Harbor Symp. Quant. Biol. 29, 213.
Kornecki-Gerrity, E. H., and Penney, D. G. (1974a), *Comp. Biochem. Physiol. B 48,* 507.
Kornecki-Gerrity, E. H., and Penney, D. G. (1974b), *Comp. Biochem. Physiol. B 49,* 15.
Kutzbach, C., Bischofberger, H., Hess, B., and Zimmermann-Telschow, H. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1473.
Nakashima, K. (1974), *Clin. Chim. Acta 55,* 245.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N.Y., Academic Press.
Schloen, L. H., Kmioteck, E. H., and Sallach, H. J. (1974), *Arch. Biochem. Biophys. 164,* 254.
Scrutton, M. C., and Utter, M. F. (1968), *Annu. Rev. Biochem. 37,* 249.
Strandholm, J. J., Cardenas, J. M., and Dyson, R. D. (1975), *Biochemistry,* preceding paper in this issue.
Susor, W. A., Kochman, M., and Rutter, W. J. (1969), *Science 165,* 1260.
Susor, W. A., and Rutter, W. J. (1971), *Anal. Biochem. 43,* 147.
Whittell, N. M., Ng, D. O. K., Prabhakararao, K., and Holmes, R. S. (1973), *Comp. Biochem. Physiol. B, 46,* 71.

1-Halo Analogs of Dihydroxyacetone 3-Phosphate. The Effects of the Fluoro Analog on Cytosolic Glycerol-3-Phosphate Dehydrogenase and Triosephosphate Isomerase†

Joan B. Silverman, Paula S. Babiarz, Kishan P. Mahajan,‡ John Buschek, and Thomas P. Fondy*

ABSTRACT: 1-Fluoro-3-hydroxyacetone phosphate (fluoroacetol phosphate) has been prepared by oxidation of 1-fluoro-3-chloro-2-propanol to 1-fluoro-3-chloroacetone, phosphorylation with silver dibenzylphosphate, and the intermediate isolation of 1-fluoro-3-hydroxyacetone phosphate dibenzyl ester, followed by catalytic hydrogenation and preparation of the stable monosodium salt. The chloro analog as the pure, stable monosodium salt has been prepared by a similar route from 1,3-dichloroacetone. 1-Fluoro-3-hydroxyacetone-P is a substrate for cytosolic NAD^+-linked glycerol-3-P dehydrogenase (EC 1.1.1.8) from rabbit skeletal muscle with an apparent K_m of 50 mM under conditions in which dihydroxyacetone-P exhibits an apparent K_m of 0.15 mM. Under these conditions the fluoro analog is 85% hydrated whereas dihydroxyacetone-P has been shown by others to be 44% hydrated. The turnover numbers are 49,000 molecules of NADH oxidized per minute per molecule of enzyme at 25° with the fluoro analog as substrate, and 60,000 with dihydroxyacetone-P as substrate. The product of the reduction of the fluoro analog has been identified as 1-fluorodeoxyglycerol-3-P. 1-Fluoro-3-hydroxyacetone-P is a comparatively weak irreversible inhibitor at 4° of rabbit muscle triosephosphate isomerase (EC 5.3.1.1) with a second-order rate constant of 2.6 M^{-1} sec^{-1}. Inhibition by pyrazole in vivo of the alcohol dehydrogenase catalyzed oxidation of 1-fluorodeoxyglycerol-3-P indicates that in mice the reduction of 1-fluoro-3-hydroxyacetone-P to L-1-fluorodeoxyglycerol-3-P is not a significant metabolic route, or that an alternative route exists when the alcohol dehydrogenase dependent pathway is inhibited.

1-Fluoro analogs of glycerol-3-P and dihydroxyacetone-3-P may be able to exert differential effects on pathways to phospholipids in normal and neoplastic cells, by virtue of their potential behavior as substrates for cytosolic glycerol-3-P dehydrogenase (Fondy et al., 1970, 1974; Ghangas and Fondy, 1971) or by inhibition of the acyldihydroxyacetone-P and acylglycerol-3-P pathways to phosphatidic acid and ether lipids. These possibilities have led us to carry out the synthesis of racemic and optically pure forms of 1-fluorodeoxyglycerol-3-P in the work cited above. Independent syntheses have been carried out by Lloyd and Harrison (1971, 1973). We have also proposed and prepared secondary fluorodeoxyketohexoses as potential intracellular precursors of the fluorotrioses (Rao et al., 1975).

In this work we present the first successful synthesis of 1-fluoro-3-hydroxyacetone-P along with alternative approaches to the synthesis of the chloro and bromo analogs. The chloro, bromo, and iodo analogs of dihydroxyacetone-P (haloacetol phosphates) were originally prepared by Hartman (1968, 1970) employing a route not readily applicable to preparation of the fluoro analog. An independent synthesis of the bromo analog has also been developed by Coulson et al. (1970). A preliminary report of our work has been presented (Fondy et al., 1971).

We have examined 1-fluoro-3-hydroxyacetone-P as a substrate for cytosolic NAD^+-linked glycerol-3-P dehydrogenase from rabbit skeletal muscle, and have identified the

† From the Department of Biology, Syracuse University, Syracuse, New York 13210. *Received August 19, 1974.* This work was supported by Public Health Service Research Grant CA-10250 from the National Cancer Institute of the National Institutes of Health, and by Grant GB-20943 from the National Science Foundation.

‡ Present address: Department of Chemistry, State University of New York, Oswego, New York.

* Recipient of Public Health Service Research Career Development Award CA-70332 from the National Cancer Institute.

reduction product. Comparative kinetic parameters of the 1-fluoro analogs of dihydroxyacetone-3-P and of L-glycerol-3-P with respect to the natural substrates offer some insight into the molecular interactions between glycerol-3-P dehydrogenase and its substrates.

The effect of 1-fluoro-3-hydroxyacetone-P on triosephosphate isomerase has been determined following procedures applied by Hartman (1971) using the higher halo analogs. Evidence concerning the metabolic fate in vivo of 1-fluoro-3-hydroxyacetone-P has been obtained by use of pyrazole as an inhibitor of the alcohol dehydrogenase catalyzed oxidation of 1-fluorodeoxyglycerol-3-P.

Experimental Section

Materials

Enzymes. Rabbit muscle glycerol-3-phosphate dehydrogenase (lot 12C-9530) and rabbit muscle triosephosphate isomerase (lot 39B-0740) were obtained from Sigma Chemical Co. (St. Louis).

Chemicals. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide), dibenzylphosphoric acid, and epichlorohydrin were products of Aldrich Chemical Co. (Milwaukee). Dibenzylphosphoric acid was also prepared by us according to the method of Lynen (1940) and Sheehan and Frank (1950). DL- and L-1-Fluorodeoxyglycerol-3-P were synthesized as previously described (Fondy et al., 1970, 1974). Potassium hydrogen fluoride was a product of ROC/RIC (Belleville, N. J.). DL-Glyceraldehyde-3-phosphoric acid was a product of Sigma Chemical Co. NADH was supplied by P-L Biochemicals (Milwaukee). Dihydroxyacetone-P as the dimethyl ketal was a product of Calbiochem (Los Angeles). Silica gel (Baker "Analyzed Reagent" 3405, 60–200 mesh) was a product of J. T. Baker Chemical Co. (Phillipsburg, N.J.).

Methods

Enzyme Assays. Glycerol-3-phosphate dehydrogenase activity was monitored according to the method of Fondy et al. (1969) using dihydroxyacetone 3-phosphate or 1-fluoro-3-hydroxyacetone-P as substrates. Activity was measured at room temperature in 50 m*M* Tris-Cl (pH 7.5), 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, and 0.1 m*M* NADH, with 3×10^{-9} *M* enzyme. Oxidation of L-1-fluorodeoxyglycerol-3-P and of L-glycerol-3-P was measured in 50 m*M* pyrophosphate buffer (pH 9.0), 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, 0.1 *M* hydrazine, 1 m*M* NAD^+, using 10^{-6} *M* enzyme for the fluoro analog and 2×10^{-8} *M* for the natural substrate. Apparent K_m was determined from duplicate or triplicate assays using a program based on the method of Wilkinson (1961), or graphically by the method of Hofstee (1952).

Triosephosphate isomerase activity was measured by the method of Norton et al. (1970), at room temperature in 20 m*M* triethanolamine hydrochloride (pH 7.9)–0.3 m*M* EDTA. The 3.0-ml system with a 1-cm light path contained 0.15 m*M* NADH, 1.0 m*M* DL-glyceraldehyde 3-phosphate, and an excess of glycerol-3-phosphate dehydrogenase (28 μg).

High Voltage Electrophoretic Analysis of Organic Phosphates. Samples containing 0.5 μmol of organic phosphate were applied in 10 μl to Whatman 3MM chromatography paper (57 × 27 cm), the paper was wetted with 18% formic acid (pH 1.65) without displacing the applied samples, and the papers were blotted. The papers were subjected to 1950 V, 0.26 A (500 W) for 2.5 hr in a Savant high-voltage electrophoresis tank under Varsol with electrode solution of 9% formic acid (pH 1.95). Organic phosphate compounds were localized on the dried papers as previously described (Fondy et al., 1970).

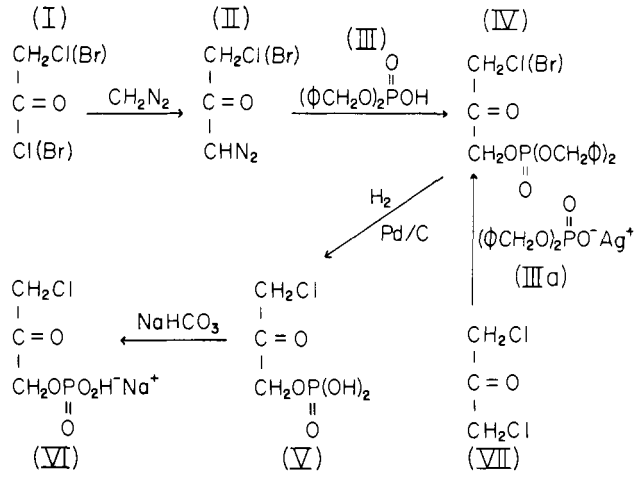

FIGURE 1: Synthesis of 1-chloro-3-hydroxyacetone-P from chloracetyl chloride and from dichloroacetone.

Analytical Determinations. Organic and inorganic chloride, bromide, and phosphate were determined as previously described (Fondy et al., 1970). Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Analyses for total phosphorus performed by Galbraith Laboratories by gravimetric procedures were consistently slightly lower than values obtained by us for total phosphate analysis. Both values are reported when available.

Inactivation of Triosephosphate Isomerase by 1-Fluoro-3-hydroxyacetone Phosphate. Following the method of Hartman (1971) 100 μg of triosephosphate isomerase in 3.99 ml of 0.1 *M* imidazolium hydrochloride (pH 6.5) was treated with 0.01 ml of 0.4 *M* 1-fluoro-3-hydroxyacetone phosphate (4 μmol) at 4°. Periodically 0.1-ml samples of this reaction mixture were diluted into 4.9 ml of cold 0.02 *M* triethanolamine hydrochloride (pH 7.9) containing 1 m*M* EDTA and 10 m*M* 2-mercaptoethanol. Aliquots of these diluted samples were assayed for triosephosphate isomerase activity within 2 min following dilution.

Syntheses of 1-Chloro- and of 1-Bromo-3-Hydroxyacetone Phosphate. The chloro analog of dihydroxyacetone-P was prepared by two independent routes, one of which was applicable to the preparation later of the fluoro analog, the other of which was also employed for the preparation of the bromo analog. The synthetic steps are shown in Figure 1. Relevant nuclear magnetic resonance (NMR) spectral characterizations are presented under Results and Discussion.

1. From Haloacetyl Chlorides and Diazomethane. 1-CHLORO(BROMO-)-3-DIAZOACETONE (II). The diazo ketones were synthesized from the corresponding haloacetyl chlorides and diazomethane by the method of Gerson and Schlenk (1968) and of Husain and Lowe (1968). Yields were 60% for the chloro diazo ketone and 84% for the bromo diazo ketone (II).

1-CHLORO(BROMO-)-3-HYDROXYACETONE PHOSPHATE DIBENZYL ESTER (IV). The reaction was modelled after procedures developed by Grundmann (1936) for acetylation and applied by Hajra and Agranoff (1968) to phosphorylation. Dibenzylphosphoric acid (III) (4.6 g, 17

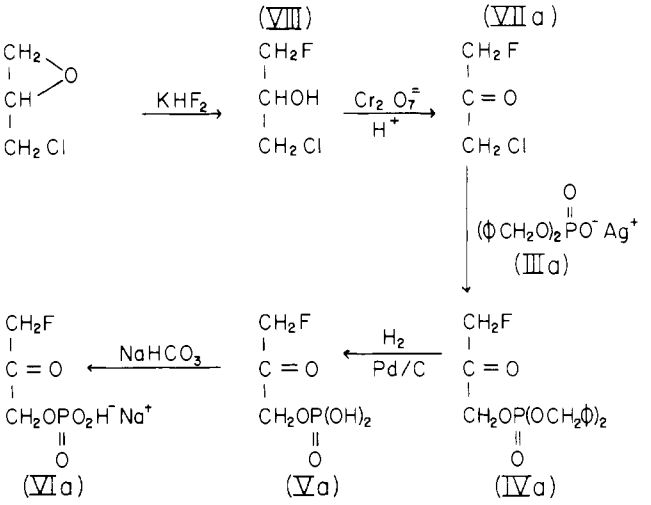

FIGURE 2: Synthesis of 1-fluoro-3-hydroxyacetone-P.

mmol) was added in five 5-ml aliquots to 3.4 g (29 mmol) of chloro diazo ketone (II) in 50 ml of benzene. The reaction was maintained at 65° in a water bath, and the amount of unreacted dibenzylphosphoric acid determined periodically by titrating a 0.1-ml aliquot diluted in 5 ml of absolute ethanol, using 0.05 *N* KOH. Dibenzylphosphoric acid uptake ceased when 30% remained unreacted. Rotary evaporation yielded a residue of 7 g. A silica gel column (3 × 55 cm) was prepared from 110 g of chloroform-washed gel, gravity packed in chloroform, and washed with one column volume of benzene. After sample addition, the column was washed with benzene until the effluent was colorless. Evaporation of the benzene gave recovery of residual unreacted chloro diazo ketone. Without interruption of flow, the column was eluted with chloroform and the position of the product determined by A_{280}. The product was recovered in the first 450 ml of chloroform eluent (5.2 g, 86%). The NMR spectrum indicated a contamination of 10–15% by dibenzylphosphoric acid. The dibenzylphosphoric acid content was reduced to 4% by washing three times with 5% $NaHCO_3$ followed by three water washes.

1-BROMO-3-HYDROXYACETONEPHOSPHATE DIBENZYL ESTER (IV) under the same conditions gave 3.8 g of product from 6.5 g (40 mmol) of bromo diazo ketone (II) and 6.9 g (26 mmol) of dibenzylphosphoric acid.

1-CHLORO-3-HYDROXYACETONE PHOSPHATE MONOSODIUM SALT (VI). The stable salt was prepared in 46% yield contaminated by 5% inorganic phosphate following procedures detailed in the subsequent section.

2. From 1,3-Dichloro-2-propanone and Silver Dibenzylphosphate. Silver dibenzylphosphate (IIIa) was prepared by the method of Sheehan and Frank (1950). To 150 ml of 50% methanol (v/v) containing 5.9 g (150 mmol) of sodium hydroxide was added 41.9 g (150 mmol) of dibenzylphosphoric acid. All the subsequent operations were performed with the exclusion of light. To 50 ml of hot water was added 25.5 g (150 mmol) of silver nitrate, followed by addition of the sodium dibenzylphosphate solution. A white precipitate formed immediately. The mixture was allowed to stand in the dark for about 46 hr, then vacuum filtered twice using a medium pore sintered glass filter. The white precipitate, silver dibenzylphosphate (IIIa), was washed with 500 ml of water, 500 ml of methanol, then 300 ml of chloroform. After drying under vacuum for about 20 hr the precipitate weighed 51.0 g (88% yield); mp 221–222° (lit. Sheehan and Frank (1950), 212–216° dec). Silver dibenzylphosphate (IIIa) could be stored at room temperature in the dark for 3 months at which time is gave a mp of 223–226°.

1-CHLORO-3-HYDROXYACETONE PHOSPHATE DIBENZYL ESTER (IV). In the dark 28.8 g (75 mmol) of silver dibenzylphosphate (IIIa) was added to 28.4 g (224 mmol) of 1,3-dichloro-2-propanone (VII) in 125 ml of dry, redistilled acetonitrile, then refluxed with constant and vigorous stirring for 5 hr. The reaction mixture was vacuum filtered and the gray precipitate washed with acetonitrile until the washings were clear. The reddish brown filtrate, when dried down on the rotary evaporator, became a solid in a viscous liquid with a combined weight of 48 g. The title compound was purified by silica gel chromatography as described above and yielded 10.8 g (39%). The NMR spectrum for the bicarbonate-washed ester is reproduced under Results and Discussion.

1-CHLORO-3-HYDROXYACETONEPHOSPHORIC ACID (V) AND MONOSODIUM SALT (VI). Hydrogenation of 8.8 g (23.9 mmol) of the chlorodibenzyl ester (IV) was carried out in 240 ml of absolute methanol with 350 mg of 10% Pd/C. Theoretical hydrogen uptake was complete after 40 min. Filtration of catalyst and removal of methanol yielded 4.5 g (23.9 mmol, 100%) of compound V.

Immediately after preparation, the 1-chloro-3-hydroxyacetonephosphoric acid (V) was dissolved in 20 ml of water and the pH adjusted to 5 with the dropwise addition of saturated $NaHCO_3$ (about 15 ml). Ethanol was added until the solution became cloudy, then a volume of acetone equal to twice the volume of ethanol was added, and a clear gelatinous precipitate collected after storage at 4° for 20 hr. Acetone was added to the supernatant and a second precipitate collected. The precipitated monosodium 1-chloro-3-hydroxyacetone phosphate (VI) totalled 2.1 g (42%). The first precipitate contained 12% inorganic phosphate, and the second contained 3.5%. Elemental analysis of the latter gave the following. Anal. Calcd for 96.5% $C_3H_5Cl_1O_5P_1Na_1$ containing 3.5% NaH_2PO_4: C, 16.50; H, 2.35; Cl, 16.27; P, 15.10; O, 38.54; Na, 11.21. Found: C, 16.69; H, 2.30; Cl, 16.11; P, 14.10 (15.20 ± 0.1).[1] Infrared spectrum established the absence of hydroxyl, excluding the possibility of ketone reduction during catalytic hydrogenation.

The synthesis of 1-chloro-3-hydroxyacetone phosphate from 1,3-dichloro-2-propanone and silver dibenzylphosphate was used as the model for the synthesis of 1-fluoro-3-hydroxyacetone phosphate because of the impracticality of beginning a synthesis with highly toxic gaseous fluoroacetyl chloride.

Synthesis of 1-Fluoro-3-hydroxyacetone Phosphate. The fluoro analog was synthesized by the sequence shown in Figure 2.

1-FLUORO-3-CHLORO-2-PROPANOL (VIII). Compound VIII was prepared by modification of the method of Bergmann and Cohen (1958). Epichlorohydrin (248 g, 2.7 mol) was mixed with 212 g (2.7 mol) of potassium hydrogen fluoride in 250 ml of dry ethylene glycol. The mixture was heated in an oil bath at 125–140° for 18 hr with vigorous stirring and then filtered and the precipitate washed with chloroform. After evaporation of the combined filtrate and washings, the residual liquid was distilled under reduced pressure (40 ± 10 mm) and the fraction distilling 50–95°

[1] Value in parentheses from quadruplicate determinations of total organic and inorganic phosphate. All other values by Galbraith Laboratories (see Methods).

(150 g) was collected with the receiver in an ice bath. This crude product was redistilled at atmospheric pressure and gave three fractions: 1,3-difluoro-2-propanol, bp 138–145°, 34.1 g, 13.2% yield; 1-fluoro-3-chloro-2-propanol (VII), bp 145–155°, 39.8 g, 13.2% yield; and a higher boiling fraction, impure 1-fluoro-3-chloro-2-propanol, bp 155–165°, 6.3 g.

1-FLUORO-3-CHLORO-2-PROPANONE (VIIA). 1-Fluoro-3-chloro-2-propanol (VIII) was oxidized using a modification of the procedure of Bergmann and Cohen (1958) in order to obtain a more complete reaction. Potassium dichromate (317.5 g, 1.1 mol) was dissolved in 1430 ml of water, 390 ml of concentrated sulfuric acid was added, and the solution was cooled to 15°. 1-Fluoro-3-chloro-2-propanol (VIII) (123.2 g, 1.10 mol) was added dropwise to the oxidizing solution over a period of 20 min with vigorous stirring and maintenance of the temperature at 15°. The reaction mixture was then stirred at room temperature for an additional 20 hr. Another 720 ml of water was added and the mixture subjected to distillation under reduced pressure (95 ± 5 mm), yielding 850 ml. Calcium chloride was added to the distillate which was then extracted with a total of 2 l. of ether. The ether extracts were combined and dried over anhydrous calcium chloride, and the ether was removed by atmospheric distillation using a Vigreux column. The dark colored residue was distilled at atmospheric pressure (without use of the Vigreux column) and gave 32 g (30% yield) of a colorless fraction distilling at 135–145°. Reported boiling point is 142–144° (Bergmann and Cohen, 1958). Anal. Calcd for $C_3H_4Cl_1F_1O_1$: C, 32.6; H, 3.6; Cl, 32.1; F, 17.2. Found: C, 32.3; H, 3.8; Cl, 31.9; F, 16.9. The NMR spectrum showed that oxidation was essentially complete.

1-FLUORO-3-HYDROXYACETONE PHOSPHATE DIBENZYL ESTER (IVA). In the dark, 82 g (210 mmol) of silver dibenzylphosphate (IIIa) and 31 g (330 mmol) of 1-fluoro-3-chloro-2-propanone (VIIa) reacted in 160 ml of acetonitrile following procedures detailed above for dichloroacetone produced 99 g of crude product. Silica gel (110 g) chromatography as detailed above gave in the first 2 l. of chloroform eluent 20.2 g of the compound (IVa) contaminated with some dibenzylphosphate. Additional product could be recovered separate from unreacted 1-fluoro-3-chloro-2-propanone by rechromatography of the residue from the benzene eluent using a silica gel to sample ratio of 7:1. After washing with 5% $NaHCO_3$ to remove dibenzylphosphate, solvent evaporation, and drying over P_2O_5, the primary product yielded 12.2 g (17%). Recovery of 25% could be obtained by dissolving the crude product in benzene, removing unreacted silver dibenzylphosphate by filtration prior to silica gel chromatography, and by use of a lower ratio of crude product per gram of silica gel. Anal. Calcd for $C_{17}H_{18}F_1O_5P_1$: C, 57.95; H, 5.16; F, 5.39; O, 22.71; P, 8.79. Found: C, 55.30; H, 5.30; F, 5.00; P, 7.46. The NMR spectrum shown under Results and Discussion demonstrated contamination of less than 5% by dibenzylphosphate.

1-FLUORO-3-HYDROXYACETONEPHOSPHORIC ACID (VA) AND MONOSODIUM SALT (VIA). Hydrogenation at atmospheric pressure of the dibenzyl ester (IVa) (2.13 g, 6 mmol) in 20 ml of absolute methanol with 135 mg of 10% Pd/C was complete after 20 min. The free phosphoric acid ester prepared as detailed above for the chloro analog (V) gave an NMR spectrum which showed the formation of some dimethylketal derivative of the fluoro ketone ester. In order to obtain a spectrum uncomplicated by ketal formation, hydrogenation was carried out on a sample of the dibenzyl ester (IVa) in benzene. Hydrogenation was slower and incomplete in benzene due to separation of the catalyst complexed with the free phosphoric acid ester. The product was separated from the catalyst by extraction with deuterium oxide and the NMR spectrum free of any dimethylketal was obtained.

The monosodium salt of 1-fluoro-3-hydroxyacetone phosphate (VIa) was prepared from 5.9 g (34.3 mmol) of the free acid prepared by hydrogenation in methanol as detailed above for the chloro analog. The yield was 2.8 g (42%) in two fractions containing contaminants of 7 and 6.4% inorganic phosphate. Analysis of the latter gave the following. Anal. Calcd for 93.6% $C_3H_5F_1O_5P_1Na_1$ containing 6.4% NaH_2PO_4: C, 17.36; H, 2.33; F, 9.16; P, 16.59; O, 38.61; Na, 11.09. Found: C, 17.30; H, 2.42; F, 9.06; P, 14.98 (16.1 ± 0.2).[2] There was essentially no detectable hydroxyl absorption in the infrared spectrum excluding any possibility of ketone reduction during hydrogenation.

Results and Discussion

Syntheses and Characterizations of 1-Halo-3-hydroxyacetone Phosphates. 1-Chloro-3-hydroxyacetone-P monosodium salt (VI) was successfully prepared beginning either from chloroacetyl chloride or from dichloroacetone with comparable yields in the range of 25%. The chloroacetyl chloride route was readily applicable to preparation of the dibenzylphosphate ester of bromo analog, but was not practical for the preparation of the fluoro analog. 1-Fluoro-3-hydroxyacetone-P monosodium salt (VIa) was produced from 1-fluoro-3-chloroacetone with a yield of 10%. The monosodium salts of the fluoro- and chlorohydroxyacetone phosphates were a convenient form in which to prepare and store the compounds for routine study. The halohydroxyacetone phosphates could not be entirely freed from inorganic phosphate as previously noted (Hartman, 1970), but the application of silica gel chromatography to the dibenzylphosphate ester, removal of residual dibenzylphosphoric acid by washing in $NaHCO_3$, and fractional precipitation of the monosodium salts after hydrogenation reduced phosphate contamination to 4–6%. The NMR spectra of the intermediates and products in this work are consistent with the assigned structures. The relevant NMR spectra are reproduced in Figure 3. The spectra of 1-fluoro-3-chloro-2-propanol (VIII) (Figure 3A) and of 1-fluoro-3-chloroacetone (VIIa) (Figure 3B) demonstrate that the modified conditions employed for oxidation produce complete oxidation, with no trace of unreacted secondary alcohol remaining. Spectra in Figure 3C and D show the dibenzyl esters respectively of 1-fluoro- (IVa) and of 1-chloro-3-hydroxyacetone phosphates (IV) with the contaminating dibenzylphosphoric acid reduced to about 4% in both cases. The NMR spectrum for 1-bromo-3-hydroxyacetone phosphate dibenzyl ester (IV) was identical with that shown for the chloro analog except for the appearance of the absorption due to the CH_2Br at 3.85 ppm (δ) rather than 4.0 ppm (δ) observed for the CH_2Cl group. The spectrum of the free phosphoric acid ester of 1-fluoro-3-hydroxyacetone-P (Va) in deuterium oxide (Figure 3E) shows the presence of both the free ketone and the corresponding hydrate as previously noted for dihydroxyacetone phosphate (Gray and Barker,

[2] Values in parentheses from six determinations of total organic and inorganic phosphate. All other values by Galbraith Laboratories (see Methods).

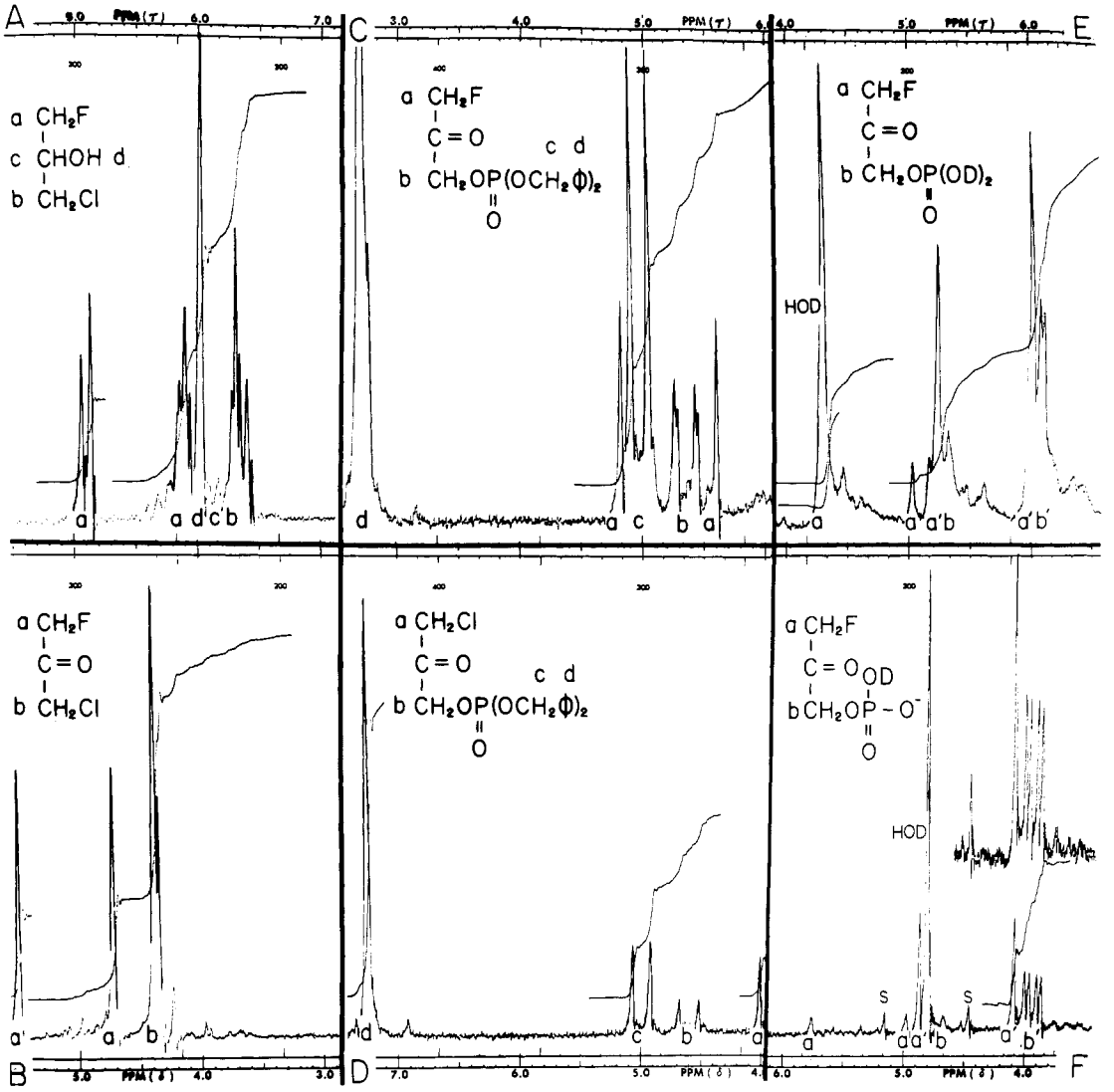

FIGURE 3: The 60-MHz nuclear magnetic resonance spectra of compounds shown in inserts. Spectra A–D in $CDCl_3$. Spectra E and F in D_2O. Absorptions are assigned by lower case letters beneath spectra peaks, corresponding to protons labeled in the structural inserts. In spectra E and F the absorption peaks designated a′ and b′ correspond to protons in the hydrated ketone structure. S designates spinning side band.

1970). Hydration was also observed in the NMR spectrum of 1-chloro-3-hydroxyacetonephosphoric acid (V). The NMR spectrum of monosodium 1-fluoro-3-hydroxyacetone-P (VIa) is shown in Figure 3F, with assignments made for the fluoromethyl protons in the free and *gem*-diol hydrate by comparison with the spectra of dichloroacetone and 1-fluoro-3-chloroacetone. There was no measurable 1-chloro analog contaminating the fluoro analog in either the dibenzyl ester or the sodium salt. At neutral pH and 25°, 1-fluoro-3-hydroxyacetone-P exists 85% in the *gem*-diol (hydrated) form. Under these conditions the chloro analog is 66% hydrated, compared to published values of about 40% for dihydroxyacetone-P (Reynolds et al., 1971; Gray and Barker, 1970). The shift in degree of hydration observed for the fluoro and chloro analogs compared to dihydroxyacetone-P is consistent with effects of electron withdrawing groups upon hydration of halo ketones discussed by Hooper (1967).

1-Fluoro-3-hydroxyacetone Phosphate as Substrate in Vitro for NAD$^+$-Linked Glycerol-3-P Dehydrogenase. 1-Fluoro-3-hydroxyacetone-P (VIa) was observed to be a substrate for the rabbit muscle cytosolic enzyme. Apparent K_m's and turnover numbers with dihydroxyacetone-P and its 1-fluoro analog as substrates measured at pH 7.5 under identical conditions are shown in Table I. The extent of hydration of the fluoro analog at neutral pH was estimated from the NMR spectrum, as detailed above, and compared to published values for the natural substrate (Table I). At 100 m*M* 1-fluoro-3-hydroxyacetone-P, the highest concentration employed in the K_m determination, the inorganic phosphate concentration was 5 m*M*, producing no more than 5% inhibition of enzyme activity. Table I also lists comparative apparent K_m's and turnover numbers for L-1-fluorodeoxyglycerol-3-P and for L-glycerol-3-P measured under identical conditions at pH 9.0

1-Chloro-3-hydroxyacetone phosphate (VI) exhibited very slight substrate activity with NAD$^+$-linked glycerol-3-P dehydrogenase as noted previously by Hartman (1972). However, it was not possible to determine whether the activity was an intrinsic property of the chloro analog, or whether generation in situ of dihydroxyacetone phosphate by chloride loss was responsible for the observed trace activity.

Generation of 1-Fluorodeoxyglycerol 3-Phosphate from 1-Fluoro-3-hydroxyacetone Phosphate. Direct demonstration of the substrate properties of the fluoro analog of dihy-

Table I: Apparent Michaelis Constants and Molecular Activities for the Natural Substrates of Rabbit Muscle Cytosolic NAD^+-Linked Glycerol-3-P Dehydrogenase and the 1-Fluorodeoxy Analogs.

Substrate	Apparent K_m (mM)	Molecular Activity (Molecules of Substrate Converted per Molecule of Enzyme per min)	Percent Non-hydrated Keto Form pH 7, 25°	Ratio of Apparent K_m for Natural Substrate Compared to Fluoro Analog
Dihydroxyacetone-P[c]	0.15	60,000	55,[a] 61[b]	83[f]
1-Fluoro-3-hydroxyacetone-P[c]	50 ± 10	49,000	15	
L-Glycerol-3-P[d]	0.5 ± 0.03	735		15
L-1-Fluorodeoxyglycerol-3-P[e]	7.5 ± 1.0	8		

[a] Gray and Barker (1970). [b] Reynolds et al. (1971). [c] Assayed at pH 7.5, 50 mM Tris-Cl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM NADH, and 3×10^{-9} M enzyme. [d] Assayed at pH 9.0, 50 mM pyrophosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 M hydrazine, 1 mM NAD^+, and 2×10^{-8} M enzyme. [e] Same as *d* above except 10^{-6} M enzyme. [f] Ratio calculated from concentrations of primary substrate, the respective nonhydrated keto forms.

droxyacetone phosphate was obtained by identification of its reduction product as the 1-fluoro analog of glycerol-3-P. A 1.1-ml reaction system containing 10 mM 1-fluoro-3-hydroxyacetone-P, 3 mM NADH, and 4.5×10^{-7} M glycerol-3-P dehydrogenase at pH 7.5 was allowed to reach equilibrium (2.5 hr). Analysis by high-voltage electrophoresis as detailed under Methods showed that the fluorohydroxyacetone phosphate (relative mobility 11.0 cm/hr) was not contaminated by any observable trace of dihydroxyacetone-P (relative mobility 12.1 cm/hr) or any other organic phosphate ester, and that there was a single organic phosphate ester product formed corresponding in its electrophoretic mobility to 1-fluorodeoxyglycerol-3-P (relative mobility 10.2 cm/hr). The relative mobility of glycerol-3-P was 9.6 cm/hr.

Implications for the Mechanism of Substrate Binding and Oxidation–Reduction in Glycerol-3-P Dehydrogenase. Reynolds et al. (1971) have established that the free keto form of dihydroxyacetone-P is the primary substrate in the reaction catalyzed by glycerol-3-P dehydrogenase. The ratios of apparent K_m values for dihydroxyacetone-P compared to 1-fluoro-3-hydroxyacetone-P corrected for differences in their respective degrees of hydration are shown in Table I, along with comparison of the apparent K_m values for L-glycerol-3-P and L-1-fluorodeoxyglycerol-3-P. The differences in apparent K_m values of between one and two orders of magnitude between the natural substrates and their respective 1-fluoro analogs suggest that the hydroxyl group at C-1 participates in hydrogen-bond donation to a group in the enzyme–coenzyme complex, a function that cannot be performed by the fluoro group substituted at C-1.

A striking similarity is observed in molecular activities of dihydroxyacetone-P and its fluoro analog, compared to a 100-fold reduction in molecular activity for the fluoro analog of L-glycerol-3-P compared to the natural reduced substrate (Table I). This observation suggests that hydride ion abstraction from C-2 is the rate-limiting step in the oxidation of L-glycerol-3-P, a step which would be disfavored by the presence of an electron-withdrawing fluoro-substituent at C-1. Reduction of dihydroxyacetone-P with hydride ion donation at C-2 as the rate-limiting step should be favored by additional positive charge at C-2 induced by the presence of the fluoro group in 1-fluoro-3-hydroxyacetone-P. Failure to observe enhanced rate of reduction in the fluoro analog suggests that additional effects other than simple induction are operating, at least in the reduction of dihydroxyacetone-P, and very likely also in the oxidative direction.

These implications constitute an interesting contrast with suggestions by Lloyd and Harrison (1974) concerning the mechanism of substrate binding of L-glycerol-3-P to the flavine-linked glycerol-3-P dehydrogenase from locust flight muscle mitochondria. A comparison of the 1-deoxy and 1-deoxyfluoro analogs of L-glycerol-3-P as substrates for the flavine-linked enzyme suggested that the hydroxy group at C-1 in the natural substrate makes little contribution to enzyme–substrate interaction. There is only a threefold difference in apparent K_m between the natural substrate and either the 1-deoxy or 1-deoxyfluoro analogs in the flavine–enzyme system, compared to a 15-fold difference between the natural reduced substrate and the 1-deoxyfluoro analog observed by us for the NAD^+-linked enzyme. Thus the hydrogen-bond donation by the C-1 hydroxyl group to the enzyme–coenzyme complex suggested by us for the NAD^+-linked enzyme, probably does not exist in the flavine–enzyme system. The contrasting results between the flavine-linked and NAD^+-linked enzyme studies suggests that these two distinct enzymes, carrying out the oxidation of the identical substrate by use of different coenzymes, differ markedly in their mechanisms of action at least at the level of substrate binding.

Inhibition of Triosephosphate Isomerase by 1-Fluoro-3-hydroxyacetone Phosphate. Hartman (1971) observed second-order rate constants for the inactivation of triosephosphate isomerase at 2° and pH 6.5 by the iodo, bromo, and chloro analogs of dihydroxyacetone-P as 260, 2300, and 2600 M^{-1} sec^{-1}, respectively, using 10 μM reagent. The rate of inactivation of triosephosphate isomerase by the fluoro analog was extremely slow with 100 μM 1-fluoro-3-hydroxyacetone-P. Inactivation of the enzyme was carried out using 1 mM fluoro analog, with other conditions identical with those employed by Hartman (1971). The observed second-order rate constant for inactivation was 2.5 M^{-1} sec^{-1}. Assays of aliquots of the inactivation reaction mixture were carried out exactly 2 min after dilution, because 2-mercaptoethanol employed to destroy unreacted reagent under conditions used by Hartman (1971) for the chloro, bromo, and iodo compounds was ineffective in completely destroying the residual fluoro analog. Further inactivation of the diluted aliquots took place because of the residual fluoro analog.

Metabolism in Vivo of 1-Fluoro-3-hydroxyacetone Phosphate and of L-1-Fluorodeoxyglycerol 3-Phosphate. The interconversion in vitro of the 1-fluoro analog of dihydroxyacetone-3-P and the 1-fluoro analog of L-glycerol-3-P catalyzed by glycerol-3-P dehydrogenase has been estab-

lished by this and by our earlier work (Fondy et al., 1970, 1974). If the reduction of 1-fluoro-3-hydroxyacetone-P to L-1-fluorodeoxyglycerol-3-P is a significant reaction under physiological conditions, inhibitors which prevent the metabolism of L-1-fluorodeoxyglycerol-3-P should also prevent the metabolism of the keto analog. We had observed (Fondy et al., 1974) that the metabolism of L-1-fluorodeoxyglycerol-3-P in BDF_1 mice produces a marked hypothermia indicating its metabolism to fluoroacetate. Intermediate steps appear to require dephosphorylation to L-1-fluorodeoxyglycerol and subsequent oxidation catalyzed by alcohol dehydrogenase. Metabolism of L-1-fluorodeoxyglycerol-3-P to fluoroacetate as evidenced by hypothermia, could be prevented by the use of pyrazole at a dose level of 50 mg/kg intraperitoneally to inhibit the alcohol dehydrogenase catalyzed oxidation of L-1-fluorodeoxyglycerol. 1-Fluoro-3-hydroxyacetone phosphate monosodium salt at a dose of 40 mg/kg intraperitoneally in BDF_1 mice produced strong hypothermia characteristic of the fluoroacetate, but pyrazole at 50 mg/kg was completely ineffective in preventing hypothermia. Pyrazole at a concentration of 60 m*M* in the enzyme assay system had no effect on the activity of pure rabbit muscle cytosolic glycerol-3-P dehydrogenase, nor on glycerol-3-P dehydrogenase activity in tissue homogenates from muscle, liver, and kidney of BDF_1 mice. Hypothermia due to 1-fluoro-3-hydroxyacetone-P was prevented and its toxicity reduced by the use of ethanol as was observed for the 1-deoxyfluoroglycerols, their 3-phosphates, and for fluoroacetate (Fondy et al., 1974) These results indicate that the reduction of 1-fluoro-3-hydroxyacetone-P to L-1-fluorodeoxyglycerol-3-P is not a significant pathway in vivo in the hybrid mice employed in this work or at least that an alternative pathway exists leading to the toxic metabolism of 1-fluoro-3-hydroxyacetone-P to fluoroacetate.

Acknowledgment

We are grateful to the Department of Chemistry, Syracuse University, for use of NMR facilities and to Richard Pero for obtaining the spectra.

References

Bergmann, E. D., and Cohen, S. (1958), *J. Chem. Soc.*, 2259–2262.

Coulson, A. F. W., Knowles, J. R., and Offord, R. E. (1970), *J. Chem. Soc. D*, 7–8.

deBoer, T. J., and Backer, H. J. (1954), *Rec. Trav. Chim. Pay-Bas 73*, 229–234.

Fondy, T. P., Ghangas, G. S., Basloe, J. L., and Mahajan, K. (1971), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 30*, 1253.

Fondy, T. P., Ghangas, G. S., and Reza, M. J. (1970), *Biochemistry 9*, 3272–3280.

Fondy, T. P., Pero, R. W., Karker, K. L., Ghangas, G. S., and Batzold, F. H. (1974), *J. Med. Chem. 17*, 697–702.

Fondy, T. P., Ross, C. R., and Sollohub, S. J. (1969), *J. Biol. Chem. 244*, 1631–1644.

Gerson, T., and Schlenk, H. (1968), *Chem. Phys. Lipids 2*, 213–220.

Ghangas, G. S., and Fondy, T. P. (1971), *Biochemistry 10*, 3204–3210.

Gray, G. R., and Barker, R. (1970), *Biochemistry 9*, 2454–2461.

Grundmann, C. (1936), *Justus Liebigs Ann. Chem. 524*, 31–48.

Hajra, A. K., and Agranoff, B. W. (1968), *J. Biol. Chem. 243*, 1617–1623.

Hartman, F. C. (1968), *Biochem. Biophys. Res. Commun. 33*, 888–894.

Hartman, F. C. (1970), *Biochemistry 9*, 1776–1782.

Hartman, F. C. (1971), *Biochemistry 10*, 146–154.

Hartman, F. C. (1972), *Methods Enzymol. 25B*, 661.

Hofstee, H. J. (1952), *Science 116*, 326–331.

Hooper, D. L. (1967), *J. Chem. Soc. B*, 169–170.

Husain, S. S., and Lowe, G. (1968), *Biochem. J. 108*, 855–859.

Lloyd, W. K., and Harrison, R. (1973), *Carbohyd. Res. 26*, 91–98.

Lloyd, W. K., and Harrison, R. (1971), *Carbohyd. Res. 20*, 133–139.

Lloyd, W. K., and Harrison, R. (1974), *Arch. Biochem. Biophys. 163*, 185–190.

Lynen, R. (1940), *Ber. 73*, 367–375.

Norton, I. L., Pfuderer, P., Stringer, C. D., and Hartman, F. C. (1970), *Biochemistry 9*, 4952–4958.

Rao, G. V., Que, L., Jr., Hall, L. D., and Fondy, T. P. (1975), *Carbohyd. Res.* (in press).

Reynolds, S. J., Yates, D. W., and Pogson, C. I. (1971), *Biochem. J. 122*, 285–297.

Sheehan, J. C., and Frank, V. S. (1950), *J. Am. Chem. Soc. 72*, 1312–1316.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324–332.