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Synthesis of 1-Halo Analogs of DL-Glycerol 3-Phosphate and Their Effects on Glycerol Phosphate Dehydrogenase*

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ABSTRACT: 1-Fluoro, 1-chloro, and 1-bromo analogs of DL-glycerol-3-P have been synthesized from the corresponding epihalohydrins with either phosphoric acid or dibenzylphosphoric acid. The 1-halodeoxyglycerol phosphates so obtained were demonstrated to be analogs of glycerol-3-P rather than glycerol-2-P by nuclear magnetic resonance spectra and by synthesis from 1-halodeoxyglycerol and dibenzylphosphorochloridate. 1-Fluorodeoxy-DL-glycerol-3-P is a substrate for rabbit muscle nicotinamide–adenine dinucleotide (NAD)-linked glycerol-3-P-dehydrogenase with an K_m (app) of 8 mM at pH 7.5 and 6.5 mM at pH 9.0. 1-Chlorodeoxy- and 1-bromodeoxy-DL-glycerol-3-P do not appear to be substrates for glycerol 3-phosphate dehydrogenase unless 0.1 M hydrazine is present. All three halo analogs are substrates for calf intestine alkaline phosphatase.

None of the three halo analogs of DL-glycerol-3-P appears to be an irreversible inhibitor of NAD-linked glycerol-3-P-dehydrogenase, at either pH 7.5 or 9.0, although nonspecific organic phosphate incorporation is observed with the chloro and bromo analogs, particularly at pH 9.0. We suggest that the 1-fluoro analogs of L-glycerol-3-P or of dihydroxyacetone-3-P or derivatives of them may be useful as agents selectively toxic to cells lacking NAD-linked glycerol-3-P-dehydrogenase, which is a characteristic of many rapidly growing cancer cells.

Cytoplasmic NAD-linked glycerol-3-P-dehydrogenase is present at a very low level or is entirely absent from a wide variety of human cancer types (Delbrück *et al.*, 1959; Boxer and Shonk, 1960; Boxer and Devlin, 1961; Angeletti *et al.*, 1960; Sacktor and Dick, 1960; Foster and Taylor, 1966; Shonk and Boxer, 1964; Shonk *et al.*, 1964, 1965a,b, 1966). Instances where the enzyme level is not depressed in cancer cells are also known (Bär *et al.*, 1963; McKee *et al.*, 1968). In the cases where there is a significantly lower level of glycerol-3-P-dehydrogenase in cancer cells when compared with corresponding normal cells, it may be possible to exploit the glycerol-3-P-dehydrogenase deficiency in the selective destruction of the cancer cell. This selective destruction might be achieved by employing potentially toxic substrate analogs that could be detoxified by the action of the enzyme. 1-Fluoro analogs of either L-glycerol-3-P or of dihydroxyacetone-3-P might be useful in this selective chemotherapeutic approach. These analogs are of particular interest because the 1-fluoro analog of glycerol is already known to have a toxicity approaching that of fluoroacetate in rats (O'Brien and Peters, 1958). Dephosphorylation of the 1-fluoro analog of L-glycerol-3-P by phosphatases might be expected to produce the toxic 1-fluoro analog of glycerol in situations where the 1-fluoro-1-deoxy-L-glycerol-3-P cannot be metabolized by alternative means. One such possible alternative fate of 1-fluoro-1-deoxy-L-glycerol-3-P might be oxidation to the 1-fluoro analog of dihydroxyacetone-3-P if the fluoro analog of glycerol-3-P were a substrate for glycerol-3-P-dehydrogenase. On the other hand, the 1-fluoro analog of dihydroxyacetone-3-P might itself be toxic, and reduction to 1-fluoro-1-deoxyglycerol-3-P, if catalyzed by glycerol-3-P-dehydrogenase, might reduce the toxicity of 1-fluorodeoxy-

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dihydroxyacetone-3-P in cells which have the requisite enzyme. Thus it becomes of interest to determine whether the 1-fluoro analogs of L-glycerol-3-P and of dihydroxyacetone-3-P are substrates for glycerol-3-P-dehydrogenase.

The 1-chloro and 1-bromo analogs of the same compounds might be useful as active-site-directed irreversible labeling agents for glycerol-3-P-dehydrogenase, especially if the 1-halo analog of dihydroxyacetone-3-P were generated directly at the active site by the action of the enzyme on the 1-halo analogs of L-glycerol-3-P. 1-Halo analogs of dihydroxyacetone-3-P have already been used for active-site-labeling studies in triosephosphate isomerase (Hartman, 1968, 1970a; Coulson *et al.*, 1970) and aldolase (Hartman, 1970b).

For these and other reasons we have been interested in carrying out the synthesis and examining the biochemical and pharmacological properties of the 1-halo analogs of glycerol-3-P and of dihydroxyacetone-3-P. This paper deals with the 1-halo analogs of DL-glycerol-3-P. Work on the corresponding analogs of dihydroxyacetone-3-P, and the stereospecific synthesis and use of the separate L and D forms of 1-halo-1-deoxyglycerol-3-P will be presented later.

Materials

Enzyme. Rabbit muscle NAD-linked glycerol-3-P-dehydrogenase was obtained from Sigma Chemical Co. (St. Louis) (lots 127B-2110 and 98B-1710).

Chemicals. Dibenzylphosphoric acid, dibenzyl phosphite, and epifluorohydrin were products of Aldrich Chemical Co. (Milwaukee). More common organic reagents were obtained from Aldrich, or from Eastman Organic Chemicals (Rochester).

Radioisotopes. Carrier-free [^{32}P]phosphoric acid in water was obtained from New England Nuclear (Boston), [*carbonyl*-^{14}C]NAD was CFA.372-1 from Amersham-Searle (Des Plaines, Ill.).

Methods

Synthesis of DL-*1-Halo-1-deoxyglycerol 3-Phosphates*

Synthetic routes are presented in Figure 1.

1. From Epihalohydrins (I) and Excess Phosphoric Acid. The procedure employed is modified from one first followed by Zetzche and Aeschlimann (1926). Phosphoric acid (31.2 ml of 86% 0.5 mole of H_3PO_4) was placed in a flask in a cold water bath. To this was added slowly and with stirring 0.2 mole of epichloro-, epibromo-, or epifluorohydrin. Temperature was maintained below 20°. After addition of epihalohydrin, the reaction was stirred overnight. Completeness of reaction was tested by analysis for P_i. The reaction mixture was poured into 600 ml of cold water and the pH was brought to 8.5 by slow addition of cyclohexylamine (approximately 90–95 ml), the temperature being maintained at 10°. A voluminous white precipitate of dicyclohexylammonium phosphate formed. The suspension was allowed to stand at 4° overnight, then filtered. The precipitate was washed with 100 ml of cold 50% ethanol and the washings were retained. The filtrate was brought to 50% ethanol (v/v) and allowed to stand overnight at 4°. Additional dicyclohexylammonium phosphate precipitated and was removed by filtration. The 50% ethanol filtrate and the washings retained from the first filtration were combined and the solvent was removed on a rotary evaporator. The solid residue was dried *in vacuo* against phosphorus pentoxide. The crude product weighing 60–70 g was recrystallized from 2 l. of boiling dioxane, with the dioxane brought to boiling before addition of the product to minimize halide loss. The filtrate was maintained just above the freezing point of dioxane for several days. In the case of the reaction with epichlorohydrin, the recrystallized product was a white powder weighing 34 g (44% yield) with a melting point of 152–152.5°. The molecular weight calculated on the basis of organic chloride was 385, and based on organic phosphate was 389 (theoretical for DL-1-chloro-1-deoxyglycerol 3-phosphate dicyclohexylammonium salt (IV) is 387). The reaction with epibromohydrin (I) gave 45 g (52% yield) with a melting point of 124–127°. The product contained 1 mmole of organic bromide/443 mg (theoretical for DL-1-bromo-1-deoxyglycerol 3-phosphate dicyclohexylammonium salt (IV) is 432 mg/mmole). The reaction with epifluorohydrin (I) gave 30 g (40% yield) of product melting at 171–173°.

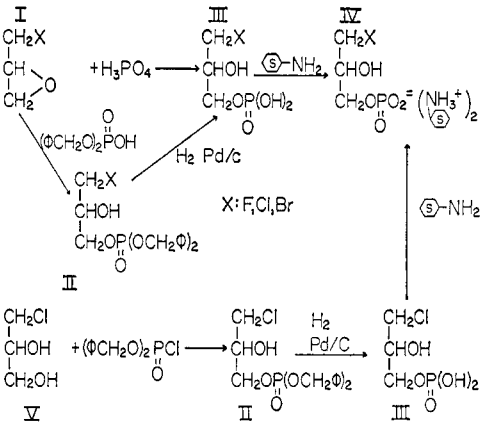

FIGURE 1: Synthesis of 1-halodeoxy-DL-glycerol 3-phosphates from epihalohydrins with phosphoric acid or dibenzylphosphoric acid, and from 1-chlorodeoxy-DL-glycerol with dibenzyl phosphorochloridate.

Anal. Calcd for $C_{15}H_{34}F_1N_2O_5P_1$: C, 48.4; H, 9.22; F, 5.10; N, 7.52; P, 8.31. Found: C, 47.9; H, 9.09; F, 4.92; N, 7.34; P, 8.08.

2. From Excess Epihalohydrins and Dibenzylphosphoric Acid. A. SYNTHESIS OF DIBENZYL ESTERS OF DL-1-HALO-1-DEOXYGLYCEROL-3-P. To 65 ml of redistilled epihalohydrin was added directly with stirring at room temperature 22.2 g of dibenzylphosphoric acid (80 mmoles). The course of the reaction was followed by titration in ethanol of unreacted dibenzylphosphoric acid to methyl red end point. The reaction was allowed to stir 9 hr, then the excess epihalohydrin was removed on a rotary evaporator and recovered if desired. In the reaction with epichlorohydrin the residue after evaporation was approximately 30 g of a yellow oil. The product was purified by chromatography on a silica gel column using benzene for packing and eluting. A column prepared from 5 g of silica gel/g of crude product was sufficient to effect purification. The product obtained after column chromatography was 23 g (78% yield based on dibenzylphosphoric acid used). Since it is known that hydroxyl groups in positions α to dibenzyl phosphate esters can catalyze hydrolysis of one

of the benzyl ester groups and lead to 2,3-cyclic phosphate formation (Brown *et al.*, 1955; Cramer *et al.*, 1957; Baddiley *et al.*, 1958; Moffatt and Khorana, 1961), the products were kept at −20°, and used without prolonged storage. Analysis of the product obtained from reaction of epichlorohydrin with dibenzylphosphoric acid showed 390 mg/mmole of organic chloride, and 382 mg/mmole of organic phosphate (theoretical for DL-1-chloro-1-deoxylglycerol 3-phosphate dibenzyl ester (II) is 370 mg/mmole). Further characterization is presented in the Results.

In the reaction of epibromohydrin with dibenzylphosphoric acid the yield of DL-1-bromo-1-deoxyglycerol 3-phosphate dibenzyl ester was 82% after silica gel chromatography.

B. HYDROGENATION OF THE DIBENZYL ESTER OF DL-1-CHLORO-1-DEOXYGLYCEROL-3-P. Crude dibenzyl 1-chloro-1-deoxyglycerol-3-P (6.1 g; 17 mmoles) that had not been purified by silica gel chromatography was subjected to catalytic hydrogenation at atmospheric pressure. Hydrogenation was carried out in 30 ml of dioxane with 250 mg of 10% palladium on charcoal. Theoretical hydrogen uptake was complete after 10 hr. The filtered hydrogenation mixture gave a negative test for inorganic chloride indicating that the chloro group was not removed by catalytic hydrogenation. Titration showed the generation of 39 mequiv of titratable protons from 17 mmoles of dibenzyl ester. The volume of the mixture was brought to 200 ml with dioxane, and 39 mmoles (3.87 g) of cyclohexylamine was added. The precipitate weighing 6.0 g was recrystallized three times from boiling dioxane as already described. The product obtained was 1.2 g (yield 20%), mp 149–151. Organic halide analysis showed 375 mg/mmole of halide (theoretical 387). The product was compared by nuclear magnetic resonance and paper chromatography to chlorodeoxyglycerol-P obtained from epichlorohydrin and phosphoric acid (see Results).

C. HYDROGENATION OF THE DIBENZYL ESTER OF DL-1-BROMO-1-DEOXYGLYCEROL-3-P. The bromo compound (25 g; 60 mmoles) purified by chromatography on silica gel was hydrogenated at low pressure on a Paar apparatus using 10% palladium on charcoal in dioxane. Hydrogenation was ended after an uptake of 2 moles of hydrogen/mole of dibenzyl ester. No evidence of inorganic bromide was detected in the hydrogenation mixture. The filtered hydrogenation product contained 107 mequiv of titratable protons. Cyclohexylamine (103 mmoles) was added to the cooled hydrogenation mixture (slightly less than theoretical cyclohexylamine was used to prevent bromide loss). After recrystallization from dioxane as described earlier, 18 g (42 mmoles) of dicyclohexylammonium 1-bromodeoxy-DL-glycerol-3-P was obtained (mp 122–124°).

Due to the limiting amount of cyclohexylamine used, this product contained some monocyclohexylammonium salt as shown by nuclear magnetic resonance and paper chromatography. The nuclear magnetic resonance spectrum of the disodium salt prepared from the barium salt showed the absence of 2,3-propanediol-3-P in the preparation, thus establishing that hydrogenation did not remove the 1-bromo group. Analysis of the barium salt gave a ratio of Br to P of 1.02.

3. From DL-1-Chloro-1-deoxyglycerol (V) and Dibenzyl Phosphorochloridate. 1-Chloro-2,3-propanediol (1-chloro-1-deoxyglycerol) (V) was obtained from Aldrich and redistilled *in vacuo* with a boiling range of 77–79° (0.23 mm). The structure was confirmed by nuclear magnetic resonance. Dibenzyl phosphorochloridate was prepared from dibenzyl phosphite and *N*-chlorosuccinimide by the method of Atherton (1957) and the identity was confirmed by nuclear magnetic resonance.

To a solution of 2.75 g (25 mmoles) of 1-chloro-1-deoxyglycerol (V) in 5 ml of anhydrous pyridine was added over a period of 5 min with stirring 7.42 g (25 mmoles) of dibenzyl phosphorochloridate. The reaction was run in an ice bath, then left in a cold room overnight. Unreacted dibenzyl phosphorochloridate was destroyed by addition of a few drops of water. Pyridine was removed on a rotary evaporator over a 10-min period. The residual syrup was taken up in 125 ml of benzene and extracted successively with 60-ml portions of the following solvents all at 4°: water, 1 N HCl, water, 1 M potassium bicarbonate, and water. The benzene layer was dried over sodium sulfate and the benzene was removed by rotary evaporation. The product weighing 4.2 g (45% yield) was characterized by nuclear magnetic resonance and compared with the dibenzyl ester of DL-1-chloro-1-deoxyglycerol-3-P (II) synthesized from epichlorohydrin (see Results).

The dibenzyl ester from 1-chlorodeoxyglycerol was debenzylated by hydrogenation with palladium on charcoal in ethanol. The dibenzyl ester (3.3 g; 8.5 mmoles) in 200 ml of anhydrous ethanol and with 0.5 g of 10% palladium on charcoal took up the theoretical volume of hydrogen in a Paar apparatus at 25 psi in 30 min. After removal of the catalyst, the amount of cyclohexylamine required to exactly neutralize the acidic protons on the 1-chloro-1-deoxy-DL-glycerol-3-phosphoric acid (III) was determined by titration of an aliquot with potassium hydroxide. The equivalent amount of cyclohexylamine was added to the cold ethanolic solution of the phosphoric acid ester, and the ethanol was removed on a rotary evaporator. The product obtained weighed 2.3 g (70% yield in the hydrogenation) (mp 149–153°). Mixture melting point was 150–151° with 1-chloro-1-deoxy-DL-glycerol-3-P dicyclohexylammonium salt prepared as described in section 1 from epichlorohydrin and phosphoric acid and melting at 152–152.5°. Phosphate determination showed 362 mg/mmole of organic phosphate (theoretical for 1-chloro-1-deoxy-DL-glycerol-3-P dicyclohexylammonium salt: 387). The product was also compared by nuclear magnetic resonance spectroscopy to that obtained from epichlorohydrin (see Results).

Preparation of Lithium, Sodium, and Potassium Salts from Dicyclohexylammonium Salts

Dowex 50 in the acid form was employed as a column for the generation of 1-halodeoxy-DL-glycerol-3-phosphoric acids from the dicyclohexylammonium salts. Use of the resin in batch form was found to leave a substantial amount of cyclohexylammonium ion associated with the compounds. The free acid was kept at 10° and brought to pH 7.5 by addition of the appropriate aqueous base. The approximate pK's for 1-chlorodeoxyglycerol-3-P were 1.85 and 6.35 at 10°. The crystalline salts were obtained by lyophilization.

Halodeoxyglycerol-3-P produced the 2,3-cyclic phosphate if permitted to remain in the acid form for any length of time. The acid-catalyzed cyclization was circumvented when desired by precipitation of the halodeoxyglycerol-3-P's as barium salts using aqueous barium chloride followed by treatment with ethanol and acetone. The precipitated

barium salts were filtered, dried, weighed, and converted into the desired salt by the addition of the appropriate sulfate. Cation-exchange columns equilibrated directly with the desired cation were not tried because of possible increased rate of halide loss on the column when the salts were in slightly alkaline aqueous medium.

Analysis for Organic and Inorganic Chloride and Bromide

Quantitative release of organic chloro and bromo groups was achieved by refluxing for 45 min in 0.5 N alcoholic potassium hydroxide, or by heating in concentrated aqueous base for 20 min in a 100° oven. The products were acidified with nitric acid and halide determined using an Aminco-Cotlove amperometric chloride titrator.

The stability of 1-chloro- and 1-bromo-1-deoxyglycerol-3-P's in aqueous solutions at pH 7.5 and 9.0 was monitored by measurement of halide loss using the chloride titrator. The buffers were 50 mM Tris-acetate–1 mM EDTA (pH 7.5) and 50 mM pyrophosphate–1 mM EDTA (pH 9.0). Mercaptoethanol was not used in stability measurements since silver mercaptide precipates along with silver halide under the conditions of the halide titration.

Analysis for Organic and P_i

Compounds containing organic phosphate were digested using the perchloric acid method of Allen (1940). Samples of about 10 μmoles were digested by heating at 200° on a sand bath in 2 ml of 70% perchloric acid until the digest appeared to be completely in solution (about 45 min). After cooling, the volume was brought to 10 ml with water and aliquots of 1 ml or less were analyzed for P_i by the method of Fiske and Subbarow (1925). We found that the presence of more than the equivalent of 0.2 ml of 70% perchloric acid in the aliquot being analyzed seriously reduced the optical density at 660 μmicrons in the Fiske–Subbarow reaction, and therefore reduced the sensitivity of the analysis. The interference by perchloric acid was eliminated in some instances by addition of aqueous potassium hydroxide to the digest. The highly insoluble potassium perchlorate was removed by centrifugation and washed with a small volume of water, and the washings were added to the supernatant for phosphate determination.

Organic Phosphate Spray Reagent

We used the method developed by Hanes and Isherwood (1949) for localization of organic phosphate on paper electropherograms and peptide maps. Perchloric acid (9 ml of 70%), concentrated HCl (1.7 ml), ammonium molybdate (2 g), and Na_2EDTA (0.2 g), in a final volume of 200 ml, were sprayed onto the papers and the papers were allowed to dry for 15 min, then heated in an oven at 100° for 3 min. Irradiation by an ultraviolet light for 5 min produced a blue color in zones containing hydrolyzed organic phosphate. The lower limit of detection was 0.1 μmole of organic phosphate. If the background color interfered it could be eliminated by exposure of the irradiated paper to the atmosphere above an open tray of concentrated ammonia.

Paper Chromatography of 1-Halodeoxyglycerol-3-P's

Whatman No. 1 chromatography paper was washed with 1 N formic acid and dried at room temperature for 2 hr. Dicyclohexylammonium salts of DL-glycerol-3-P, glycerol-2-P, and of 1-halodeoxy-DL-glycerol-3-P's were spotted (0.5 μmole/sample). The papers were developed by successive descending chromatography with 1-propanol–ethyl acetate–water (7:1:2, v/v) then air-dried and developed again using 1-butanol–formic acid–water (32:1:9, v/v). Organic phosphate compounds were localized on the dried paper by using the phosphate spray previously described.

Enzyme Assays

For assays at pH 7.5 the buffer routinely used was 50 mM triethanolamine acetate, 1 mM EDTA, and 1 mM 2-mercaptoethanol. In cases where indicated 1 mM phosphate was used in place of triethanolamine acetate. Assays at pH 9.0 were done in 10 mM pyrophosphate, 1 mM EDTA, and 1 mM 2-mercaptoethanol. In cases where indicated when analyzing for halide loss, 2-mercaptoethanol was not used. Coenzyme concentrations were 0.1 mM for NADH, or 0.3 mM for NAD^+. All assays were performed at 23–25° by monitoring absorbance at 340 mμ. Activity in the direction of glycerol phosphate oxidation proceeds at a rate considerably slower than reduction of dihydroxyacetone phosphate, especially at pH 7.5. Therefore activity in the direction of glycerol phosphate oxidation was monitored either by using 100-mm cells in the Perkin-Elmer Model 137 spectrophotometer, or by using 10-mm cells in a Model 2400 Gilford spectrophotometer with the recorder scale expanded to read 0.1-absorbance unit full scale.

Reaction with Alkaline Phosphatase

For testing DL-1-halodeoxyglycerol 3-phosphates as substrates for alkaline phosphatase, a 3-ml system was used comprised of 1 ml of 50 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 1 ml of an aqueous solution of calf intestine alkaline phosphatase (0.5 mg/ml), and 1 ml of 20 mM substrate. Aliquots were assayed for P_i at various times and compared to a blank which lacked enzyme. Hydrolysis of all the added organic phosphate was complete after 2 hr in all cases tested.

Alkaline phosphatase catalyzed dephosphorylation was employed on the preparative level by using 2 mmoles of disodium DL-1-fluorodeoxyglycerol-3-P or disodium DL-1-chlorodeoxyglycerol-3-P in 10 ml of water. To this was added 8 ml of 50 mM Tris-acetate (pH 7.5), 20 mM magnesium acetate, and 2 ml of an aqueous solution of enzyme (2 mg/ml). Complete hydrolysis on this scale took approximately 2 days.

Results

Structure of 1-Chlorodeoxyglycerol-P Synthesized from Epichlorohydrin and from 1-Chlorodeoxyglycerol. Figure 2 shows the relevant portion of the nuclear magnetic resonance spectrum (given in δ values) of the dicyclohexylammonium salt 1-chlorodeoxyglycerol-P synthesized from epichlorohydrin with either phosphoric acid (Figure 2D) or with dibenzylphosphoric acid followed by catalytic hydrogenation (Figure 2E). Shown also are the spectra of authentic disodium glycerol-3-P (Figure 2A) and disodium glycerol-2-P (Figure 2B). The latter is characterized by a complex weak absorption pattern centered at 4.1 and due to the proton on carbon-2 of glycerol-2-P. The absorption due to this proton is shifted downfield by the phosphate group and split by the protons on carbons-1 and -3 and also by the phosphate group which

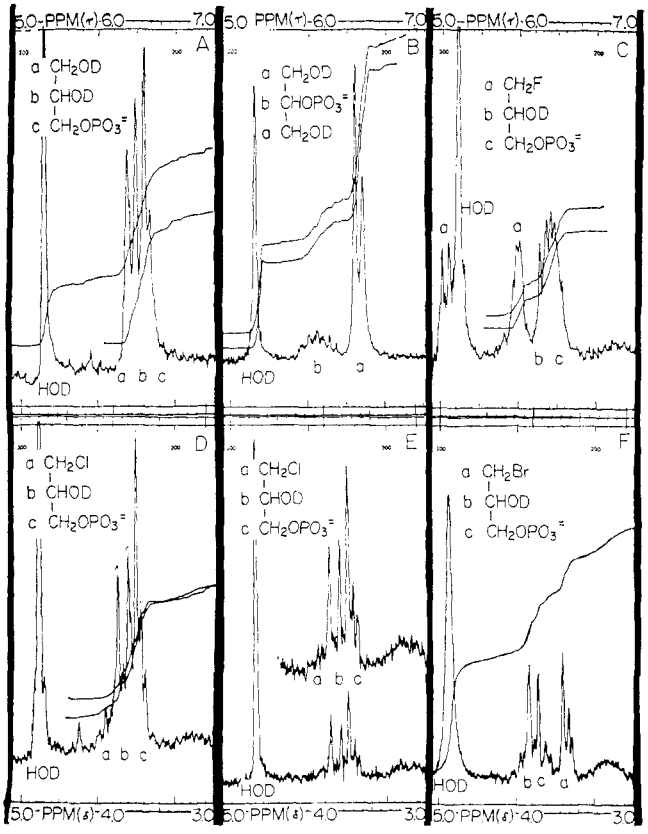

FIGURE 2: 60-MHz nuclear magnetic resonance spectra in D_2O of salts of DL-glycerol phosphates and 1-halodeoxy-DL-glycerol 3-phosphates. Spectra A and B are disodium salts of glycerol-3-P and glycerol-2-P. Spectra C and D are of disodium salts of DL-1-fluorodeoxy- and DL-1-chlorodeoxyglycerol-3-P synthesized from epihalohydrins with phosphoric acid and prepared from dicyclohexylammonium salts by batch treatment with cation-exchange resin. Broad absorption in range of 3.1 is due to some cyclohexylammonium ion not removed by ion exchange. Spectrum in E is of dicyclohexylammonium salt of DL-1-chlorodeoxyglycerol-3-P prepared from DL-1-chlorodeoxyglycerol and dibenzyl phosphorochloridate. Spectrum in F is of dicyclohexylammonium salt of DL-1-bromodeoxyglycerol-3-P prepared from epibromohydrin and dibenzylphosphoric acid and followed by hydrogenation. Broad absorption at 3.1 is due to cyclohexylammonium ion. Only the relevant portions of these spectra are shown. Additional absorption due to cyclohexylammonium ion occurred at higher field.

is on carbon-2. The spectrum of glycerol-3-P, on the other hand, does not show this absorption pattern centering at 4.1 since carbon-2 does not bear the phosphate group. The major absorption in glycerol-2-P appears as a doublet in keeping with its symmetrical structure whereas glycerol-3-P shows a complex pattern consisting of at least four absorption peaks. The 1-chlorodeoxyglycerol-P synthesized from epichlorohydrin and phosphoric acid does not show the complex weak multiplet centered at 4.1 and characteristic of glycerol-2-P. It does show the four or more major absorption peaks expected of a glycerol-3-P analog.

Definitive proof of the structure of the halodeoxyglycerol-3-P's synthesized from epihalohydrins is obtained by carrying out the synthesis beginning with 1-chlorodeoxyglycerol. Phosphorylation with equimolar dibenzyl phosphorochloridate followed by catalytic hydrogenation gives a cyclohexylammonium salt of 1-chlorodeoxyglycerol-3-P whose nuclear magnetic resonance spectrum is indistinguishable for the 1-chlorodeoxyglycerol-P obtained from epichlorohydrin. Since dibenzyl phosphorochloridate used in amounts equimolar with 1-chlorodeoxyglycerol would preferentially phosphorylate the primary alcohol (Ballou and MacDonald, 1963; Maley and Lardy, 1956; Atherton *et al.*, 1948), the product obtained from such a phosphorylation would be 1-chlorodeoxyglycerol-3-P.

Paper chromatography of the 1-chlorodeoxy-glycerol-P's obtained from epichlorohydrin by use of either phosphoric acid or dibenzyl phosphate, or from 1-chlorodeoxyglycerol by use of dibenzyl phosphorochloridate showed that the products were in all cases essentially homogeneous and identical with one another in their chromatographic mobility in mixed samples. The R_F value for dicyclohexylammonium 1-chlorodeoxy-glycerol-3-P was 0.80 and of the bromo analog was 0.77 in the double solvent system described under Methods. Under these conditions the salts of glycerol-3-P and glycerol-2-P had R_F's of 0.21 and 0.29, respectively.

Structure of 1-Fluorodeoxyglycerol-P Synthesized from Epifluorohydrin. Figure 2C shows the relevant portion of the nuclear magnetic resonance spectrum of the dicyclohexylammonium salt obtained from the reaction of epifluorohydrin with phosphoric acid. The absorptions appearing as doublets at 4.2 and 5.0 are due to the CH_2F group which is split with a coupling constant (J_{FCH_2}) of approximately 60 cycles. This splitting is consistent with spectra of other fluoro organic compounds which we have examined such as fluoroacetone, in which the coupling constant for the fluoro group and a proton on the same carbon is about 55 cycles. The complex absorption pattern obtained at 3.8 indicates that the compound is an analog of glycerol-3-P where the protons on carbon-3 are split by the neighboring proton, by the phosphate group on carbon-3, and perhaps by the fluoro group on carbon-1.

Paper chromatography of the dicyclohexylammonium salt of 1-fluorodeoxyglycerol-3-P showed that the compound was free of both glycerol-2-P and glycerol-3-P, and was also free of any contaminants with R_F values differing from the fluoro compound itself. The 1-fluoro analog of glycerol-3-P migrates behind the 1-chloro analog. The R_F value observed for the fluoro analog was 0.74, while the chloro analog had an R_F of 0.80.

Structure of 1-Bromodeoxyglycerol-P Synthesized from Epibromohydrin. Figure 2F shows the relevant portion of the nuclear magnetic resonance spectrum of the dicyclohexylammonium salt of 1-bromodeoxyglycerol-P obtained by reaction of dibenzylphosphoric acid with epibromohydrin followed by catalytic hydrogenation. The spectrum shows that the compound is an analog of glycerol-3-P and has a close similarity to the spectrum of DL-1-chlorodeoxyglycerol-3-P (Figure 2D,E). The spectrum shows the expected slight shift upfield to 3.6 of the absorption peaks due to the bromomethyl group as compared with the absorption due to the chloromethyl group in Figure 2D,E. The nuclear magnetic resonance spectrum of the sodium salt of 1-bromodeoxyglycerol-3-P showed unequivocally the absence of any 2,3-propanediol-3-P in the preparation of 1-bromodeoxyglycerol-3-P thus establishing that under the conditions of the hydrogenation the bromo group is not removed.

Structure and Stability of the Dibenzyl Esters of DL-1-Halodeoxyglycerol-3-P's. Reaction of epichlorohydrin with

dibenzylphosphoric acid produced the dibenzyl ester of DL-1-chlorodeoxyglycerol-P. Reaction of 1-chlorodeoxyglycerol with dibenzyl phosphorochloridate produced dibenzyl DL-1-chlorodeoxyglycerol-3-P. The identity of the dibenzyl esters produced by the two different routes was confirmed by nuclear magnetic resonance, thus demonstrating that epichlorohydrin and dibenzylphosphoric acid produced the ester of glycerol-3-P and not of glycerol-2-P. The synthetic routes beginning with epihalohydrins are of more general use than synthesis from the 1-halodeoxyglycerols since only chlorodeoxyglycerol is readily available commercially, whereas epifluoro-, epichloro-, and epibromohydrins are available.

The nuclear magnetic resonance spectra were also useful in determining the stability of the dibenzyl esters of the halodeoxyglycerol-3-P's. Figure 3 shows the relevant portion of the spectra of the dibenzyl ester of 1-chlorodeoxyglycerol-3-P, taken immediately after purification and again after standing in chloroform at room temperature for 2 weeks. The intramolecular cyclization and monodebenzylation of dibenzyl esters of vicinyl glycols has been reported as a significant problem in their preparation (Brown *et al.*, 1955; Cramer *et al.*, 1957; Baddiley *et al.*, 1958; Moffatt and Khorana, 1961). Such debenzylation would produce benzyl alcohol for which the hydroxymethyl group absorbs at 4.6 (Bhacca *et al.*, 1962). The appearance of an absorption at 4.6 becomes a direct measure of the extent of cyclization. Figure 3 shows that the intramolecular cyclization has proceeded to the extent of 50% after 2 weeks in chloroform at room temperature. The dibenzyl ester of 1-chlorodeoxyglycerol-3-P, which is initially practically free of absorption due to benzyl alcohol at 4.6, shows that after 2 weeks the absorption due to free benzyl alcohol is 25% of the total absorption due to both free benzyl alcohol and to that still esterified to the glycerol-3-P (the doublet at 5.0).

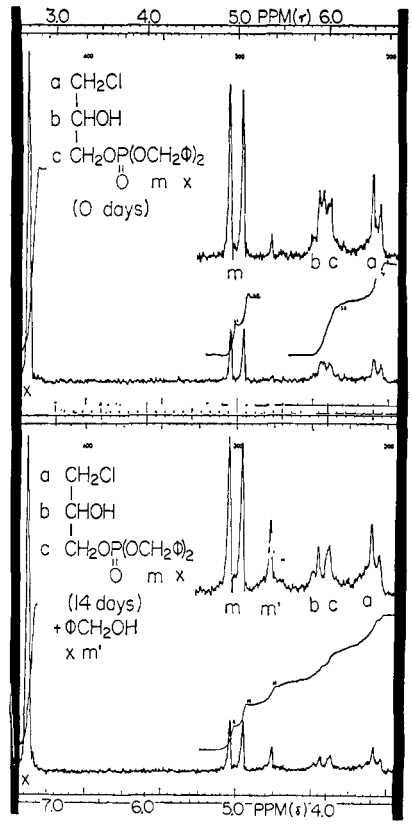

FIGURE 3: 60-MHz nuclear magnetic resonance spectra in deuteriochloroform of the dibenzyl ester of DL-1-chlorodeoxyglycerol-3-P synthesized from epichlorohydrin and dibenzylphosphoric acid. Upper spectrum: immediately after synthesis and purification on silica gel. Lower spectrum: after standing at room temperature in deuteriochloroform for 2 weeks. Only the relevant portion of the spectra are shown. No other absorption appeared in the range from 0 to 10.

*Stability to Halide Loss of 1-Chlorodeoxy-*DL*-glycerol-3-P and 1-Bromodeoxy-*DL*-glycerol-3-P in Aqueous Solutions.* Since we intended to employ the 1-halo analogs of glycerol-3-P as possible substrates and inhibitors for glycerol-3-P-dehydrogenase, it was necessary to determine the effect on the halide group of the aqueous buffers being used. In 0.1 M Tris-acetate (pH 9.0)—1 mM EDTA at 22°, 0.1 M 1-chlorodeoxy-DL-glycerol-3-P lost only 4% of the chloro groups present in 24 hr. Incubation under the same conditions but at 38° produced 30% halide loss after 24 hr.

The bromo analog at 22° lost 20% of the bromo group in 24 hr at pH 9.0. At pH 7.0 the bromide loss was 5%.

DL-*1-Fluorodeoxyglycerol-3-P as Substrate for Glycerol-3-P-dehydrogenase.* The 1-fluoro analog of DL-glycerol-3-P acted as a substrate for rabbit muscle NAD-linked glycerol-3-P dehydrogenase at pH 7.5 and 9.0. In 50 mM triethanolamine acetate (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.3 mM NAD^+, using 3×10^{-7} M enzyme, the K_m (app) estimated from Lineweaver-Burk plots with substrate concentrations ranging from 0.2 to 10 mM was 8 mM. With the same buffer and coenzyme concentration, using 2×10^{-8} M enzyme, the K_m (app) for DL-disodium glycerol-3-P was 0.2 mM. The reaction rates obtained at the substrate concentrations equal to the respective K_m's and coenzyme concentration 0.3 mM produced an increase in the optical density at 340 mμ of 0.015/min for DL-glycerol-3-P using 2×10^{-8} M enzyme and 0.0024/min for the fluoro analog using 2×10^{-7} M enzyme. The extent of oxidation of 10 mM DL-1-fluorodeoxyglycerol-3-P at pH 7.5 was about 1% at equilibrium. The initial reaction rates and K_m's (app) were not affected by the presence of 0.1 M hydrazine, which can be employed in the glycerol phosphate dehydrogenase assay system as a trapping agent for dihydroxyacetone-P.

The enzyme preparation employed in these assays was obtained commercially. In order to confirm that the observed activity was indeed due to the catalytic function of glycerol-3-P-dehydrogenase and not to some undetected contaminant in the enzyme preparation, the enzyme was purified by isoelectric focussing. Details of the purification will be presented elsewhere. The enzyme resolved into three catalytically active zones. All three forms of the enzyme catalyzed the oxidation of the fluoro analog. The K_m (app) for the fluoro analog obtained at pH 7.5 using the enzyme fraction from the center of the main enzyme zone was again 8 mM.

At pH 9.0, 10 mM pyrophosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.3 mM NAD^+, using 2×10^{-7} M enzyme, the K_m (app) for the lithium salt of DL-1-fluorodeoxyglycerol-3-P was 6.5 mM. The K_m (app) for the sodium salt of DL-glycerol-3-P under these conditions using 3×10^{-9} M enzyme was 0.2 mM. At substrate concentrations equal to the respective K_m's and at the enzyme and coenzyme concentrations

used in the K_m determinations, the fluoro analog reacted at 25% of the rate obtained for DL-glycerol-3-P. Neither the reaction rate nor the K_m (app) was affected by the presence of 0.1 M hydrazine.

DL-1-Chlorodeoxyglycerol-3-P Tested for Activity with Glycerol-3-P-dehydrogenase. The dicyclohexylammonium salt of the 1-chloro analog of DL-glycerol-3-P was examined as a possible substrate for rabbit muscle glycerol-3-P-dehydrogenase at pH 7.5 and 9.0 under conditions previously described for the fluoro analog in the absence of hydrazine. In addition, a second buffer was used at pH 7.5 consisting of 1 mM phosphate, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Reduction of NAD was observed in all three assay systems, but in all cases that observed activity appeared to be due to a 2% contaminant of glycerol-3-P in the preparation of the chloro analog and possibly to the *in situ* generation of glycerol-3-P from the chloro analog under the assay conditions. For example, the reaction rate at pH 7.5 using 3×10^{-9} M enzyme did not remain linear for more than 1 min and stopped altogether after 8 min even though equilibrium was not reached using the fluoro analog under these conditions for a much longer time. With 3×10^{-8} M enzyme the reaction with the chloro analog was complete after 4 min, and with 3×10^{-7} M enzyme, after 30 sec. The observed activity at various enzyme concentrations with 0.2 mM chloro analog was identical with that obtained when we used 5×10^{-6} M DL-glycerol-3-P dicyclohexylammonium salt directly as substrate.

Cyclohexylamine did not affect reaction rates at concentrations from 0.1 to 2 mM. The K_m (app) obtained in triethanolamine acetate buffer at pH 7.5 for dicyclohexylammonium DL-glycerol-3-P was 0.17 mM, which is the same K_m (app) obtained under these conditions for the disodium salt.

At pH 9.0 in pyrophosphate buffer (10 mM)–1 mM EDTA (no 2-mercaptoethanol), a mixture of 10 mM NAD^+ and 10 mM chloro analog with 10^{-6} M enzyme did not show enzyme-catalyzed halide loss when compared with controls without enzyme or without NAD^+. Identical results were obtained in triethanolamine acetate (50 mM) (pH 7.5)–1 mM EDTA. These results indicate that the chloro analog is not a substrate for the enzyme since the 1-chloro analog of dihydroxyacetone-3-P that would be formed should lose chloride in an aqueous environment at a faster rate than does 1-chlorodeoxyglycerol-3-P.

When buffers at pH 7.5 or 9.0 containing 0.1 M hydrazine were used for assay of the chloro analog, prolonged linear reaction rates were obtained. With 3×10^{-7} M enzyme and 0.2 mM chloro analog at pH 7.5, the change in optical density was 0.010/min and continued linearly for 15 min. With 1 mM chloro analog the initial rate was 0.074/min. At pH 9.0 the corresponding initial rates were 0.005 and 0.032 per min. The observed activity was not due to any affects of hydrazine on NAD^+ in the presence or absence of enzyme. The presence of the chloro analog was required.

DL-1-Bromodeoxyglycerol-3-P Tested for Activity with Glycerol-3-P-dehydrogenase. The bromo analog gave results similar to those obtained with the chloro analog when assayed at pH 7.5 or 9.0 in the absence of hydrazine. The observed activity appeared to arise from contaminating DL-glycerol-3-P in the preparation of the analog, or from the generation *in situ* of DL-glycerol-3-P from the bromo analog. With 1 mM bromo analog in the pH 7.5 assay system containing 0.1 M hydrazine, a sustained linear reaction rate with an increase in optical density of 0.040/min was obtained. This rate was about 10-fold faster than that obtained under the same conditions in the absence of hydrazine. At pH 9.0 the initial rate obtained was 0.028/min in the presence or absence of hydrazine.

1-Fluorodeoxy- and 1-Chlorodeoxy-DL-glycerol-3-P as Reversible Inhibitors of Glycerol-3-P-dehydrogenase. Both the fluoro and chloro analogs were weak reversible inhibitors under assay conditions at pH 7.5 and 9.0. Initial rate of oxidation of DL-glycerol-3-P at 0.2 mM was inhibited by 37% in the presence of 5 mM 1-fluorodeoxyglycerol-3-P, when assayed at pH 9.0. The per cent inhibition was somewhat less at pH 7.5. In these assays, enzyme was added last to reduce any effects due to time-dependent irreversible inhibition. In the presence of 2.5 mM chloro analog the initial rate of oxidation of 0.2 mM DL-glycerol-3-P was inhibited 40% at pH 7.5 and 15% at pH 9.0. This inhibition by the fluoro and chloro analogs of glycerol-3-P could be overcome by increasing the concentraton of glycerol-3-P, indicating that the inhibition was reversiible and probably competitive.

Attempted Irreversible Inhibition on Glycerol-3-P-dehydrogenase with 1-Chloro and 1-Bromo Analogs of DL-Glycerol-3-P. The enzyme was dialyzed against 10 mM triethanolamine acetate (pH 7.5)–1 mM EDTA, centrifuged to remove any denatured protein, assayed, and then added to five different test tubes in 3-mg portions. The enzyme was incubated at either pH 7.0 or 9.0 at room temperature in a final reaction volume of 3 ml. The buffer for the pH 7.0 incubations was 10 mM triethanolamine acetate–1 mM EDTA; for the pH 9.0 incubations the buffer was 10 mM pyrophosphate–1 mM EDTA. The incubations included the enzyme alone, at a final concentration of 1 mg/ml (1.6×10^{-5} M), enzyme and 25 mM NAD, enzyme and 25 mM each of coenzyme and DL-glycerol-3-P, enzyme and 25 mM DL-1-halodeoxyglycerol-3-P, and finally enzyme with a 25 mM concentration of one of the halo analogs along with 25 mM NAD^+. The incubations were done for the most part on duplicate samples, from which aliquots were removed immediately after mixing the components, and at daily intervals for several days. The aliquots were diluted 1:100 in cold triethanolamine acetate buffer (pH 7.5)–1 mM EDTA and 2-mercaptoethanol, and assayed with NADH and dihydroxyacetone phosphate as described earlier (Fondy *et al.*, 1968).

At the conclusion of the incubations, the various samples were dialyzed against 8 M urea for 24 hr, then twice against distilled water for 12-hr periods. After freeze-drying, the samples were digested with perchloric acid and analyzed for organic phosphate as detailed under Methods. P_i controls were carried out on buffers after dialysis and on samples of the enzyme which were not subjected to perchloric acid digestion.

Studies on the rate of dialysis of 25 mM NAD alone indicated that it took approximately 40 hr for the optical density at 260 mμ to approach equality inside and outside the membrane. Similar studies with ^{32}P-labeled DL-1-chlorodeoxyglycerol-3-P showed that about 22 hr were required for equilibrium. When 25 mM NAD labeled with ^{14}C at the carbonyl group was incubated with 1.6×10^{-5} M enzyme at pH 7.0, no radioactivity remained bound to the enzyme after dialysis for three 24-hr periods against 6 M guanidine hydrochloride. Dialysis of rabbit muscle glycerol-3-P-dehydrogenase against 8 M urea for 24 hr, followed by dialysis against distilled water left from one to three molecules of organic phosphate bound per enzyme subunit. This tightly bound organic phosphate

could be removed by alkylation with iodoacetate and dialysis against ammonium bicarbonate under conditions described in earlier work (Fondy *et al.*, 1968).

Neither the 1-chloro nor the 1-bromo analog of DL-glycerol-3-P in the presence or absence of coenzyme and at pH 7 or 9 has a marked effect on the activity of the enzyme even after 5 days. There was no evidence of rapid striking losses in activity such as might be associated with active-site-directed irreversible inhibition. Analysis for covalently bound phosphate indicated that the enzyme incorporated approximately 2 to 4 moles of phosphate per subunit in the 5-day incubation at pH 7 with the chloro or bromo analogs, but little or no incorporation when the coenzyme was also present along with the analogs. At pH 9, the same low level of phosphate incorporation was observed in the presence of the chloro analog, while incorporation during incubation with the bromo analog reached 7 to 10 moles of organic phosphate per subunit in the presence and in the absence of coenzyme.

Discussion

Structure of the 1-Halodeoxy-DL-glycerol Phosphate Synthesized from Epihalohydrins. Nuclear magnetic resonance spectra of the salts of 1-halodeoxy-DL-glycerol phosphate, synthesized from epihalohydrins by use of either phosphoric acid or of dibenzylphosphoric acid, when compared to the spectra of glycerol-2-P and glycerol-3-P show that the synthetic compounds are analogs of glycerol-3-P. The synthesis of the identical 1-chlorodeoxy-DL-glycerol phosphate starting from 1-chlorodeoxyglycerol and using dibenzyl phosphorochloridate provides strong confirmation for the conclusions derived from nuclear magnetic resonance. An alternative means of structural confirmation would be oxidation of halodeoxyglycerol phosphates prepared from epihalohydrins to the corresponding 1-halodeoxydihydroxyacetone-3-P or the reduction of the latter to the former. This work is in progress in connection with studies on the preparation and biochemical properties of 1-halo analogs of dihydroxyacetone-3-P.

1-Fluorodeoxy-DL-glycerol-3-P As Substrate for Glycerol-3-P-dehydrogenase and Potential Value as Anti-cancer Agent. We have observed that the 1-fluoro analog of glycerol-3-P is a substrate for NAD-linked glycerol-3-P-dehydrogenase. This is an indispensable requirement for any further testing of the rationale presented in the introduction for the use of 1-fluorodeoxy-DL-glycerol-3-P as an agent selectively toxic to cells lacking glycerol-3-P-dehydrogenase. All the work discussed here involves racemic mixtures of the halo analogs, whereas the rationale requires that only the L enantiomorph of 1-fluorodeoxyglycerol-3-P would be acted upon by glycerol-3-P-dehydrogenase or would enter into lipid synthesis. Therefore, the D enantiomorph would affect cells indiscriminantly whether they have glycerol-3-P-dehydrogenase or not. We are proceeding with the stereospecific synthesis of separate L- and D-1-fluorodeoxyglycerol-3-P in order to determine if the L enantiomorph may be less toxic than the DL mixture. Furthermore, the presence and function of mitochondrial flavin-linked glycerol-3-P-dehydrogenase cannot be ignored in the rationale for the use of halo analog of glycerol-3-P or of dihydroxyacetone-3-P as agents selectively toxic to cells lacking NAD-linked glycerol-3-P-dehydrogenase. The flavin enzyme and its effects on the halo analogs are also under investigation. Finally, we have observed a relatively low toxicity for 1-fluorodeoxy-DL-glycerol-3-P[1] suggesting that perhaps a more likely anti-cancer agent might be the 1-fluoro analog of dihydroxyacetone-3-P. If the 1-fluorodeoxydihydroxyacetone-3-P were itself toxic, for example by inhibiting triosephosphate isomerase, its reduction to 1-fluorodeoxy-L-glycerol-3-P might be a means of selective detoxification in cells which have NAD-linked glycerol-3-P-dehydrogenase. This approach has the added advantage of working in the direction of ketone reduction which is strongly favored by the equilibrium constant for the cytoplasmic enzyme.

Attempted Active-Site Labeling with 1-Halodeoxy-DL-glycerol-3-P. It was our hope that if the 1-halo analogs of glycerol-3-P were substrates for glycerol-3-P-dehydrogenase, they would produce the corresponding 1-halo analogs of dihydroxyacetone-3-P precisely at the active site. The generation of a potent α-halo ketone alkylating agent at the active site might be likely to give very specific active-site labeling. However, neither the fluoro, chloro, or bromo analogs of glycerol-3-P were potent time-dependent irreversible inhibitors, and only the fluoro analog appeared to be substrate. Alkylation was observed on prolonged incubation using the chloro and bromo analogs, but this alkylation was not enhanced by the presence of the coenzyme, and did not appear to involve specific reaction at the active site. It thus seems likely the direct active-site labeling of NAD-linked glycerol-3-P-dehydrogenase will require the use of 1-halo analogs of dihydroxyacetone-3-P that are synthesized chemically rather than generated *in situ* by the action of the enzyme on the corresponding halo analogs of glycerol-3-P.

Substrate Properties of 1-Chlorodeoxy- and 1-Bromodeoxy-DL-glycerol-3-P in the Presence of Hydrazine. Hydrazine has been used often in assaying glycerol-3-P-dehydrogenase in the direction of NADH generation. The dihydroxyacetone-P formed is bound to the hydrazine and prevented from reaching equilibrium concentration, thus pulling the reaction continuously in the normally unfavorable direction. When we employed hydrazine in the assay for oxidation of the 1-chloro or 1-bromo analogs of DL-glycerol-3-P, these analogs which did not appear to reduce NAD^+ in the absence of hydrazine, did so in its presence. The effect of hydrazine may have been to react with any 1-halodeoxydihydroxyacetone-3-P that was being formed and perhaps prevent it from inhibiting further oxidation of 1-halodeoxy-DL-glycerol-3-P. We have no evidence that oxidation proceeds at all, and if it does it is not known whether 1-halodeoxydihydroxyacetone-3-P that would be formed is such a potent inhibitor of the glycerol-3-P-dehydrogenase-catalyzed oxidation of 1-halodeoxyglycerol-3-P. If halo analogs of dihydroxyacetone-3-P are indeed formed, they are not potent inhibitors of glycerol-3-P-dehydrogenase when assayed for reduction of dihydroxyacetone-3-P.

A more likely explanation for the effect of hydrazine is that it functions in converting the 1-chloro and 1-bromo analogs of DL-glycerol-3-P into something which is a substrate for the enzyme. This possible substrate might be 1-hydrazine-substituted glycerol-3-P generated by the reaction of hydrazine with the α-halo alcohol. This explanation is consistent with the fact that the bromo analog is affected more strongly at pH 7.5 than the chloro analog by the presence of hydrazine, and the fluoro analog is not affected at all.

[1] T. P. Fondy and C. R. Ross, unpublished observations.

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