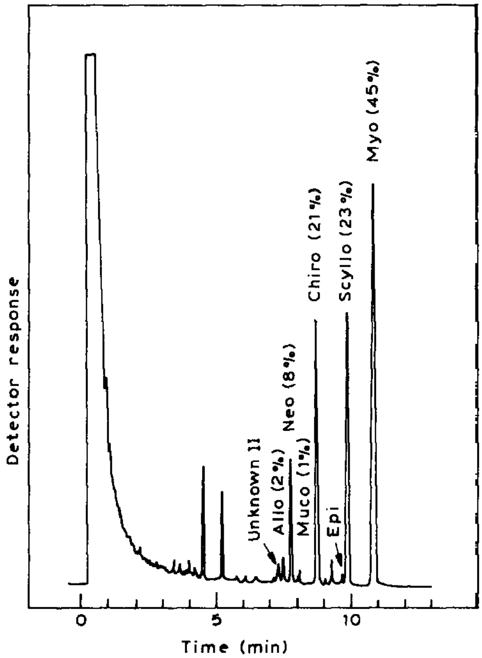


Fig. 1. Complete separation of inositol epimers produced from *myo*-inositol boiled in water with Raney nickel for 3 h, by g.l.c. on a capillary column of SE-30. Numbers in parentheses are relative peak areas.

increased relative to the inositols after 3, 5, and 8 h of reaction time with Raney nickel. After 24 h, no inositols were detected by g.l.c. The relative proportion of the inositol epimers changed only slightly between 3 and 8 h (see Table II).

Identification of inositol epimers by g.l.c. using a capillary column and by g.l.c.–m.s. — Seven per(trimethylsilyl)ated standard inositols (*myo*-, *scyllo*-, *epi*-, *chiro*-, *muco*-, *neo*-, and *allo*-inositol) were completely resolved by g.l.c. using a capillary column of SE-30 or SE-54. The identity of each inositol in the reaction products was confirmed by co-chromatography with a standard sample, and by g.l.c.–m.s.[14]. *epi*-Inositol as observed in a concentration estimated at <1% (see Fig. 1). The relative peak areas (Fig. 1) were similar to those obtained after chromatography using a column packed with SE-33 (see Table II).

At least one component (unknown II), which emerged immediately before *allo*-inositol (see Fig. 1), was not resolved from it on a packed column of SE-33. Its formation was confirmed by isolation of the unknown II, using a column of Dowex 1 (see Fig. 2) and by g.l.c. analyses of the per(trimethylsilyl)ated compound using the two different columns. The proportion of unknown II relative to other inositols increased with the time of the Raney-nickel reaction up to a limit of 8 h.

Separation of inositol epimers produced during treatment with Raney nickel in water by Dowex 1 anion-exchange chromatography. — There were two peaks eluted from the column with water (see Fig. 2), neither of which contained inositol as a major component. G.l.c. analysis of the first peak (combined third and fourth

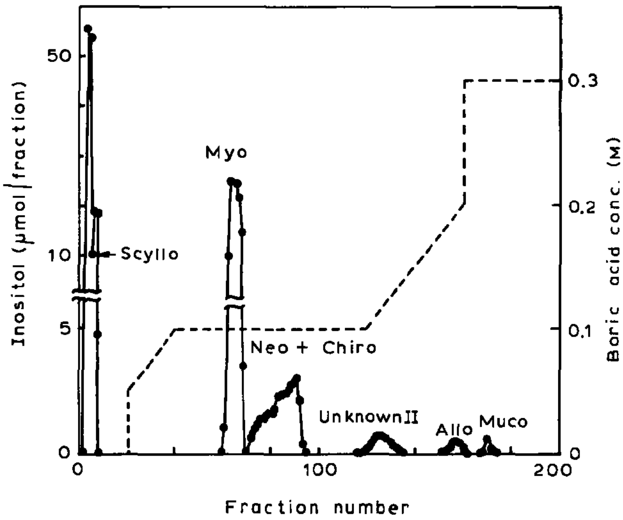


Fig. 2. Separation of inositol epimers produced from *myo*-inositol boiled in water with Raney nickel for 5 h, by Dowex 1 anion-exchange chromatography. Key: ●—●, inositol; -----, boric acid concentration. Inositols were assayed[13] with *myo*-inositol as a colorimetric standard.

fractions) revealed at least six components, all of which had shorter retention-times than *allo*-inositol. Analysis of per(trimethylsilyl)ated compounds in the second peak (combined sixth to eighth fractions) revealed the presence of five unknown components and a small proportion of *scyllo*-inositol. Most of the *scyllo*-inositol was in the fifth fraction, with small proportions of unknown components, and it could be purified to yield a single peak in g.l.c. on a capillary column of SE-30, by crystallization from water and ethanol. A longer column of Dowex 1 resolved three peaks in the water fraction, the second of which was *scyllo*-inositol (data not shown).

myo-Inositol was eluted in 0.1M boric acid after the first linear gradient. An unknown compound detected by g.l.c. analysis of the *myo*-inositol was also removed by crystallization from water and ethanol. The second inositol peak eluted at this stage showed an asymmetry that arose from incomplete resolution of *neo*- (the first component) and *chiro*-inositols.

The second boric acid gradient provided clear resolution of *allo*-inositol. A further unknown compound emerged between the combined *neo*- and *chiro*-inositol peak and *allo*-inositol. This compound was inseparable from *allo*-inositol by g.l.c. on a column packed with SE-33, but the two were clearly resolved by using a capillary column of SE-30. This compound corresponded to unknown II, immediately before the *allo*-inositol peak in Fig. 1.

muco-Inositol was eluted from the Dowex 1 column with 0.3M boric acid. The colored products remained on top of the column throughout the chromatography.

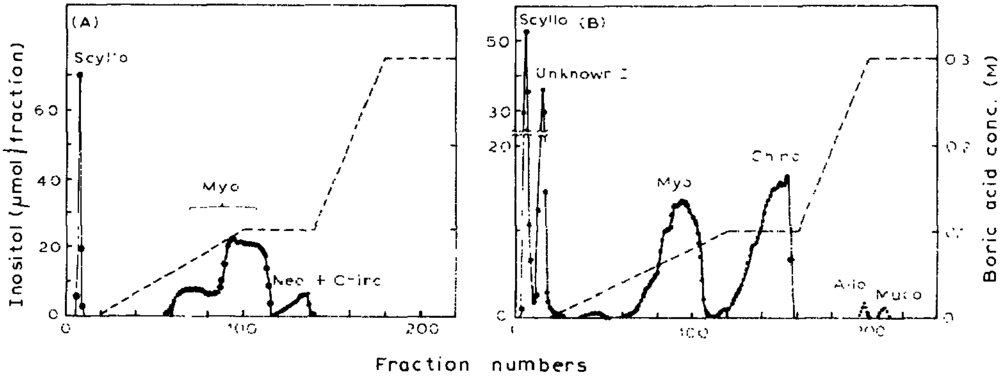


Fig. 3. Preparative-scale separation of deuterium-labeled inositols by Dowex 1 anion-exchange chromatography. Inositols were assayed[13] with non-labeled *myo*-inositol as a standard. A: ppt IV; B: sup III + sup V + ppt V. Key: ●—●, inositol; -----, boric acid concentration.

Large-scale separation of deuterium-labeled inositols using a column of Dowex 1. — When precipitate IV (see Scheme 1 and Table I) was chromatographed on a longer column of Dowex 1 (see Fig. 3A), a well-resolved peak of pure *scyllo*-inositol was obtained by elution with water. Three peaks emerged during chromatography with the first linear gradient and 0.1M boric acid. *myo*-Inositol was the only compound found in the first two peaks. Overloading of the column led to the double peaks of *myo*-inositol, whereas the third peak contained a mixture of *neo*- and *chiro*-inositols.

Chromatography of the combined sup III, V, and ppt V (see Scheme 1 and Table I) resulted in separation of two components in the water fraction (see Fig. 3B). The first peak was identified by g.l.c. as that of *scyllo*-inositol, whereas the per(trimethylsilyl)ated compound in the second peak had the same retention time as the unknown I noted in Table I. Pure *myo*-inositol emerged as a single peak during elution with the first gradient. A large peak of *chiro*-inositol emerged during elution with 0.1M boric acid. The absence of *neo*-inositol from this part of the chromatogram indicated that it had been removed during crystallizations prior to chromatography on Dowex 1. The last two components were *allo*- and *muco*-inositol, respectively.

Thus, combinations of crystallization and anion-exchange chromatography allowed for complete resolution of inositol epimers produced during the deuterium-labeling of *myo*-inositol in deuterium oxide with Raney nickel. More than 400 mg of deuterium-labeled *myo*-inositol was purified from precipitate IV. As much as 600 mg of deuterium-labeled *myo*-inositol was obtained from a mixture that also contained *scyllo*-inositol (76 mg) and *neo* + *chiro*-inositol (80 mg). Preliminary washing of the crude product with methanol or ethanol served to remove substantial contaminants from inositols, but purification of *allo*- and *muco*-inositols was achieved by direct application of the crude product to the anion-exchange column.

It is noteworthy that the color intensity after reaction of deuterium-labeled inositols by the method reported by Isaaks *et al.*[13] was less than one third of that formed with equivalent amounts of nonlabeled inositols. This may arise from a slower reaction due to an isotope effect. There was no difference between the peak areas after g.l.c. analysis of per(trimethylsilyl)ated deuterium-labeled and nonlabeled insotiols. This method is, therefore, recommended for accurate determination of deuterium-labeled inositols, unless deuterium-labeled inositols are used as standards in the colorimetric analysis.

DISCUSSION

The ^{1}H–^{2}H exchange reaction of carbohydrates with deuterated Raney nickel leads to isomerization, although it is generally slower than the exchange reaction[1–5]. We have identified *myo*-inositol and six epimers (*scyllo-, chiro-, neo-, muco-, allo-*, and *epi*-inositol) in the reaction mixture by g.l.c. of their per-(trimethylsilyl) derivatives in a capillary column of SE-30 and by g.l.c.–m.s. In their ^{13}C-n.m.r.-spectral analysis after reaction of *myo*-inositol with Raney nickel, Angyal and Odier[6] assigned the extra signals observed to *scyllo-, neo-, chiro-*, and *epi*-inositols. The presence of *scyllo*-inositol was detected after only 15 min of exchange. Furthermore, Angyal and Odier[6] observed deoxygenation of inositol during the exchange reaction at 60°, and we observed even more degradation at 100°. The complete absence of inositols from the reaction product with Raney nickel in water after 24 h contrasts with a recovery of ~20% of crude inositol mixture after 24 h of boiling with Raney nickel in deuterium oxide. The exchange reaction, epimerization, deoxygenation, and other reactions would be slower in deuterium oxide than in water, but the reactivity of Raney nickel is also affected by its age[6]. It is, therefore, necessary to determine the optimum exchange-reaction time for each Raney nickel preparation. Analysis of per(trimethylsilyl)ated products by g.l.c.–m.s. using a fused silica capillary column of SE-30 or SE-54 is a convenient and sensitive method by which to observe the epimer formation, and also to estimate the extent of the exchange reaction.

Thus, the epimerization and degradation reactions constitute serious hindrances to the preparation of deuterium-labeled inositols, using Raney nickel as a catalyst. The purification of inositol epimers may be achieved by repeated recrystallization, but it is very time-consuming and leads to major losses of products that, in the case of deuterium exchange, are very expensive. Cellulose-powder chromatography could be used for the preparative-scale separation, but the process is still cumbersome and the epimers remain poorly resolved. Other column-chromatographic systems resolved only mixtures of two inositol epimers[9,10].

Anion-exchange chromatography with Dowex 1 (borate) resin using gradients of boric acid concentration provides complete separation of *myo-, scyllo-, allo-*, and *muco*-inositol, and partial separation of *neo-* from *chiro*-inositol. The latter could be separated by a recrystallization that relies on the much greater crystallizability of *neo*-inositol from water. The method described allows purification of as much as 600 mg of deuterium-labeled *myo*-inositol. Larger columns may be used in order to separate more material in a single experiment. The Dowex 1 column can be used repeatedly after it has been washed with water. Boric acid is preferred over potassium or sodium borate solutions, which are commonly used for chromatography of neutral sugars[15–18], because it is easily removed by repeated evaporation with methanol, thereby eliminating the need for cation-exchange chromatography for the recovery of pure inositols.

Thus Dowex 1 anion-exchange chromatography provides a purification protocol on a preparative scale for all of the inositol epimers produced during the exchange reaction with Raney nickel or by other methods. Our results also give promise for the application of this method in the much quicker separation of inositol epimers by h.p.l.c.

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