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Substrate Properties of Analogs of *myo*-Inositol

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SUMMARY

The hydrolysis of the minor cell membrane lipid phosphatidylinositol-4,5-bisphosphate mediates the action of many growth factors and hormones. As an approach to the development of specific inhibitors of this process, we have synthesized a series of analogs of *myo*-inositol and have evaluated their ability to serve as substrates for phosphatidylinositol synthetase. Modification at the 2-, 3-, or 4-positions produced compounds unable to serve as substrates, but several 5-modified analogs retained activity as substrates of phosphatidylinositol synthetase. The product formed from 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol by phosphatidylinositol synthetase was hydrolyzed by phospholipase D and gave 5-deoxy-5-fluoro-*myo*-inositol as the radiolabeled product. Two analogs, 5-deoxy-*myo*-inositol and 5-deoxy-5-fluoro-*myo*-inositol, were shown to permeate L1210 leukemia cells and be incorporated into cellular phospholipid. Analysis of the radiolabeled lipids formed on incubation of L1210 cells with 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol indicated that the fraudulent lipid formed was further phosphorylated to the monophosphate but not to the diphosphate form.

Many hormones and growth factors produce their effects on cells by stimulating the hydrolysis of PIP_2 present in the plasma membrane (see Refs. 1 and 2 for two recent reviews). This hydrolysis yields two second messengers, diacylglycerol and inositol trisphosphate (reaction 3 in Fig. 1). These second messengers modulate a number of processes including cell secretion, production of prostaglandins, and cell division. Although the central role of this process suggests that it would be an attractive target for pharmacologic intervention, no specific inhibitors of the formation or hydrolysis of PIP_2 have been identified. Inhibitors of this process are of potential value in modulating cell division and would be invaluable as biochemical tools to elucidate the role of PIP_2 hydrolysis in regulation of cell function.

One approach to the design of such inhibitors is the synthesis of inositol analogs that could be incorporated into PI (reaction 1 in Fig. 1) but could not be further phosphorylated to PIP_2 (reaction 2 in Fig. 1). Such fraudulent PI species may inhibit the phospholipase C directly or, alternatively, reduce the rate of PIP_2 hydrolysis simply by reducing the availability of PIP_2. As the initial step in the development of such inhibitors, we have synthesized the series of *myo*-inositol analogs shown in Fig. 2 and evaluated their ability to serve as substrates for PI synthetase (EC 2.7.8.11) and to be incorporated into cellular phospholipid.

Materials and Methods

Triton X-100, *myo*-inositol, PI, and dipalmitoyl CDP-diglyceride were purchased from Sigma Chemical Co., St. Louis, MO. The CDP-diglyceride was prepared as a 5 mM stock solution in 1% Triton X-100. *myo*-[2-^{3}H]Inositol, 14.2 Ci/mmol, was purchased from Amersham Corp., Arlington Heights, IL. 4-Deoxy-*myo*-inositol (Ref. 3; 2-deoxy-DL-*epi*-inositol [488-74-4]) was a generous gift of Dr. S. J. Angyal of the University of New South Wales, Kensington, Australia. Reagents for the protein assays were from Pierce Chemical Co., Rockford, IL.

All other inositol analogs were prepared as described (4). The purity of all the compounds was examined by thin layer chromatography on cellulose plates (Kodak, Rochester, NY) eluted with acetone/water, 4:1. This system resolved each analog from *myo*-inositol. Compounds were detected by the silver nitrate system described by Posternak (5). In addition, each compound was analyzed as its trimethylsilyl derivative by gas chromatography and was >99% pure and free of detectable *myo* inositol (<0.2%).

5-Deoxy-*myo*-[5-^{3}H]inositol (100 mCi/mmol) was synthesized from 5-amino-*neo*-inositol by a previously described method (6). 5-Deoxy-5-fluoro-*myo*-[^{3}H]inositol was obtained from Moravek Biochemicals, Brea, CA. The procedure used for tritium labeling was a modification of that used for the Raney nickel/deuterium oxide deuteration of cyclitols in general (7) and 5-deoxy-5-fluoro-*myo*-inositol in particular (8). Short times of exchange and moderate temperatures were shown to minimize formation of isomeric products (8) when compound **2** was exchanged with either deuterium oxide or tritium oxide. Studies by ^{1}H NMR spectroscopy (8) of the exchange 2 with deuterium oxide (60–65°, 40 min) showed replacement of H by D principally at C-2 (95%), with lesser amounts (65%) at C-1 and C-3. The prepared radiolabeled 5-deoxy-*myo*-inositol co-chromatographed with the unlabeled standard in the following systems: cellulose plates, *n*-butanol/pyridine/H_2O, 6:4:3, cellulose plates, acetone/H_2O, 5:1; silica G plates, butanol/acetic acid/H_2O, 2:1:1. The radiolabel was >98% associated with 5-deoxy-*myo*-inositol or 5-deoxy-5-fluoro-*myo*-inositol in all three systems and

ABBREVIATIONS: PIP_2, phosphatidylinositol-4,5-bisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-monophosphate.

<1% *myo*-[^{3}H]inositol; none was detected in the first two systems, which resolve *myo*-inositol from 5-deoxy-*myo*-inositol.

L1210 murine leukemia cells were obtained from American Type Culture Collection, Rockville, MD, and maintained in stationary cultures in Fischer's medium with 10% horse serum in an atmosphere of 10% CO_2 and 90% air.

Molecular modeling was carried out using an Evans and Sutherland PS300 system and SYBYL software, a product of Tripos Associates, St. Louis, MO. The geometries were optimized by SYBYL's molecular mechanics program, MAXIMIN, followed by RINGSEARCH to locate the lowest energy conformer. Such a procedure was demonstrated to work satisfactorily for both cyclohexanes and fluorine-substituted alkanes before its application in this work. Molecular volumes and difference volumes were calculated using a combination of the FIT and MVOLUME routines in SYBYL.

Assay of PI synthetase. PI synthetase was prepared from rat brain microsomes and solubilized by 0.5% Triton X-100 as described by Rao and Strickland (9). The enzyme used in this study had a specific activity of 25–41 nmol/min/mg of protein when assayed as described with 5 mM *myo*-inositol and protein determined by the method of Smith *et al.* (10). The assay (modified from Ref. 9) was performed in a volume of 0.2 ml of 48 mM $MgCl_2$, 50 mM glycylglycine, pH 8.4, 0.5% Triton X-100, 0.5 mM CDP-diglyceride (dipalmitoyl), 50 μl of enzyme preparation (90 μg of protein), and the indicated concentration of substrate. The reaction was initiated by addition of enzyme and proceeded at 37°. Aliquots of 40 μl were removed at 5, 10, 15, and 20 min and mixed with 0.76 ml of chloroform/methanol (2:1) to terminate the reaction. Water (0.16 ml) was added and the upper aqueous phase was removed to analyze for CMP formation. When incorporation of *myo*-[^{3}H]inositol into lipid was measured, the lower chloroform-rich phase was washed twice with 0.2 ml of preequilibrated upper phase. A portion (200 μl) of the lipid-containing (lower) phase was then counted in 10 ml aqueous counting scintillant (Amersham) with a scintillation counter.

The generation of CMP from CDP-diglyceride was used as a measure of PI synthetase (11). The formation of CMP was measured by chromatography of 75 μl of the aqueous phase on a Spherisorb SAX column, 25 × 0.46 cm (Alltech, Deerfield, IL), eluted with 1.5 ml/min of 0.02 M NH_4PO_4, pH 4.5. CMP eluted at 3.8 min and was quantified by electronic integration of the absorbance at 285 nm. Peak area was proportional to amount injected over the range of 0.075 to 2.0 nmol.

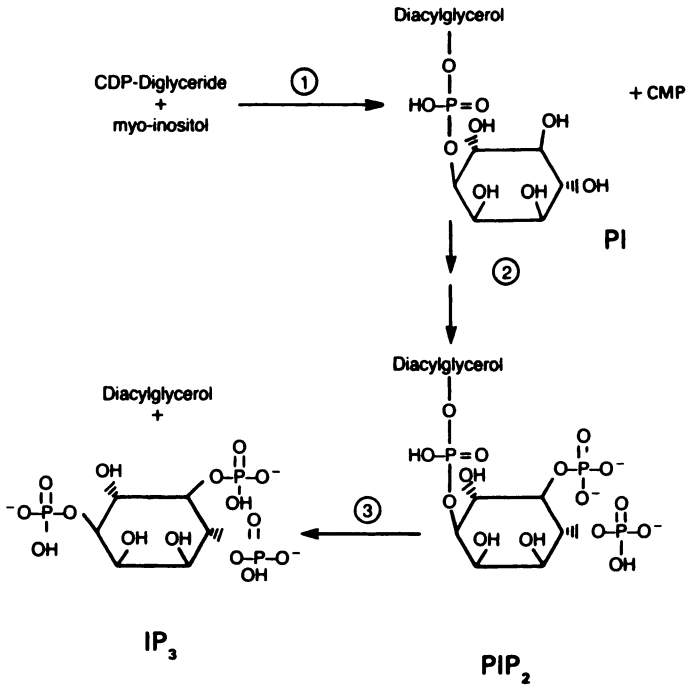

Fig. 1. The reactions of phosphatidylinositol bisphosphate synthesis and hydrolysis. The reactions are catalyzed by the following enzymes: *1*, PI synthetase (EC 1.7.8.11); *2*, PI kinase (EC 2.7.1.67) then PIP kinase (EC 2.7.1.68); *3*, phospholipase C (EC 3.1.4.10).

Assay by either the incorporation of [^{3}H]*myo*-inositol or formation of CMP gave linear rates for 30 min and was absolutely dependent on the addition of CDP-diglyceride. The radiolabeled product in the chloroform-rich phase was shown to be >95% PI by chromatography on silica G thin layer plates as described (12).

Uptake and incorporation of [^{3}H]*myo*-inositol by L1210 cells. This procedure is modified from Wohlhueter *et al.* (13). L1210 cells were collected by centrifugation and resuspended at 2 × 10^7 cells/ml in inositol-free Fischer's medium. The cells were incubated with precursor as indicated at 37°. At the indicated times, 0.5-ml samples were removed and placed in a 1.5-ml conical tube containing 0.5 ml of inositol-free Fischer's medium overlayed on 0.5 ml of Versilube F50 silicon oil (Read Plastics, Rockville, MD). This tube was then centrifuged at 10,000 × *g* for 10 sec to separate cells from the medium by spinning through the oil layer. The cell pellet was then isolated and extracted with 0.4 ml of chloroform/methanol, 2:1. This solution was then separated into aqueous and lipid-soluble fractions by addition of 0.1 ml of water, followed by centrifugation. The amount of radiolabel in each fraction was then determined by scintillation counting. In parallel incubations the total water volume and extracellular water volumes were determined with ^3H_2O and [^{14}C]inulin as described by Wohlhueter *et al.* (13). Typical values for total water volume and extracellular volume were 7.1 ± 1.2 and 0.96 ± 0.42 μl, respectively, for 10^7 cells (mean ± standard deviation, six determinations).

Phospholipase D hydrolysis. The radiolabeled lipid (30 μl, 0.02 μCi) formed from 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol by rat brain microsomes was sonicated in 0.5 ml of 0.3 M Tris/maleate, pH 7.0, with 5 mM calcium chloride and 8 mM sodium dodecyl sulfate. Phospholipase D (*Streptomyces chromofucus*, 2500 units/mg) was added (50 μl, 675 units) followed by 0.2 ml of anhydrous diethyl ether. After mixing well, this was incubated at 30° for 3 hr. The reaction mixture was then mixed well with 0.9 ml of chloroform followed by 0.45 ml of methanol. The mixture was shaken and the phases were separated after centrifugation. The water-soluble product was identified by chromatography of a 100-μl portion of the aqueous phase in four thin layer chromatography systems. The plates were cut into 1-cm strips and radioactivity was determined by scintillation counting. The systems and R_F values are as follows: silica G plates, *n*-butanol/acetic acid/H_2O, 2:1:1—R_F, *myo*-inositol = 0.41, 5-deoxy-5-fluoro-*myo*-inositol = 0.48; cellulose plates, acetone/water, 4:1—R_F, *myo*-inositol = 0.21, 5-deoxy-5-fluoro-*myo*-inositol = 0.57; cellulose plates, *n*-butanol/pyridine/water, 6:4:3—R_F, *myo*-inositol = 0.2, 5-deoxy-5-fluoro-*myo*-inositol = 0.47; cellulose plates, ethanol/water/concentrated ammonium hydroxide, 20:4:1—R_F, *myo*-inositol = 0.4, 5-deoxy-5-fluoro-*myo*-inositol = 0.53.

Incorporation of [^{3}H]inositols into L1210 cell phospholipid. L1210 cells in Fischer's medium, initially at 1 × 10^5 cells/ml, were incubated as indicated in the legend for Fig. 6. The cells in 10-ml suspension were collected by centrifugation and extracted with 0.3 ml of 15% trichloroacetic acid. The pellet containing the lipids was extracted with 0.3 ml chloroform/methanol/concentrated HCl (200:100:1) for 20 min at 25°. The lipid-containing layer was then deacylated as described (14). The deacylated lipids were chromatographed on a Whatman Partisphere SAX anion exchange high performance liquid chromatography column (4.0 × 12.5 cm), with a precolumn containing Whatman pellicular anion exchange resin. The column was eluted isocratically with 0.01 M ammonium phosphate buffer, pH 3.3, for 5 min (flow rate, 1 ml/min), followed by a linear gradient from 0.01 M to 1.0 M ammonium phosphate in 23 min. Then the column was eluted isocratically with 1.0 M NH_4PO_4 buffer for 2.0 min. Fractions (0.5 ml) were collected for 30 min. Each fraction was mixed with 6 ml of aqueous counting scintillant (Amersham) containing 5% water and radioactivity was determined by liquid scintillation counting.

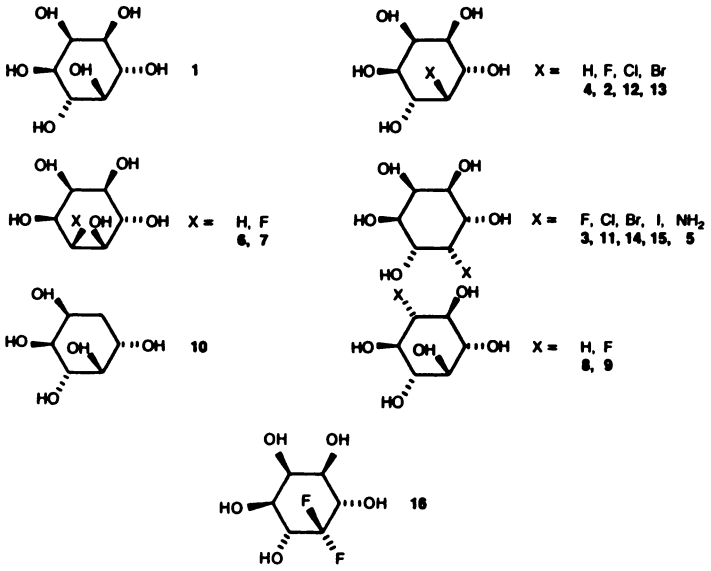

Fig. 2. Analogs of *myo*-inositol.

Results and Discussion

We have synthesized a systematic series of analogs of *myo*-inositol modified at the 2-, 3-, 4-, and 5-positions numbered relative to the *myo*-inositol moiety of PI (Fig. 2). These compounds were evaluated as inhibitors and substrates of PI synthetase from rat brain (Table 1). Two assays were used to evaluate the compounds, inhibition of the incorporation of radiolabeled *myo*-inositol into PI or ability of the analogs to serve as substrates as measured by formation of CMP from CDP-diglyceride. The former serves to identify compounds that bind to the active site but does not indicate if these compounds are substrates. The latter assay identifies substrates, as no CMP is formed from CDP-diglyceride in the absence of an effective substrate. All analogs examined were less effective than *myo*-inositol as substrates for PI synthetase. Loss of the hydroxyl at the 2-position (compound **8**), the 4-position (compound **6**), or the 3-position (compound **10**) produced very poor or completely inactive substrates. Replacement of the 5-hydroxyl with a hydrogen (compound **4**) or fluoride (compound **2**) produced compounds that were substrates for PI synthetase. Compound **2**, which retained the original *myo*-configuration, was the most effective alternative substrate in the series, with a rate equal to 26% of that seen with *myo*-inositol at equal concentrations (5 mM). Interestingly, the isomeric 5-deoxy-5-fluoro-*neo*-inositol (compound **3**) was less effective. *myo*-Inositol analogs with chlorine, bromine, or iodine substituents at the 5-position (compounds **11–15**) were inactive or very poor substrates. The rank order of analogs by the inhibition assay was similar to that found by measurement of the CMP release, with the exception of the 5-difluoro-compound **16**, which was an effective inhibitor but inactive as a substrate (Table 1).

Molecular modeling techniques were used to more quantitatively evaluate the role of steric factors, particularly as stereochemistry at the 5-*myo* position is concerned, in determining substrate activity for PI synthetase. Using the "MVOLUME" routine[1] of SYBYL, molecular volume differences were calculated (Table 1) for the 5-modified inositols. The results show (Table 1) that those compounds (i.e., **2**, **3**, and **4**) with volumes smaller than *myo*-inositol (compound **1**) exhibit appreciable substrate activity with PI synthetase, as measured by either inhibition of *myo*-inositol uptake or CMP formation. These volumes are shown graphically in Fig. 3, a–c. (Note: projections are numbered counterclockwise, beginning at the *lower right* carbon.) The grids, which represent differences in volumes [*(−)* indicates smaller volume than *1*], show that both the 5-deoxy (compound **4**; Fig. 3a) and the 5-deoxy-5-fluoro-*myo*-inositols (compound **2**; Fig. 3b) have voids in electron density in the region above and to the "left" of the 5-position. In the case of the 2-deoxy-2-fluoro-*neo*-inositol (**3**), the F atom at C-5 ("down") is virtually isosteric with the 5-H of compound **1** (Fig. 3c), and a significant void is created above the ring. Substitution at the 5-position with the larger halogen atoms, however, produced large positive volume differences with **1** as illustrated with the 5-chloro-5-deoxy-*myo* (**12**, Fig. 3d) and 2-chloro-2-deoxy-*neo*-inositol (**11**, Fig. 3e). The other *neo*-analogs **14** and **15** (i.e., those with 5-substituents "down," *myo* numbering) produced very pronounced positive volume differences from **1** and were invariably very poor substrates for PI synthetase (Table 1). Proceeding in a predictive fashion, a number of substituents at C-5 were modeled and evaluated. Of those as yet unsynthesized compounds modeled, which include the *myo*-5-C-methyl (volume difference = 3.1 Å^3), —SH (4.0), —CN (5.4), and -I (9), all but the 5-NH_2 compound showed volume differences that are likely too large for acceptable substrate activity with PI synthetase. The modeled 5-amino-5-deoxy-*myo*-inositol shows a volume difference of only +1 Å^3 (Fig. 3f), possibly indicating its need for synthesis and evaluation as a substrate for PI synthetase.

A previous study of the specificity of guinea pig brain PI synthetase showed that none of the eight diastereomers of *myo*-inositol can serve as alternative substrates (11). Our results, together with this earlier study, indicate that PI synthetase has stringent requirements for the cyclitol substrate. The results with the 5-modified compounds (Table 1), however, show that modification, within limits, at the 5-position can be made with retention of substrate activity. This encouraged further inves-

[1] Volume difference calculations in SYBYL reflect the electron density differences in Å^3 between a given analog and the parent **1**. For volumes smaller than **1**, **1** − (analog) routines were used; these negative values appear "<>". For volumes larger than **1**, (analog) − **1** routines were used. All structures were minimized using a combination of "MAXIMIN" and "RINGSEARCH" techniques before volume calculations were made.

TABLE 1

myo-Inositol analogs as inhibitors and substrates for PI synthetase

Each compound was tested at 5 mM in the assays described under Materials and Methods. The structures are given in Fig. 2. Refer to specific footnotes below regarding nomenclature. All compounds are cross-referenced to numbering as *myo*-inositol analogs.

	Compound [*Chem. Abstr.* Reg. No.]	Inhibition[a]	CMP Formation[b]	Difference molecular volume[c]
		%	% of *myo*-inositol	Å^3
1	*myo*-Inositol [87-89-8]	68	100	0
2	5-Deoxy-5-fluoro-*myo*-inositol	30	26 ± 4	⟨3.3⟩
3	2-Deoxy-2-fluoro-*neo*-inositol[d]	15	16 ± 2	⟨3.0⟩
4	5-Deoxy-*myo*-inositol [26671-58-9]	22	15 ± 4	⟨3.0⟩
5	2-Amino-2-deoxy-*neo*-inositol [488-53-9][e]	<10	7 ± 1	
6	2-Deoxy-DL-*epi*-inositol [488-74-4][f]	13	<5	
7	2-Deoxy-2-fluoro-DL-*epi*-inositol[g]	<10	<5	
8	2-Deoxy-*myo*-inositol [527-42-4]	<10	<5	
9	1-Deoxy-1-fluoro-*scyllo*-inositol [85716-59-2][h]	<10	<5	
10	1-Deoxy-DL-*chiro*-inositol [81623-43-0][i]	12	6 ± 1	
11	2-Chloro-2-deoxy-*neo*-inositol[d]	<10	10 ± 1	9.1
12	5-Chloro-5-deoxy-*myo*-inositol	<10	5 ± 2	4.0
13	5-Bromo-5-deoxy-*myo*-inositol	<10	<5	4.5
14	2-Bromo-2-deoxy-*neo*-inositol[d]	<10	5 ± 2	10.2
15	2-Deoxy-2-iodo-*neo*-inositol[d]	<10	<5	15.4
16	5-Deoxy-5,5-difluoro-*myo*-inositol[j]	75 ± 1	<5	⟨2.9⟩

[a] Inhibition of incorporation of 0.04 mM *myo*-[^{3}H]inositol (specific activity, 10 Ci/mol) into lipid; the control rate was 1.8 nmol/10 min. *myo*-Inositol (1) (5 mM unlabeled) is shown for comparison. Average of duplicate determinations, which agreed within 10%, are presented.

[b] CMP formation for the control (5 mM *myo*-inositol) was 38 nmol/10 min. The mean ± standard deviation of four independent determinations is shown.

[c] Difference volumes (in Å^3) as calculated using SYBYL's "MVOLUME" command. The values reflect a computer-generated electron density difference between *myo*-inositol and a given analog. Negative values (i.e., where *myo*-inositol has the larger volume) are indicated ⟨ ⟩.

[d] Considered as the 5-deoxy-5-halo-*neo*— analog.

[e] Considered as the 5-amino-5-deoxy-*neo*— analog. Prepared according to Allen (19). *Chemical Abstracts* has named this as 1-amino-1-deoxy-*neo*-inositol.

[f] Considered as the (±)-4-deoxy-*myo*— analog.

[g] Considered as the (±)-4-deoxy-4-fluoro-*myo*— analog.

[h] Considered as the 2-deoxy-2-fluoro-*myo*— analog. The compound was prepared according to the procedure reported by Yang *et al.* (20).

[i] Considered either the 1-deoxy-*myo*— or the 3-deoxy-*myo*— analog.

[j] See Ref. 8.

tigation of these compounds as precursors of cellular phospholipids.

Synthesis of 5-deoxy-*myo*-[^{3}H]inositol by the recently published method of Auchus *et al.* (6) and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol as described under Materials and Methods allowed direct measurement of incorporation of these analogs into phospholipid. Incubation of 1 mM 5-deoxy-*myo*-[^{3}H]inositol under the conditions of the PI synthetase assay as described under Materials and Methods produced a rate of incorporation of 0.16 nmol/min versus 0.91 nmol/min for 1.0 mM *myo*-[^{3}H]inositol under the same conditions. The incorporation of 5-deoxy-*myo*-[5-^{3}H]inositol into phospholipid was directly proportional to time over the period 0 to 30 min, and the rate was directly proportional to the amount of enzyme added. The incorporation of 0.1 mM 5-deoxy-*myo*-[5-^{3}H]inositol **4** was absolutely dependent on the presence of CDP-diglyceride in the incubation mixture and was reduced 81% and >95% by 5 mM and 25 mM *myo*-inositol, respectively. Similarly the incorporation of 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol into lipid by rat brain microsomes was absolutely dependent on addition of CDP-diglyceride and inhibited by *myo*-inositol. These results provide strong evidence that 5-deoxy-*myo*-inositol and 5-deoxy-5-fluoro-*myo*-inositol can serve as a substrate for PI synthetase although at a lower rate than *myo*-inositol.

The inhibition of incorporation of analog inositols by *myo*-inositol together with the relatively poorer incorporation of the analog suggests that competition may limit the extent of analog incorporation *in vivo*. The plasma concentrations of *myo*-inositol are in the range of 40–80 μM (15, 16). Thus, considerably higher concentrations of analog may be necessary for activity *in vivo*.

For biological activity it is essential for these analogs to enter cells. Auchus *et al.* (6) recently reported that 5-deoxy-*myo*-inositol was not accumulated in the cytoplasm of mouse fibrosarcoma cells, suggesting that these cells have a specific inositol transport system capable of excluding this analog. To examine this point further, we measured the uptake of *myo*-inositol and 5-deoxy-*myo*-inositol by L1210 murine leukemia cells (Fig. 4). Both compounds reached an intracellular concentration by 1 hr of incubation approximately equal to the extracellular concentrations and were incorporated into cell lipids in a time-dependent manner. The incorporation of 25 μM *myo*-[^{3}H]inositol into cell lipid was more rapid than that of 25 μM 5-deoxy-*myo*-[^{3}H]inositol (Fig. 4). Similar incubations of 5-fluoro-5-deoxy-*myo*-[^{3}H]inositol with L1210 cells were performed and indicated that this analog also enters cells and is incorporated into lipid. At 1 hr 5-fluoro-5-deoxy-*myo*-[^{3}H]inositol incubated with L1210 cells as for Fig. 4 obtained intracellular concentrations similar to that of *myo*-[^{3}H]inositol and incorporation into lipid was 43 ± 18% (mean ± SD, three experiments) of that with *myo*-[^{3}H]inositol (data not shown). Further investigation of the transport of *myo*-inositol by L1210 cells suggest that uptake may be by simple diffusion or a low affinity carrier.[2]

As an initial characterization of the radiolabeled lipid formed from 5-deoxy-*myo*-[5-^{3}H]inositol, the products formed on incubations with rat brain PI synthetase or L1210 cells in cultures were chromatographed in a thin layer system for the separation of phospholipids (Fig. 5). In both conditions the product formed from 5-deoxy-*myo*-[5-^{3}H]inositol had a mobility slightly greater than that of PI. Similarly when these samples were chromatographed with a second solvent system ($CHCl_3$/methanol/acetic acid/0.9% NaCl, 50:25:8:2.5; Ref. 17) the lipid products of two independent incubations from both brain extracts and L1210 cells chromatographed as a single component with a somewhat faster mobility than PI.

To further characterize the lipid formed from 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol we incubated brain microsomes, CDP-diglyceride (5 mM), and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol (5 mM, 0.8 mCi/mmol) at 37° for 2 h, and extracted the radiola-

[2] Nancy Malinowski and James Moyer, unpublished results.

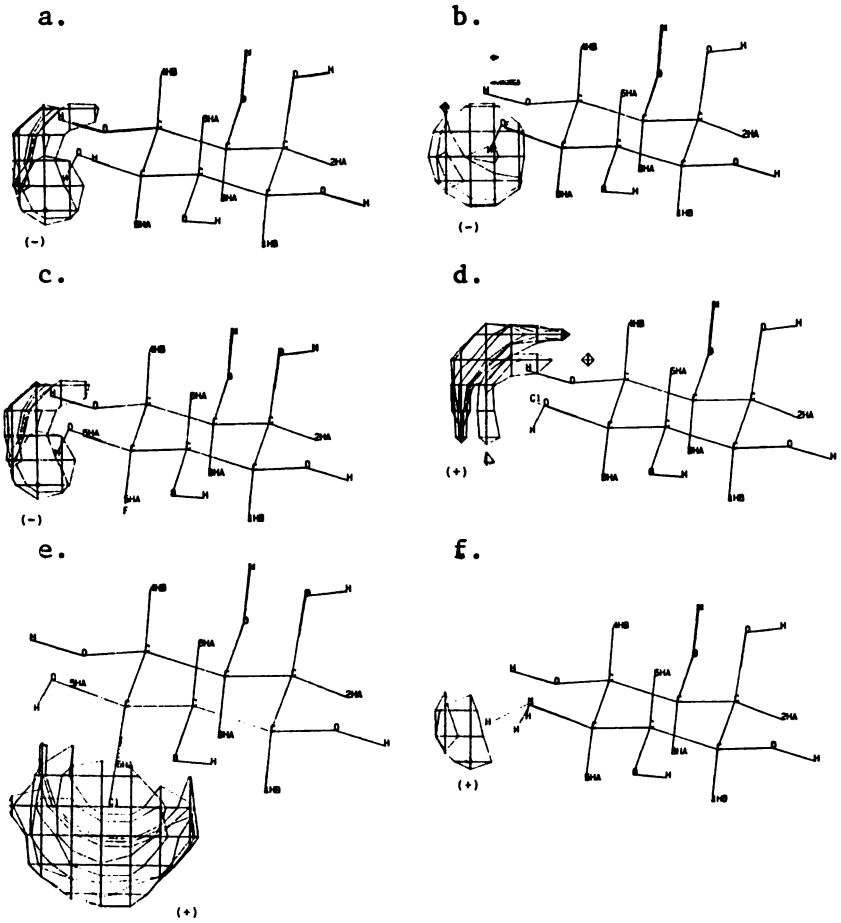

Fig. 3. Computed molecular volume differences for selected analogs. a. 1 – 4; b. 1 – 2; c. 1 – 3; d. 12 – 1; e. 11 – 1; f. 5-amino-5-deoxy-*myo*-inositol – 1. See Discussion for details of the calculations.

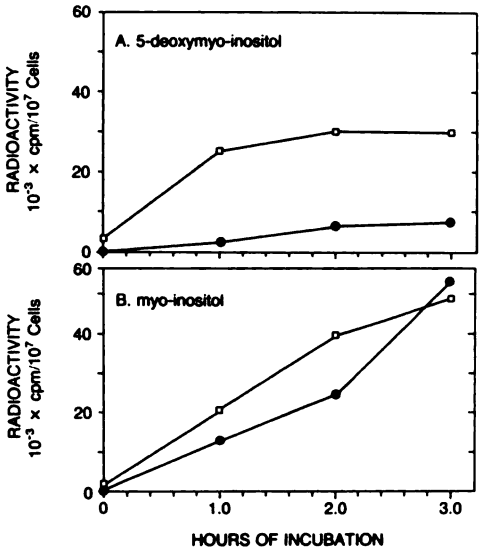

Fig. 4. Comparison of uptake and metabolism of 5-deoxy-*myo*-inositol and *myo*-inositol by L1210 cells. A suspension of L1210 cells (3 ml, 2 × 10^7 cells/ml) in Fischer's medium (GIBCO, Grand Island, NY) was incubated with 4 μCi/ml, 25 μM, *myo*-[^{3}H]inositol or 5-deoxy-*myo*-[5-^{3}H] inositol (Moravek) at 37°. At the indicated times a 0.5-ml aliquot was removed and water-soluble (□) and lipid-soluble (●) radioactivity determined as described under Materials and Methods. These values are not corrected for the radiolabel contained in the extracellular water entrapped in the cell pellet, which is 1.4 × 10^3 cpm/10^7 cells. This result is representative of two independent experiments.

beled lipid product as described for Table 1. Two chemical transformations were performed to characterize the radiolabeled product. First, deacylation under mild alkaline hydrolysis (14) converted >95% of the radiolabeled lipid to water-soluble form. This is consistent with the presence of ester linkages to fatty acids. Secondly, this radiolabeled lipid was incubated with phospholipase D as described under Materials and Methods. This procedure converted 65% of the radiolabel to a water-soluble form in 1 hr and 81 ± 10% in 3 hr (three experiments). No water-soluble product was released in the absence of enzyme or the presence of enzyme heated at 95° for 10 min before addition. The radiolabeled product of this enzyme-catylyzed hydrolysis was identified as 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol by identical chromatographic behavior in four chromatographic systems as described under Materials and Methods. This result shows that the radiolabeled lipid contains 5-deoxy-5-fluoro-*myo*-inositol in phosphoester linkage. Further characterization of this fraudulent phospholipid, particularly determination of the site of the phosphodiester bond to the cyclitol, must await a large-scale preparation and studies by NMR spectroscopy and by mass spectrometry.

The incorporation of 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol into cellular phospholipid was compared with that of *myo*-[^{3}H] inositol by chromatography of lipid extracts deacylated by the method of Hawkins *et al.* (14) as shown in Fig. 6. *myo*-Inositol was incorporated into the PI, PIP, and PIP_2 with PI constituting >90% of the total, as expected from studies of other cell types. 5-Deoxy-5-fluoro-*myo*-inositol was incorporated into

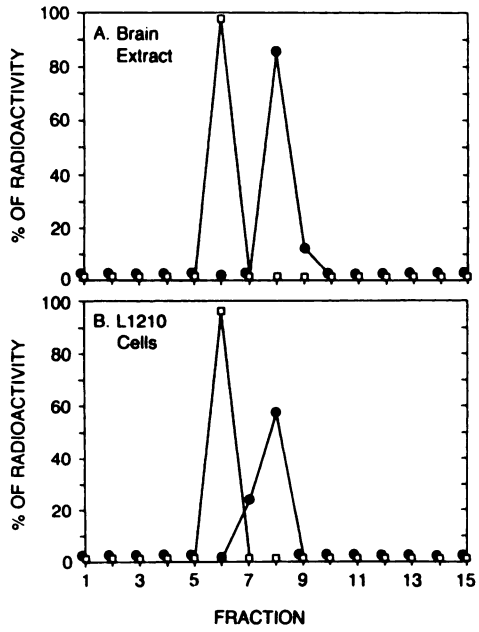

Fig. 5. Chromatography of the lipid formed from 5-deoxy-*myo*-inositol. Aliquots of the products formed from incubation of *myo*-[^{3}H]inositol (○) or 5-deoxy-*myo*-[5-^{3}H]inositol (●) with solubilized brain microsomes and CDP-diglyceride (A) or with intact L1210 cells (B) as described for Fig. 1 were chromatographed by the method of Shukla and Hanahan (12). One-cm strips of each plate from the origin (fraction 1) to the solvent front (fraction 15) were scraped into 10 ml of aqueous counting scintillant and radioactivity was determined by scintillation counting. Results are representative of two independent experiments.

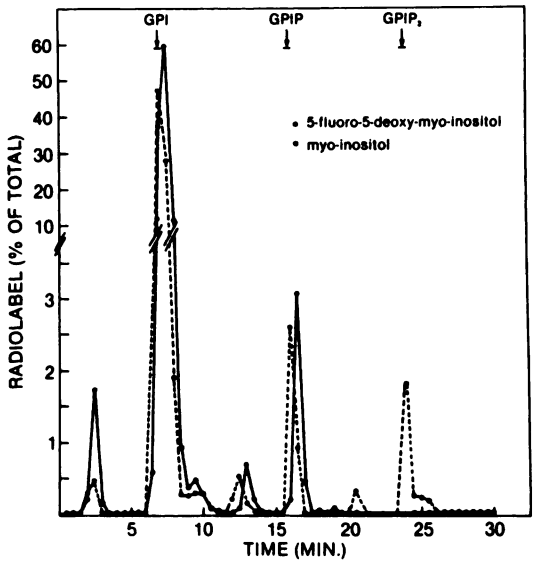

Fig. 6. Separation of deacylated phospholipids from L1210 cells incubated with *myo*-[^{3}H]inositol and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol. L1210 cells were grown in inositol-free Fischer's medium with 10% dialysed horse serum, 200 μg/ml streptomycin, and 20 U/ml penicillin. Cells were incubated with 1 μCi/ml 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol or *myo*-[^{3}H]inositol for 48 hr. Phospholipids were extracted, deacylated, and chromatographed as described under Materials and Methods. The chromatogram shown is representative of four independent incubations and is presented as percentage of total radiolabel chromatographed. Total radiolabel for *myo*-[^{3}H]inositol (– – –) and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol (——) was 2.1 × 10^5 cpm and 0.35 × 10^5 cpm, respectively. The elution positions of glycerophosphoinositol (*GPI*), glycerophosphoinositol-4-phosphate (*GPIP*), and glycerolphosphoinositol-4,5-diphosphate (GPIP$_2$), prepared as described (18), are indicated.

TABLE 2

Incorporation of *myo*-[^{3}H]inositol and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol into L1210 cell phospholipids

L1210 cells were grown, incubated, and chromatographed as described in the legend to Fig. 6. Data shown are the mean of four determinations ± standard error.

	PI	PIP	PIP$_2$
		pmol/10^6 cells	
5-deoxy-5-fluoro-*myo*-[^{3}H] inositol	46 ± 2	1.0 ± 0.1	ND[a]
myo-[^{3}H]inositol	250 ± 14	10. ± 1.4	4.5 ± 0.8

[a] ND, not detected, <0.06 pmol.

species with chromatographic properties similar to PI and PIP, but no more phosphorylated species corresponding to PIP$_2$ was observed (Fig. 6). Thus, phosphorylation by PIP kinase did not occur *in vivo*, as expected, because 5-deoxy-5-fluoro-*myo*-inositol lacks the necessary hydroxyl group at the 5-position. Comparison of the relative incorporation of *myo*-[^{3}H]inositol and 5-deoxy-5-fluoro-*myo*-[^{3}H]inositol (Table 2) indicated the latter compound was incorporated into phospholipid by L1210 cells at about 15% the efficiency of *myo*-inositol.

In order to study the effects of 5-deoxy-5-fluoro-*myo*-inositol on cellular phospholipids, L1210 cells were incubated with *myo*-[^{3}H]inositol (4 μCi/ml, 0.2 μM) for 48 hr in the presence and absence of unlabeled 5-deoxy-5-fluoro-*myo*-inositol (2 mM). Phospholipids from these cells were then extracted and chromatographed as described in the legend for Fig. 6. In the cells incubated with 2 mM 5-deoxy-5-fluoro-*myo*-inositol, *myo*-inositol incorporation into PI, PIP, and PIP$_2$ decreased 62 ± 6%, 56 ± 3%, and 37 ± 2%, respectively (mean ± SD for three independent incubations).

The present results, although confirming the high specificity of PI synthetase for the cyclitols, show that conservative modification at the 5-position yields analogs that are substrates. Because such analogs cannot be converted to the corresponding analog PIP$_2$, as they lack the site for phosphorylation, they may disrupt cell signaling processes mediated by PIP$_2$ hydrolysis if sufficient amounts can be incorporated into cellular phospholipid. Further investigation of these analogs, particularly 5-deoxy-5-fluoro-*myo*-inositol (compound **2**), the most active substrate, should establish the feasibility of this approach. A possible extension of this work would be the synthesis of the phosphatidyl analogs incorporating these fraudulent inositols.

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