Damadian, R. (1969), *Science 165*, 79.
Heidrich, H., Stahn, R., and Hannig, K. (1970), *J. Cell. Biol. 46*, 137.
Heldt, H. W., Jacobs, H., and Klingenberg, M. (1965), *Biochem. Biophys. Res. Commun. 18*, 174.
Henderson, P., and Lardy, H. (1970), *J. Biol. Chem. 245*, 1319.
Henderson, P., McGivan, J. D., and Chappell, J. B. (1969), *Biochem. J. 111*, 521.
Hopfer, U., Lehninger, A. L., and Lennarz, W. (1970), *J. Membrane Biol. 2*, 41.
Hoppel, C., and Cooper, C. (1968), *Biochem. J. 107*, 367.
Klingenberg, M., Grebe, K., and Heldt, H. (1970), *Biochem. Biophys. Res. Commun. 39*, 344.
Klingenberg, M., and Pfaff, E. (1966), *in* The Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 180.
Lamprecht, W., and Trautschold, I. (1958), *Hoppe Seyler's Z. Physiol. Chem. 311*, 245.
Lardy, H., and Wellman, H. (1953), *J. Biol. Chem. 201*, 357.
Lee, C. P., and Ernster, L. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 218.
Lev, A. (1964), *Nature (London) 201*, 1132.
Ling, G. (1960), A Physical Theory of the Living State, Waltham, Mass., Blaisdell Publishing Co.
Low, H., Vallin, I., and Alm, B. (1963), *in* Energy Linked Functions of Mitochondria, Chance, B., Ed., New York, N. Y., Academic Press, p 1.
McLaughlin, S., Szabo, G., Eisenmen, G., and Ciani, S. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1268.
Meisner, H. (1970), *Biochim. Biophys. Acta 205*, 27.
Meisner, H., and Klingenberg, M. (1968), *J. Biol. Chem. 243*, 3631.
Meisner, H., and Wenner, C. (1970), *Biochim. Biophys. Acta 223*, 46.
Mitchell, P. (1966), *Biol. Rev. 41*, 445.
Pfaff, E., Heldt, H., and Klingenberg, M. (1969), *Eur. J. Biochem. 10*, 484.
Pfaff, E., and Klingenberg, M. (1968), *Eur. J. Biochem. 6*, 66.
Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965), *Biochim. Biophys. Acta 104*, 312.
Pressman, B. (1958), *J. Biol. Chem. 232*, 967.
Vignais, P. V., and Vignais, P. M. (1970), *Biochem. Biophys. Res. Commun. 38*, 843.
Weidemann, M. J., Erdelt, H., and Klingenberg, M. (1970), *Eur. J. Biochem. 16*, 313.

neo-Inositol in Mammalian Tissues. Identification, Measurement, and Enzymatic Synthesis from Mannose 6-Phosphate*

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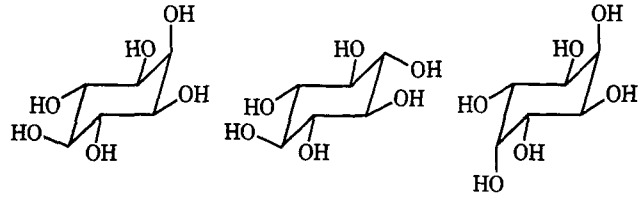
ABSTRACT: *neo*-Inositol has been isolated from calf brain and identified, as its trimethylsilyl ether and as its acetate ester, by gas chromatography and by combined gas chromatography–mass spectrometry. *neo*-Inositol has been found to be present in micromolar amounts in the brain, heart, kidney, testis, and spleen of the rat, but absent from liver. We have shown that the same enzyme preparation which converts D-glucose 6-phosphate into L-*myo*-inositol 1-phosphate is capable of transforming D-mannose 6-phosphate to a substance which, by mass spectrometry, as its trimethylsilyl ether ester is an inositol phosphate. By analogy with the cyclization of D-glucose 6. phosphate this product should be L-*neo*-inositol 1-phosphate. We have found that this enzyme preparation displays typical Michaelis–Menten kinetics with D-mannose 6-phosphate giving a K_m value of 5.6×10^{-3} as compared to a K_m of 6.2×10^{-3} with D-glucose 6-phosphate. A comparison of the V_{max} values for the two products using the same enzyme preparation shows *myo*-inositol to be formed at a rate about 200-fold faster than *neo*-inositol.

During a study in which we identified *myo*-inosose-2 by the gas chromatography of a lyophilized and trimethylsilylated extract of calf brain an unknown substance was found to have been inadvertantly isolated along with the inosose. A mass spectrum of the unknown was obtained at the same time that trimethylsilyl- (Me_3Si) *myo*-inosose-2 was identified by combined gas chromatography–mass spectrometry (Sherman *et al.*, 1968). The unknown substance has now been identified as *neo*-inositol.



myo-inositol *scyllo*-inositol *neo*-inositol

neo-Inositol hexaphosphate has been recognized as a soil constituent where it is found accompanied by *myo*-inositol hexaphosphate and *scyllo*-inositol hexaphosphate (Cosgrove, 1963; Cosgrove and Tate, 1963).

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In this paper we present evidence that *neo*-inositol often accompanies *myo*- and *scyllo*-inositols in mammalian tissues in the same relative proportions (*myo*- > *scyllo*- >> *neo*-inositol) as they occur in soil. We also present evidence that *neo*-inositol is synthesized from D-mannose-6-P by the same enzyme preparation, containing D-glucose-6-P–L-*myo*-inositol-1-P cyclase (hereafter simply called cyclase), which converts D-glucose-6-P into L-*myo*-inositol-1-P. We also show that the first product obtained from the enzymatic reaction is a *neo*-inositol phosphate.

Materials and Methods

Preparative Gas Chromatography to Isolate neo-Inositol from Calf Brain (Sherman *et al.*, 1968). In this study water extracts of calf brain were deproteinized, lyophilized, and then treated with pyridine–hexamethyldisilazane (HMDS)[1]–trimethylchlorosilane (Me_3ClSi) in a ratio of 17:2:1, v/v. Preparative gas chromatography of this mixture was first carried out on a 12 mm × 5 ft column of 20% CDMS coated on Gas Chrom Q (Applied Science Laboratories, State College, Pa.). Following this the pooled fractions collected from the CDMS column were further purified by chromatography on 3% SE-30 on the same support. Gas chromatography of the pooled SE-30 fractions on a column of 3% EGSS-X showed it to contain the Me_3Si ethers of α-glucose, *myo*-inosose-2, some *myo*-inositol, and the, then unknown, substance which we have found to be *neo*-inositol.

Me_3Si–Inositols. allo-, *cis-*, *epi,-* and *neo*-inositols were the gift of S. J. Angyal, University of New South Wales. *muco*-Inositol was the gift of C. E. Ballou, University of California, Berkeley. *scyllo*-Inositol was the gift of L. Anderson, University of Wisconsin. *myo*- and (+)-*chiro*-inositol were commercial samples. Additional amounts of *neo*-inositol were synthesized (Angyal and Matheson, 1955). Me_3Si ethers of the inositols were obtained by the method of Sweeley *et al.* using about 200 μg of each inositol and 100 μl of the HMDS-silylating reagent.

Trimethylsilylation was carried out using three reagent mixtures. When preparing the Me_3Si derivatives of pure inositols the reagent used was anhydrous pyridine:HMDS:Me_3SiCl in the ratio 17:2:1, v/v. When preparing tissue extracts, or incubation mixtures containing nonphosphorylated inositols the same reagent was used, but in the ratio 7:2:1. Where the phosphorylated carbohydrates were of interest the reagent used was pyridine–BSA–Me_3SiCl in the ratio of 7:2:1, v/v (Sherman *et al.*, 1971). We have also used BSTFA in place of BSA with identical results. Pyridine was dried over Molecular Sieve 5A (Linde) activated by heating for 1 hr at 450° before use. The pyridine was stored over the sieve. A convenient check for water in the pyridine is to add a small amount of HMDS and Me_3SiCl to the pyridine in question. Only a slight haze should develop, a precipitate indicates the presence of excessive moisture.

Tissue extracts were prepared as described in Sherman *et al.* (1967). The essentials of this procedure are: quick freezing of organs removed from animals; addition of a fivefold excess of water to the frozen tissue sample followed by heating at 100° for 3 min; addition of $ZnSO_4$; centrifugation and lyophilization; addition of the trimethylsilylating reagent to the dried tissue and chromatography.

Tissue Recovery Experiment. Triplicate samples of rat cerebral hemispheres containing 0.5 g of tissue were prepared as described previously (Sherman *et al.*, 1967) except that 0.5 μg of *neo*-inositol was added to each sample and the sample was homogenized with 3 ml of water using a Ten-Broeck glass homogenizer. After heating at 100° for 3 min 60 μl of $ZnSO_4$ was added and the samples centrifuged. Aliquots of 1.75 ml were removed from each tube and lyophilized. A similar set was prepared without tissue using 3.5 ml of water, again 1.75-ml aliquots were removed and lyophilized. The dried samples were reacted for 48 hr at room temperature with 200 μl of BSTFA–pyridine 1:1 containing 10% Me_3SiCl. Analysis was carried out using the selective ion detection technique. Aliquots of 2 μl were gas chromatographed at the mass spectrometer and complete scans were made of the Me_3Si-*neo*-inositol–Me_3Si-D-mannose mixture (brain samples) or the Me_3Si-*neo*-inositol from the water samples. Scans of standard Me_3Si-*neo*-inositol were made under identical mass spectrometric and gas chromatographic conditions. The abundances of ions *m/e* 318 and 432 from samples and standard were compared and the recoveries of *neo*-inositol from the samples were calculated from these abundances.

Acetylation. A sample known by gas chromatography to contain *neo*-inositol phosphate (see *Incubations of Cyclase with Hexose Phosphate*) was heated at 70° overnight with 50% methanol in water to remove all Me_3Si groups. The sample was then incubated with *Escherichia coli* alkaline phosphatase and Mg^{2+} (again, see *Incubations of Cyclase with Hexose Phosphate*) to convert the *neo*-inositol-P into free *neo*-inositol. The free *neo*-inositol was converted into its acetate by reacting with acetic anhydride containing 1% of 70% $HClO_4$ at room temperature for 2 hr. The acetate was chromatographed on a 3% OV-17 column at the mass spectrometer and identified by comparison of the spectrum obtained with authentic *neo*-inositol acetate.

Analytical gas chromatography of the known and unknown Me_3Si-inositols was carried out with glass columns of 3% SE-30 and 3% OV-17 on Gas Chrom Q (0.25 in. × 6 ft at 165° with 70-cc/min carrier flow) and 15% EGS on Gas Chrom P (0.25 in. × 12 ft at 150° and 70 cc/min). All packings were obtained from Applied Science Labs, State College, Pa. Gas chromatography was on an F & M (Hewlett Packard) Model 402 with a hydrogen flame ionization detector.

Combined gas chromatography–mass spectrometry was carried out using an LKB-9000 under the following conditions: 70-eV ionizing potential; trap current 60 μA and 120 eps filtering; carrier gas separator 240°; source 270°; scans were at 60 average mass units/sec carried out through gas chromatograph peak centers. In the identification of Me_3Si-*neo*-inositol phosphate our LKB-9000 was coupled with an 8K memory PDP-12 computer (Holmes, 1971; Holmes *et al.*, 1971). In this case, scans were made at 115 average mass units/sec, other instrumental conditions remained the same. The chromatographic conditions (carrier flow 30 cc/min, 160–180° oven temperature) were such that, at half-height, peaks were about 45 sec wide. The Me_3Si-*neo*-inositol phosphate was chromatographed on columns of 0.5 or 3% OV-17 or OV-25 on Gas Chrom Q.

The mass spectral peaks were assigned *m/e* values by reference to perfluorokerosene in the early work, and later, using the LKB Hall-effect mass marker with and without the PDP-12 computer.

Mass Spectrometer as Chemically Selective Gas Chromato-

[1] Abbreviations used are: HMDS, hexamethyldisilazone; CDMS, cyclohexanedimethanol succinate; BSA, *N,O*-bis(trimethylsilyl)acetamide; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DEGS, diethylene glycol succinate; EGS, ethylene glycol succinate.

graphic Detector. The mass spectrometer was operated using the standard conditions except, rather than sweeping the magnetic sector, it was held at a selected field strength which brought the appropriate fragment ion into focus on the electron multiplier. The sample was then injected onto the gas chromatographic column and the ion of interest monitored by observing the oscillograph galvanometers. At the appropriate time the oscillograph was turned on at its slowest chart speed and the record thus obtained compared to a calibration chart obtained with dilutions of Me_3Si-*neo*-inositol. Over the ranges used the calibration chart was linear and proportional to the concentration of Me_3Si-*neo*-inositol. In the identification of Me_3Si-*neo*-inositol phosphate the LKB-9000-PDP-12 system was set to scan repeatedly through the region suspected to contain the *neo*-inositol phosphate and the collected spectra reviewed to obtain the data we present.

Incubations of cyclase with hexose phosphate were carried out in 50 mM Tris·HCl (pH 7.5). A typical incubation consisted of 0.1 ml of the enzyme in the presence of 5 mM D-glucose-6-P or D-mannose-6-P with 10 mM ammonium chloride, with 1 mM DPN and in the presence of 1 mM dithiothreitol. Incubations at 37° were linear in *myo*-inositol production for at least 4 hr.

For the preparation of *neo*-inositol phosphate, 1 ml of a highly active enzyme preparatio was incubated with D-mannose-6-P (10 mM), DPN (2 mM), and other additions as above in a final volume of 1.3 ml. In this case, incubations were carried out for 24 hr. In order to ensure that the *neo*-inositol phosphate was not formed by bacterial contamination over the 24-hr period of the incubation a separate incubation was carried out in the presence of 0.02% sodium azide to ensure asepsis. The results were essentially the same as those from incubation in the absence of sodium azide.

When the nonphosphorylated inositol was desired the incubation of cyclase with the desired hexose-6-P was followed by a 1-hr incubation with *E. coli* alkaline phosphatase (10 μl of a 1:1 dilution of type III obtained from Sigma Laboratories, St. Louis) and Mg^{2+} (final concentration 2 mM) in a 1-ml incubation volume. This step is eliminated in the preparation of *neo*-inositol phosphate. After completion of the appropriate incubation the reaction was heated at 100° for 3 min. After cooling about 0.5 cc of washed Dowex 50 (H form) was added to remove the Tris buffer which complicated silylation and chromatography. After mixing, the sample was centrifuged and the clear supernatant pipetted from the residue of Dowex and denatured protein. After two washes of the residue the combined supernatants were lyophilized, silylated with pyridine–BSA (or BSTFA)–Me_3SiCl (7:2:1, v/v), and stored until used. Storage at −70° in a sealed jar in the presence of anhydrous $CaSO_4$ (Drierite, Hammond Co., Xenia, Ohio) protected the samples until used.

When examining Sephadex filtration fractions for cyclase, 100–200 μl of each fraction collector tube was incubated with the buffered substrate in 1 × 75 cm disposable test tubes in a final volume of 1 ml. After incubating for 1 hr the samples were dephosphorylated with the alkaline phosphatase. All of the tubes were then frozen and lyophilized together in a Virtis freeze-drying flask. When dry, the samples were silylated with 100–200 μl of pyridine–BSA–Me_3SiCl (7:2:1, v/v) and stored at −70° until used.

Partial Purification of Cyclase. Fresh or fresh-frozen decapsulated bovine testis (150 g) was homogenized with two parts by volume of 0.154 M KCl. The suspension was made 1 mM in dithiothreitol and centrifuged at 100,000*g* for 1 hr. The clear pink supernatant was then brought to 20% of saturation with ammonium sulfate at 4°. Following this the precipitate was removed by centrifugation and discarded. The supernatant was then brought to 30% of saturation with ammonium sulfate and the precipitate collected. The precipitate was then suspended in 25 ml of 50 mM Tris·HCl buffer (pH 7.5), centrifuged, and the insoluble portion discarded. For precolumn activity an aliquot of this solution (1 ml) was dialyzed overnight against the 50 mM Tris·HCl buffer and the remainder was pumped on to a 5 × 75 cm bed of a Sephadex G-100 column at a rate of 50 ml/hr. This column was eluted with the buffer described above but containing 0.002% sodium azide (to retard growth in the buffer) using the same pumping rate. Fractions of 17 ml were taken. Each fraction was then made 1 mM in dithiothreitol and frozen until assayed. After analysis the tubes containing the cyclase activity were pooled and brought to 30% of saturation with ammonium sulfate. The precipitate was collected by centrifugation, redissolved in 9 ml of buffer, and dialyzed before use. Protein concentrations were measured by the Lowry method. Storage at −20° with the preparation of 1 mM in dithiothreitol protected the enzyme activity for at least five freeze-thaw cycles.

Gas Chromatographic Analyses of Sephadex Column Samples for Cyclase Activity. Aliquots of 2–4 μl of the silylated column fractions were injected onto a 0.25 in. × 6 ft glass column of 3% OV-17 on Gas Chrom Q. The chromatograph was then temperature programmed at a rate of 10°/min from 190 to 250° and held at this temperature. Under these conditions Me_3Si-*myo*-inositol eluted in about six minutes. During the next 6 min a group of long-retention time impurities eluted which appeared to be related to DPN. Following this the column oven was cooled for the next analysis. Using a Varian Model 1200 gas chromatograph which has a small, low-mass oven, the entire cycle could be repeated at 15-min intervals. The Me_3Si-*myo*-inositol was measured by comparison of the gas chromatographic peak heights with that of standard Me_3Si-*myo*-inositol.

Results and Discussion

During the isolation of *myo*-inosose-2 from calf brain (Sherman *et al.*, 1968) an unknown substance was inadvertently carried through the purification process. In brief, the purification process was (1) aqueous extraction of tissue, (2) deproteinization and lyophilization, (3) trimethylsilylation, (4) removal of silylating reagent followed by hexane extraction, (5) gross separation of volatile and nonvolatile substances by preparative gas chromatography on CDMS, (6) a second, higher resolution, gas chromatographic collection from SE-30, (7) combined gas chromatography–mass spectrometry (GC–MS) on columns of EGSS-X and of OV-17. By chance, Me_3Si-*myo*-inosose-2 has a retention time relative to Me_3Si-α-glucose (R_g) on SE-30 of 1.02, close to the R_g of the unknown (1.09, see Table I) and was thus collected simultaneously on this column. At the combined GC–MS stage a column of EGSS-X resolved the Me_3Si-inosose (R_g 1.48) from the unknown (R_g 1.15), thus providing a relatively clean spectrum. Similarly, OV-17 separated Me_3Si-inosose (R_g 1.14) from the unknown (R_g 0.65, Table I), giving a confirmatory spectrum.

At this stage, we had two lines of evidence at hand which could be applied in the identification of the new compound: gas chromatographic retention times on different columns and the mass spectrum of the substance.

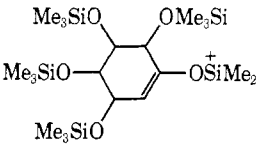
The mass spectrum of the unknown compound was made up of the same fragment ions as are present in Me_3Si-*myo*-

TABLE I: Gas Chromatographic Retention Times of Me_3Si-inositols Relative to α-D-Glucose.

Inositol	Liquid Phase		
	3% OV-17[a]	3% SE-30[a]	EGS[b]
	165°	165°	150°
Unknown	**0.65**	**1.09**	**0.96**
allo-	0.58	1.02	0.90
neo-	**0.65**	**1.09**	**0.96**[c]
muco-	0.73	1.17	0.98
(+)-*chiro-*	0.97	1.42	1.19
epi-	1.18	1.80	1.84
cis-	1.27	1.98	2.07
scyllo-	1.53	1.97	1.74
myo-	1.86	2.47	2.48

[a] On Gas Chrom Q. [b] On Gas Chrom P. [c] The value for the R_g of *neo*-inositol on EGS in Wells *et al.* (1965) is incorrect (see text).

TABLE II: Isotope Abundances of Fragment Ion *m/e* 507[a] from the Mass Spectrum of *myo-*, *scyllo-*, and *muco*-Inositols Compared to the Unknown from Calf Brain.



m/e 507

Inositol	*m/e*				
	507	508	509	510	511
myo-, *scyllo-*, and *muco-*	1	0.51	0.29	0.10	0.04
Unknown	1	0.50	0.30	0.11	0.03

[a] Proof of this structure is given in Sherman *et al.* (1970). The ion abundances given for *myo-*, *scyllo-*, and *muco*-inositols are averaged in the table.

inositol but with differing relative intensities. The highest mass ion in the spectrum of the unknown was *m/e* 612, the molecular ion of the Me_3Si-inositols. Furthermore, the ratios of the peaks in the spectrum of the unknown which result from the statistical combinations of naturally occurring heavy isotopes (^{13}C, ^{2}H, ^{18}O, ^{29}Si, and ^{30}Si) indicated that there were five silicon atoms in the fragment ion *m/e* 507 (M − 105 resulting from the loss of $\cdot CH_3$ and Me_3SiOH). The ratios of the isotopically generated ions related to *m/e* 507 are given in Table II. This data suggests that *m/e* 507 in the spectrum of the unknown has the same elemental composition as does *m/e* 507 in Me_3Si-*myo-*, *scyllo-* and *muco*-inositols, and presumably the others as well.

We then obtained samples of all of the inositols (only one of the dl pair (+)- and (−)-*chiro*-inositol) and subjected them to gas chromatography and to GC–MS. Retention times for all of the Me_3Si-inositols on DEGS have been reported by Lee and Ballou (1965) and for all but *cis-* and *allo*-inositol on SE-30 and EGS by Wells *et al.*[2] (1965). On the basis of these papers. and our data on OV-17, SE-30, and EGS (Table I) the unknown appeared to be *neo*-inositol; however, *allo-* and *muco*-inositols also have R_g values close to the unknown.

It was thus necessary to obtain supporting data for the rigorous identification of the compound as *neo*-inositol. The fact that the mass spectrum of Me_3Si-*myo*-inositol and the unknown had a similar but distinguishable ion abundance pattern under electron impact raised the question of whether it was possible that all of the isomeric inositols might, as their Me_3Si derivatives, be sufficiently unique with respect to the fragment ion abundances to be distinguishable from one another. The fact that they *are* distinguishable as their Me_3Si ethers (Sherman *et al.*, 1970), at a mass spectrometric ionization source temperature of 270° and under bombardment with 70 eV is remarkable considering the energy delivered to the sample under these conditions. An abridged list of ion abundances for the eight inositols is given in Table III. A comparison of our unknown from calf brain with authentic Me_3Si-*neo*-inositol showed conclusively that it was identical with authentic Me_3Si-*neo*-inositol (Figure 1) and different from the other seven inositols as shown in Table III.

As further proof that the substance we had isolated was *neo*-inositol we prepared a sample of *neo*-inositol phosphate enzymatically from D-mannose-6-P and identified it by combined gas chromatography–mass spectrometry. The sample was then detrimethylsilylated and dephosphorylated followed by acetylation with acetic anhydride and perchloric acid. The gas chromatogram of the resulting mixture of acetylated sugars was shown to contain *neo*-inositol acetate by gas chromatography–mass spectrometry. The spectrum of the inositol acetate from this mixture was found to be identical with authentic *neo*-inositol acetate (Sherman *et al.*, 1970). In addition to providing further evidence that the substance under study is *neo*-inositol, this experiment is consistent with the idea that the phosphorylated compound obtained on incubation of the cyclase with D-mannose-6-P is a *neo*-inositol phosphate (see below for further proof).

Having identified *neo*-inositol from brain we then wished to measure its concentration in that tissue and compare it to levels in other tissues. As Table IV shows, *neo*-inositol and α- or β-D-mannose elute unresolved from one another on many gas chromatographic liquid phases. We were unable to find a gas chromatographic column that could resolve Me_3Si-*neo*-inositol from D-mannose and were therefore unable to measure it in tissues by simple gas chromatography. We then turned to a technique which has been named mass chromatography by Hites and Biemann (1970). In this technique an ion in the mass spectrum of a compound of interest is selected and monitored by the mass spectrometer during the course of a chromatographic run. The appearance and disappearance of this ion is translated into a gas chromatographic record. If the ion is unique to a compound or to a family of compounds, the chromatographic record will show the location of these to the exclusion of other substances. A series of ions which are characteristic of the inositols is: *m/e* 612, 597, 507, 432, and 318 (Figure 1). All of these ions except the last probably carry the basic nucleus of the inositols, the cyclohexane ring (Sherman *et al.*, 1970). Ion *m/e* 432, which is probably a cyclo-

[2] The R_g value for Me_3Si-*neo*-inositol on EGS was mistakenly reported as 1.51 in this paper. A sample of the *neo*-inositol used was obtained from Dr. W. W. Wells and found to have an R_g on EGS of 0.96 as the Me_3Si derivative.

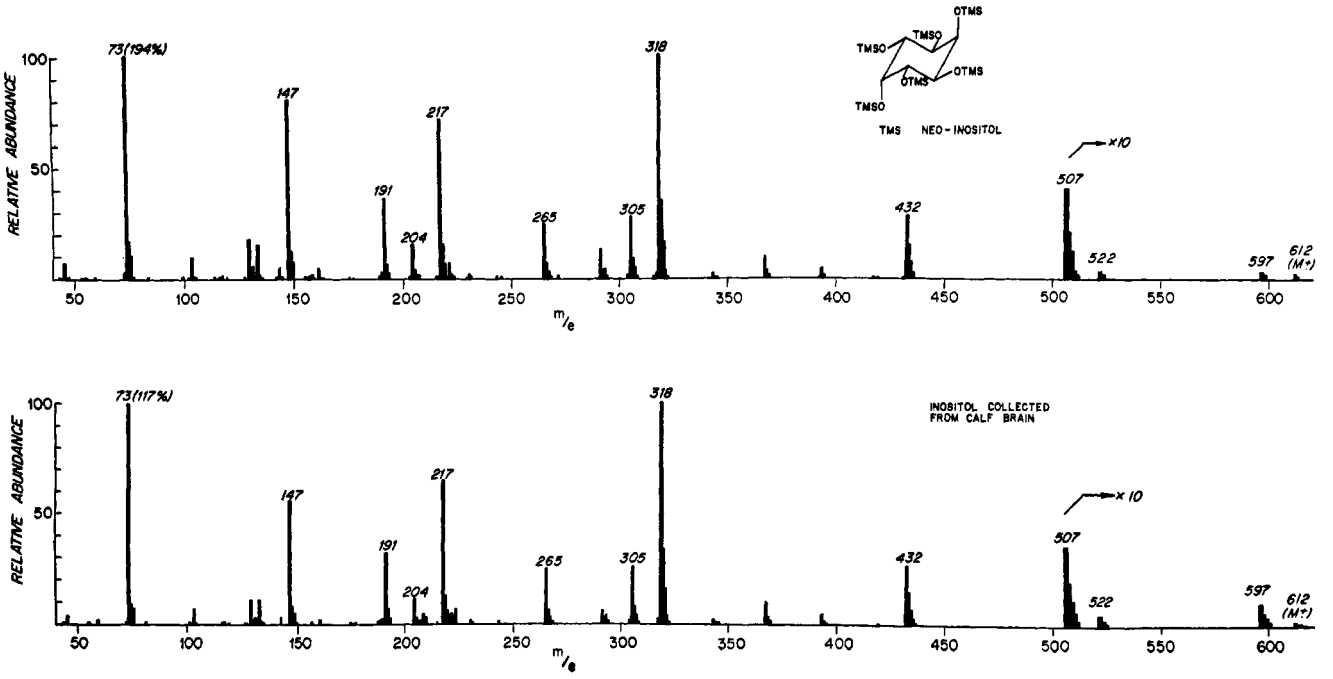


FIGURE 1: 70-ev mass spectrum of authentic Me_3Si *neo*-inositol (top) and that of the Me_3Si unknown obtained from calf brain.

TABLE III: Me_3Si-inositol Fragment Ion Relative Abundances. Mass Spectral Fragment Ion Abundances in the Spectra of the Trimethylsilyl Inositols.[a]

Me_3Si-inositol	73	147	217	305	318	432	433	597	612
scyllo-	112	51	74	73	100	2.5	2.5	0	0.5
myo-	122	65	100	95	52	12	11	0.2	2.0
(+)-*chiro*	248	96	89	84	100	8.7	10	0.3	0.6
epi-	137	91	100	69	51	1.6	4.4	0.2	0.2
neo-	194	81	72	28	100	29	16	0.2	0.15
muco-	230	72	94	98	100	1.3	3.9	0.02	0.5
allo-	225	84	79	58	100	5.6	8.3	0.3	0.2
cis-	184	100	93	65	26	1.3	3.6	0.7	0.1

[a] An abridged table of values. A complete table of values can be found in Sherman *et al.* (1970).

hexadiene and thus highly specific to the inositols, has a fortuitously high abundance in the spectrum of Me_3Si-*neo*-inositol. Since Me_3Si-D-mannose has no ion at *m/e* 432 (for the spectrum of Me_3Si-D-glucose, essentially that of Me_3Si-D-mannose, see DeJongh *et al.*, 1969) this was selected for the quantitative analysis of *neo*-inositol in tissue extracts and *in vitro* reactions.

Table V lists the levels of *neo*-inositol found in several tissues of the rat using mass chromatography. It can be seen that *neo*-inositol is present in these tissues in micromolar quantities in contrast with the millimolar levels of *myo*- and *scyllo*-inositol which are found in mammalian tissues (Sherman *et al.*, 1967). The high degree of sensitivity inherent in the mass chromatographic method is testified to by the data in this Table. Recovery of *neo*-inositol from brain samples of the size used in Table V was found to be 48%. When no tissue was used (water controls) the recovery was still only 60%, probably due to surface adsorption of the small samples used (0.5 μg/3.5 ml of tissue homogenate or water). In the mass chromatographic measurement of *neo*-inositol in these samples *m/e* 318 was used as the detector ion, justified by the low concentration of mannose in the brain samples, and the small abundance (0.4%) of *m/e* 318 in the spectrum of Me_3Si-D-mannose.

TABLE IV: Retention Distances of Me_3Si-*neo*-Inositol and Me_3Si-D-Mannose.[a]

neo-Inositol	D-Mannose	Column
6.8	(α) 6.8	3% ECNSS-M
10.9	(β) 10.8	3% JXR
7.8	(β) 8.0	3% QF-1
6.8	(α) 6.7	3% OV-17
1.09	(β) 1.04	3% SE-30

[a] Distances on chromatographic chart in centimeters from injection.

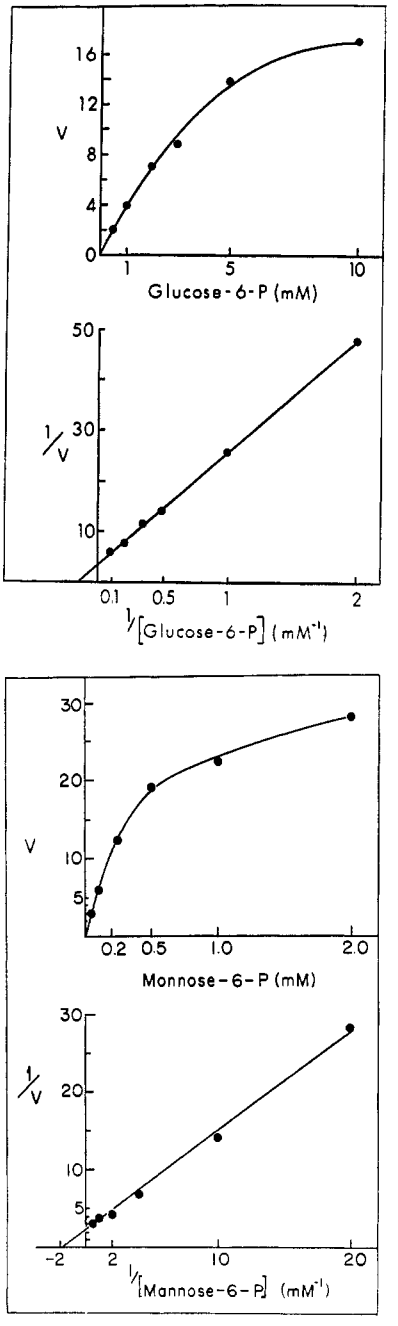


FIGURE 2: Effect of substrate concentration on the rate of reaction of D-glucose-6-P and D-mannose-6-P with D-glucose-6-P-L-*myo*-inositol-1-P cyclase. In the case of D-glucose-6-P, V = moles of *myo*-inositol produced per mg of protein/hr $\times 10^{-8}$. With D-mannose-6-P, V = moles of *neo*-inositol formed per mg of protein/hr $\times 10^{-10}$.

In an early phase of this work we attempted to measure *neo*-inositol in tissues by conversion of aldo- and ketohexoses into their oximes followed by trimethylsilylation and gas chromatography (for the method of oxime formation and chromatography of the Me_3Si derivatives of the oximes, see Sweeley *et al.* 1963). When this is done, the Me_3Si sugar oximes have longer retention times on gas chromatography and are thus removed from the region of the chromatogram containing the hydroxylamine-inert *neo*-inositol. As part of this study we obtained complete spectra of chromatographically resolved Me_3Si-*neo*-inositol from rat brain, kidney, testis, and spleen. The spectra were identical with authentic Me_3Si-*neo*-inositol thus confirming its presence in these tissues. As a quantitative analytic method this technique overestimated the amount of *neo*-inositol in tissues by fivefold when compared with the mass chromatographic measurement of Me_3Si-*neo*-inositol.

TABLE V: *neo*-Inositol in Rat Tissues.[a]

Tissue	μmoles/k Wet Wt		
Testis	14.0	6.5	7.5
Brain	6.5	2.5	1.9
Kidney	8.1	6.2	9.6
Heart	3.5	<0.8	3.1
Liver	<0.8	<0.8	<0.8
Spleen	4.4	4.3	1.2

[a] Approximately 500 mg of tissue extracted with water, deproteinized, lyophilized (Sherman *et al.*, 1967), and treated with 200 μl of BSA–Me_3SiCl–pyridine. Twenty microliters injected into combined GC–MS measured at *m/e* 432. Values are corrected for 48% recovery in this experiment (see Results).

Our interest then turned to the biosynthetic pathway by which *neo*-inositol might be formed. Two possibilities which have precedent in the inositol family are (1) dehydrogenation reduction as in the enzymatic reaction in which *myo*- is converted into *scyllo*-inositol *via* *myo*-inosose-2 (Candy, 1967) and (2) cyclization of D-mannose-6-P in a manner analogous to the cyclization of D-glucose-6-P to *myo*-inositol-1-P (Eisenberg, 1967; Chen and Charalampous, 1966).

In the first instance *myo*-inositol might be dehydrogenated to the 5-keto compound (*myo*-inosose-5) and the carbonyl group reduced to produce *neo*-inositol which is epimeric with *myo*-inositol at C-5. Our attempts to detect *neo*-inositol in incubation mixtures of *myo*-inositol with a low-speed brain and liver supernatant, in the presence of DPN or TPN, gave no indication of the conversion of *myo*- into *neo*-inositol.

We then examined the second possibility, that of whether the conversion of D-mannose-6-P into *neo*-inositol was catalyzed by the same system which cyclizes D-glucose-6-P. At this stage of our work we were using a 30–40% ammonium sulfate cut from a 100,000*g* rat testis supernatant (Eisenberg, 1967). All of our incubations of this preparation (as well as later preparations from bovine testis) with D-glucose-6-P appeared to contain phosphohexoisomerases as evidenced by the formation, in addition to L-*myo*-inositol-1-P, of an amount of α- and β-D-mannose-6-P and D-fructose-6-P close to that of the final substrate level of D-glucose-6-P (Sherman *et al.*, 1969). In later work it was found that these incubations also contained *neo*-inositol after enzymatic dephosphorylation.

Since the incubation mixture quickly equilibrated to the three hexose phosphates *via* the phosphohexoisomerases, this complicated the identification of the precursor of *neo*-inositol. Further purification of the enzyme by Sephadex filtration gave a partial separation wherein the cyclase eluted before, but incompletely resolved from the isomerase activity. However, ammonium sulfate (30% of saturation) was found to precipitate the cyclase while the isomerase activity remained in solution. Table VI shows the extent of purification which we obtained in this way. Once separated from the isomerases the cyclase preparation when incubated with mannose-6-P gave

TABLE VI: D-Glucose-6-P–L-*myo*-Inositol-1-P Cyclase Purification from Bovine Testis.

Purifcn Step	Total Protein (mg)	Protein Recov (%)	Total Act. (μmoles/mg of Protein per hr)	Act. Recov (%)	Sp Act. (μmole/mg of Protein per hr)	Purifcn
100,000*g* Supernatant, 20–30% $(NH_4)_8SO_4$[a]	72		4.8		0.066	
Sephadex G-100 Pool	37	51	5.8	120	0.16	2.6
0–30% $(NH_4)_2SO_4$[a]	4.7	13	2.9	50	0.61	9.2

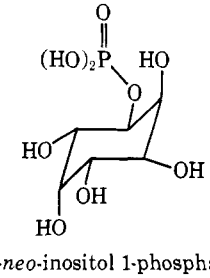
[a] Precipitate. Redissolved and dialyzed before measuring.

only trace amounts of D-fructose-6-P, D-glucose-6-P, and L-*myo*-inositol-1-P.

In the case of enzyme incubations with D-mannose-6-P the D-mannose generated by the phosphatase reaction completely obscured the *neo*-inositol (Table IV). However, Me_3Si-*neo*-inositol was identified as a companion eluent of Me_3Si-D-mannose by the presence, in the composite mass spectrum, of ions characteristic of the inositols (*e.g.*, *m/e* 612, *m/e* 597, *m/e* 507, *m/e* 432, and *m/e* 318, Sherman *et al.*, 1970). The gas chromatographic peak as located by the maximum intensities of these ions had a retention time identical with authentic *neo*-inositol.

To further substantiate the conversion of D-mannose-6-P into *neo*-inositol we incubated graded concentrations of D-mannose-6-P with the cyclase preparation for one hour. From the Michaelis curve and reciprocal plot in Figure 2, the mannose–*neo*-inositol cyclase is clearly saturable, thus enzymatic. It is interesting to note that the K_m of D-mannose-6-P (5.6 mM) is very close to that of D-glucose-6-P (6.2 mM) when incubated under the same conditions (DPN 1 mM, Figure 2). In contrast, the maximum velocity of *neo*-inositol production is 4.5×10^{-9} mole/mg of protein per hr while *myo*-inositol is produced at 2.5×10^{-7} mole/mg per hr. Since it appears from our work and that of others that at least testis contains the phosphohexoisomerases necessary for the interconversion of D-glucose-6-P, D-fructose-6-P, and D-mannose-6-P, the low tissue levels of *neo*-inositol are probably a reflection of the low V_{max} of the enzyme with D-mannose-6-P.

If the D-glucose-6-P–L-*myo*-inositol-1-P cyclase is responsible for the cyclization of D-mannose-6-P, or if a second cyclase, specific for D-mannose-6-P is present, the initial product of this reaction should be L-*neo*-inositol-1-P. Since the spectra



L-*neo*-inositol 1-phosphate

of the Me_3Si inositols are similar with respect to fragment ion composition (Sherman *et al.*, 1970) and the spectra of the Me_3Si-aldohexose 6-phosphates are similarly related (Zinbo and Sherman, 1970) it should follow that the spectra of the Me_3Si-inositol phosphates are also related in this sense. We have found this to be the case for Me_3Si-*myo*-inositol-1-, -2-, -4-, and -5-P as well as (−)-*chiro*-inositol-3-P (M. Zinbo and W. R. Sherman, unpublished results). We have therefore used the ion *m/e* 749 (M − 15 of the monophosphorylated inositols) as a marker ion for the location of Me_3Si-inositol phosphates. Incubations of D-mannose-6-P with the cyclase-containing enzyme preparation (with and without added sodium azide for asepsis in the 24-hr incubation) were trimethylsilylated directly and chromatographed at the inlet of the mass spectrometer. The total ion current chromatogram (the gas chromatographic record obtained from the mass spectrometer) of this sample is given in Figure 3. The presence of Me_3Si-D-glucose- and D-fructose-6-P as well as Me_3Si-L-*myo*-inositol-1-P results from phosphohexoisomerase activity in this highly active but less pure cyclase than the preparation used for the synthesis of *neo*-inositol and for the Michaelis–Menten study reported above. The 24-hr incubation period also contributed to the high levels of these products.

Peak 4 in Figure 3 was found to possess ion *m/e* 749 as does peak 7. A similar chromatogram on OV-25 also has *m/e* 749 occurring in two places. We present evidence below that the shorter retention peak in each case is a Me_3Si-*neo*-inositol-P. The second, longer retention peak has been shown by direct comparison with authentic standard to be Me_3Si-*myo*-inositol-1-P. The retention times relative to Me_3Si-α-D-glucose-6-P for

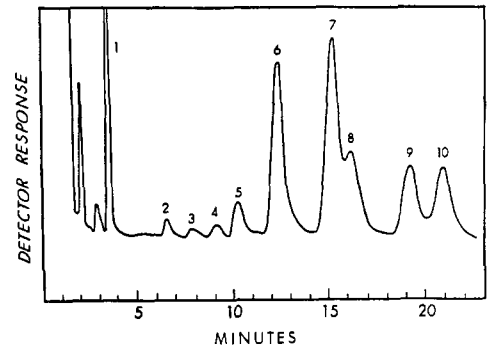


FIGURE 3: Combined gas chromatograph–mass spectrometer total ion current gas chromatographic record of the trimethylsilylated products obtained from the incubation of D-mannose-6-P with partially purified bovine testis cyclase. Retention times on this column of 3% OV-17 (on Gas Chrom Q) relative to D-glucose-6-P are given as follows: chromatogram peak number, compound, followed by R_{G-6-P}: 1, free *myo*-inositol (0.16); 2, D-ribose-5-P (0.32); 3, unknown (0.39); 4, *neo*-inositol-P (0.48); 5, D-fructose-6-P (0.53); 6, α-D-mannose-6-P (0.64); 7, L-*myo*-inositol-1-P (0.79); 8, β-D-mannose-6-P (0.84); 9, α-D-glucose-6-P (1.00); 10, β-D-glucose-6-P (1.09).

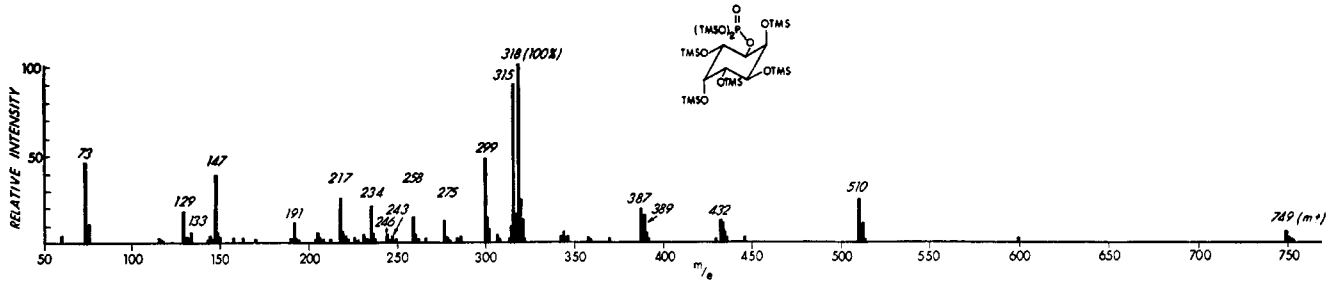


FIGURE 4: 70-eV mass spectrum taken through the center of peak 4 (Figure 3). Ions *m/e* 749, 389, 387, 318, 315, 299, 246, 243, 217, 191, 147, 133, 129, and 73 are characteristic of the spectra of Me_3Si-inositol phosphates (Zinbo and Sherman, 1969, unpublished work from this laboratory). Ions *m/e* 510, 275, 258, and 234 are from an unknown substance eluting within the same chromatographic peak and partially resolved from Me_3Si-*neo*-inositol-P (see Figure 5).

Me_3Si-*neo*-, and Me_3Si-*myo*-inositol-P are, respectively, OV-17, $R_{G\text{-}6\text{-}P}$ 0.48 and 0.79; OV-25, 0.42 and 0.74.

Peak 4 in Figure 3 is small when compared with Me_3Si-*myo*-inositol-1-P presumably because of the 200-fold difference in the V_{max} of formation of the two inositol phosphates.

The spectrum of peak 4, which contains what we believe is L-*neo*-inositol phosphate, is shown in Figure 4. A comparison of this spectrum to that of D-*myo*-inositol-1-P (Sherman *et al.*, 1969) reveals that there are four ions present in the peak 4 spectrum which are not in Me_3Si-L-*myo*-inositol-1-P. These are at *m/e* 510, 275, 258, and 234. We have found that these ions represent an unknown substance which elutes from the gas chromatograph later than and slightly resolved from the Me_3Si-*neo*-inositol-P. Figure 5 is a plot of the elution envelope of the Me_3Si-*neo*-inositol-P represented by *m/e* 749 (M − 15) and of the unknown compound as represented by *m/e* 510. It can be seen that there is a clear separation of the two amounting to 33 sec under these conditions. The ions at *m/e* 275, 258, and 234 all follow the same elution envelope as *m/e* 510. All of the remaining major ions in Figure 4 are observed in the spectrum of Me_3Si-L-*myo*-inositol-1-P. The base peak *m/e* 318 is the most abundant ion in the spectrum of Me_3Si-*neo*-inositol-P (excluding *m/e* 73) as in the spectrum of Me_3Si-L-*myo*-inositol-1-P. As in unphosphorylated Me_3Si-*neo*-inositol *m/e* 432 is of greater abundance in Me_2Si-*neo*-inositol-P (12.4% abundance relative to *m/e* 318) than in Me_3Si-L-*myo*-inositol-1-P (6% relative to *m/e* 318). Otherwise the spectrum is quite similar to the other Me_3Si-inositol phosphates we have examined.

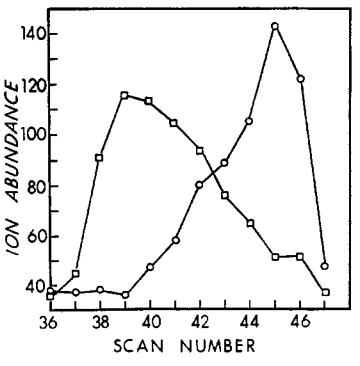


FIGURE 5: Elution envelopes of the ions *m/e* 749 (□) and *m/e* 510 (○) in the mass spectra from gas chromatographic peak 4 (Figure 3). The spectra were taken every 5.5 sec and stored by a PDP-12 computer on magnetic tape. Analysis of the spectra obtained through peak 4 revealed two families of ions, one from Me_3Si *neo*-inositol-1-P, represented here by *m/e* 749, and the other from an unknown substance whose spectrum is represented by *m/e* 510. The latter spectrum is made up in part of peaks *m/e* 510, 275, 258, and 234 (Figure 4).

In order to support the identification of peak 4 of Figure 3 as a Me_3Si-*neo*-inositol-P, we have taken the sample from which Figure 3 was obtained to dryness and heated it in 50% methanol in water at 75° for 24 hr. The detrimethylsilylated sample was then incubated with *E. coli* alkaline phosphatase for 2 hr at 38° and the product lyophilized and retrimethylsilylated. Analysis of the sample before removal of the Me_3Si groups and the phosphate showed 1.8 μg of free *neo*-inositol to be present. Following phosphatase action and trimethylsilylation a total of 80 μg of *neo*-inositol (as its Me_3Si derivative) was obtained, an increase of 78.2/1.8 or 43-fold. This showed conclusively that the inositol phosphate in the original incubation of D-mannose-6-P with the cyclase was a *neo*-inositol-P. By analogy with the cyclization of glucose-6-P with the cyclase the phosphate group should be at the 1 position of the *neo*-inositol, and the substance should be L-*neo*-inositol-1-P.

Conclusions

The occurrence of *neo*-inositol in tissues which normally contain *myo*- and *scyllo*-inositol suggests a metabolic relationship between these three inositols. The fact that they also occur together in soil (Cosgrove and Tate, 1963; Cosgrove, 1963) supports this idea. Our work seems to eliminate the possibility that *neo*-inositol is formed from *myo*-inositol by an oxidation–reduction process because in more than one experiment where we had the glucose-6-P-*myo*-1-P cyclase essentially free of the phosphohexoisomerases, only trace amounts of *myo*-inositol were found following treatment of D-mannose-6-P incubations with bacterial alkaline phosphatase. These preparations contained considerable amounts of *neo*-inositol which, if formed from L-*myo*-inositol-1-P must have done so in a way which virtually depleted the L-*myo*-inositol-1-P. Assuming that such an oxidation–reduction would be an equilibrium process the *myo*-inositol-1-P would not be exhausted and the *neo*-inositol must have some other origin. The Michaelis–Menten curve and Lineweaver–Burk reciprocal plot show a clear dependence of *neo*-inositol production on D-mannose-6-P concentration. What is not known at this time is whether the *neo*-inositol results from a lack of specificity of the cyclase for the 2 position of the aldohexose-6-P resulting in the accidental production of *neo*-inositol-1-P or whether it results from the presence of a second enzyme unseparated by us from the D-glucose-6-P–L-*myo*-inositol-1-P cyclase.

We have been unable to cyclize 2-deoxy-D-glucose-6-P,

2-deoxy-2-amino-D-glucose-6-P, and D-galactose-6-P to their respective inositols using the same system that is active toward D-mannose-6-P. Whether these negative results support the idea that a second cyclase is present in the preparations, which is specific for D-mannose-6-P, or whether the D-glucose-6-P–L-*myo*-inositol-1-P cyclase is nonspecific only with respect D-mannose-6-P cannot be determined with the data at hand.

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References

Angyal, S. J., and Matheson, N. K. (1955), *J. Amer. Chem. Soc. 77*, 4343.
Candy, D. J. (1967), *Biochem. J. 103*, 666.
Chen, I.-W. and Charalampous, F. C. (1966), *J. Biol. Chem. 241*, 2194.
Cosgrove, D. J. (1963), *Aust. J. Soil Res. 1*, 203.
Cosgrove, D. J., and Tate, M. E. (1963), *Nature (London) 200*, 568.
DeJongh, D. C., Radford, T., Hribar, J. D., Hannessian, S., Bieber, M., Dawson, G., and Sweeley, C. C. (1969), *J. Amer. Chem. Soc. 91*, 1728.
Eisenberg, F., Jr. (1967), *J. Biol. Chem. 242*, 1375.
Hites, R. A., and Biemann, K. (1970), *Anal. Chem. 42*, 855.
Holmes, W. (1971), Biochemical Applications of Mass Spectrometry, Waller, G., Ed., New York, N. Y., Wiley (in press).
Holmes, W., Holland, W. H., and Parker, J. A. (1971), *Anal. Chem.* (in press).
Lee, Y. C., and Ballou, C. E. (1965), *J. Chromatog. 18*, 147.
Sherman, W. R., Eilers, N. C., and Goodwin, S. L. (1970), *Org. Mass Spectrom. 3*, 829.
Sherman, W. R., Goodwin, S. L., and Zinbo, M. (1971), *J. Chromatog. Sci. 9*, 363.
Sherman, W. R., Stewart, M. A., Kurien, M. M., and Goodwin, S. L. (1967), *Biochim. Biophys. Acta 158*, 197.
Sherman, W. R., Stewart, M. A., Simpson, P. C., and Goodwin, S. L. (1968), *Biochemistry 7*, 819.
Sherman, W. R., Stewart, M. A., and Zinbo, M. (1969), *J. Biol. Chem. 244*, 5703.
Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W. (1963), *J. Amer. Chem. Soc. 85*, 2497.
Wells, W. W., Pittman, T. A., and Wells, H. J. (1965), *Anal. Biochem. 10*, 450.
Zinbo, M., and Sherman, W. R. (1970), *J. Amer. Chem. Soc. 92*, 2105.

Yeast Hexokinase. IV. Multiple Forms of Hexokinase in the Yeast Cell*

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ABSTRACT: Using improved methods for the preparation of hexokinase from baker's yeast without autolysis by any of the yeast proteases, the extract was shown to contain four hexokinase isoenzymes, A, A′, B, and C, and a specific glucokinase. The latter is extracted in a form having molecular weight about one million. Other active hexokinase species, arising by proteolysis of native forms, are distinct from these, and are suppressed when initial removal of proteases is efficient. Conditions are described for obtaining hexokinases A, B, and C in pure form, each homogeneous in starch gel electrophoresis. Hexokinases A and B are the previously described forms, and differ in that A has a greater relative activity on fructose, a lower specific activity, and a higher negative charge (at pH above 5) than B. Their measured activity differences are essentially a reflection of differences in the Michaelis–Menten maximum velocities on glucose and on fructose. Hexokinase C is a newly described isoenzyme, identical with B in most properties but having a different mobility in anion-exchange chromatography. It can be converted to B when a high ionic strength is applied at pH 4.6 or 8, followed by gel filtration. Immediately upon rupture, yeast cell lysates contain most or all of their hexokinase B in the form of C. Also present in the fresh lysates is hexokinase A′, which appears to be a hybrid of A and B. This hybrid splits to give A and B in the conditions of purification. The course of release of the hexokinases was followed in the melting of frozen yeast. Sixty per cent of the total activity was readily liberated as soluble forms, these being characterized as A, A′, and C. The remainder is probably osmotically trapped in the cell debris, and is not bound to the cell walls.

In part I of this series (Lazarus *et al.*, 1966) a procedure was described for the preparation of hexokinase from yeast under conditions designed to protect the enzyme against degradation by the proteases readily available in cell homogenates of baker's yeast. This was achieved by liberating the enzyme from the cells by a freeze-thawing method, by inactivating the

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