tivity of A and B to hydrolysis, column chromatography or preparative paper chromatography are not successful. The half-life in aqueous solution is about 30 min at pH 7.0 and decreases rapidly at pH levels above 8.

Inhibition of Glucosidases with Bromoconduritol. α-Glucosidase from yeast[15] and β-glucosidases from *Cicer arietum* and *Alocasia macrorrhiza*[11] show a time-dependent, irreversible inhibition with very low concentrations of bromoconduritol. From experiments with preparations partially enriched with respect to A or B, it was found that isomer A reacts with α-glucosidase whereas the above β-glucosidases react preferentially with B.

The contents of a reactive isomer in a given preparation can be determined from the degree of inhibition observed when a known molar excess of enzyme is allowed to react with the inhibitor. Based on the concentration of this isomer, we observed second-order kinetics for the reaction between α-glucosidase and one enantiomer of bromoconduritol A. The rate constants ranged from $8.7 \times 10^4\ M^{-1}\ \text{min}^{-1}$ at pH 6 to $2.2 \times 10^4\ M^{-1}\ \text{min}^{-1}$ at pH 5.0 and 7.4.

[41] Glycidol Phosphates and 1,2-Anhydrohexitol 6-Phosphates

By Edward L. O'Connell and Irwin A. Rose

The preparation of epoxide analogs was undertaken in the expectation that inactivation of an enzyme would signify that a nucleophile was positioned for a back-side attack on one of the ring carbons and that an acid group might be required for protonation of the ring oxygen. Such an antarafacial positioning of basic and acidic functional amino acids had been postulated in the aldose-ketose isomerase reactions for which the epoxide substrate analogs proved to be successful active site reagents in two cases tested: triosephosphate isomerase[1,2] and phosphoglucose isomerase.[3] In addition glycidol phosphate (glycidol-P) was effective against enolase.[1,2] In each case, a carboxyl oxygen on the enzyme was the nucleophile, leading to formation of an ester.

That the inactivators show *enantio*meric and diastereomeric specificities is seen in the table.

[1] I. A. Rose and E. L. O'Connell, *J. Biol. Chem.* **244,** 6548 (1969).
[2] K. J. Schray, E. L. O'Connell, and I. A. Rose, *J. Biol. Chem.* **248,** 2214 (1973).
[3] E. L. O'Connell and I. A. Rose, *J. Biol. Chem.* **248,** 2225 (1973).

STEREOSPECIFICITIES OF INACTIVATION

Enzyme	Compound	K_{inact} (mM)	V_{inact} (min^{-1})
Triosephosphate isomerase	*R*-Glycidol P	4	3.8×10^{-1}
	S-Glycidol P	5.2	3.8×10^{-2}
Enolase	*R*-Glycidol P	9	2.35×10^{-2}
	S-Glycidol P	15	6.70×10^{-3}
Phosphoglucose isomerase	(2*R*)-1,2-Anhydro hexitol-6-P	0.27	2.5×10^{-2}
	(2*S*)-1,2-Anhydro hexitol-6-P	0.47	5.0×10^{-4}

Preparation of Glycidol-P[1], -^{32}P, or -^{3}H Labeled

The phosphorylation reaction was carried out under anhydrous conditions. Use of *N,N*-dimethylaniline rather than pyridine gave a much purer and higher yield of glycidol-P by preventing acid catalyzed ring opening of the epoxide by Cl^-.

Reagents. These were purified as described below and stored under anhydrous conditions.

Trimethyl phosphate, dried over magnesium sulfate, filtered, and distilled at atmospheric pressure using a vacuum-jacketed distilling column; the fraction boiling at 193°–195° was collected

Dimethylaniline, dried over calcium hydride, filtered, and distilled from fresh calcium hydride at atmospheric pressure; b.p. 193°–195°

Phosphorus oxychloride, redistilled at atmospheric pressure before use; b.p. 106°–107°

Glycidol, fractionally distilled[4]

In an ice bath are mixed 0.5 ml of trimethylphosphate, 0.25 ml of *N,N*-dimethylaniline, and 0.1 ml of $POCl_3$ or $^{32}POCl_3$ (1 mmole). To the stirred solution is added a mixture of 0.5 ml of trimethyl phosphate containing 0.07 ml (1 mmole) of glycidol. After 1 hr at 0°, the reaction is added dropwise to 10 ml of cold water while maintaining the pH between 5 and 9 with 2 *N* NaOH. After final adjustment to pH 7.0, the dimethylaniline is extracted twice with 6 ml of ether. The aqueous solution is adjusted to pH 7.5, 2 ml of 1 *M* barium acetate are added, and

[4] The glycidol is freshly distilled at 11 mm of pressure and 56° [J. C. Sowden and H. O. L. Fischer, *J. Am. Chem. Soc.* **64,** 1291 (1942)]. The tritiated form is prepared by reduction of glycidaldehyde (see following) with NaB^3H_4 [modified to a small scale from P. H. Williams, G. B. Payne, W. J. Sullivan, and P. R. Van Ess, *J. Am. Chem. Soc.* **82,** 4883 (1960)]. *R,S*-glycidol is available commercially, or the two epimers may be prepared as indicated next.

the resulting precipitate of barium phosphate is discarded. Ethanol (2.5 volumes) is added, and the precipitate of barium glycidol phosphate formed after 30 min in ice is collected. The precipitate is dissolved in 10 ml of water, 2.5 volumes of ethanol are added, and the mixture is kept at 30° until shiny crystals begin to form. After standing overnight at 4°, the barium glycidol-P salt is obtained by filtration (180 mg, 63% yield).

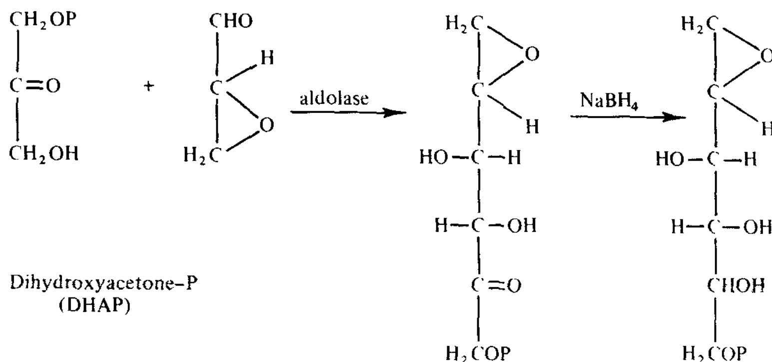
R,S-Glycidol-P was assayed by heating a ~0.1 m*M* solution in 1 *N* $HClO_4$ at 100° for 20 min, neutralizing with KOH, and assaying the L-glycerol-P formed.[5] Correcting for formation of glycerol-2-P, 14% at equilibrium, only 43% of *R,S*-glycidol-P can be expected as L-glycerol-P.

Preparation of *R,S*-Glycidaldehyde for Synthesis of *R,S*-[^{3}H] Glycidol-P and 1,2-Anhydro-*D,L*-hexitol-P's

R,S-Glycidaldehyde is available commercially (Aldrich) and should be vacuum-redistilled just before use. A simple preparation from acryl aldehyde with H_2O_2 at pH ~8 has been described.[6]

Preparation of Mixed 1,2-Epoxides of Hexitol-6-P's by Condensation of Glycidaldehyde with Dihydroxyacetone-P and Muscle Aldolase[3]

Use of *R,S*-glycidaldehyde yields a mixture of four diastereoisomers, epimers at C-2 and C-5. *R*-Glycidaldehyde gives 1,2-anhydro-D-mannitol-



6-P and 1,2-anhydro-L-gulitol-6-P (the 2*R* mixture). *S*-Glycidaldehyde gives the D-glucitol and L-iditol-6-P's (the 2*S* mixture).

One hundred micromoles of DHAP, 200 μmoles of triethanolamine-

[5] H. U. Bergmeyer, ed., "Methods of Enzymatic Analysis." Academic Press, New York, 1965.

[6] G. B. Payne, *J. Am. Chem. Soc.* 81, 4901 (1959).

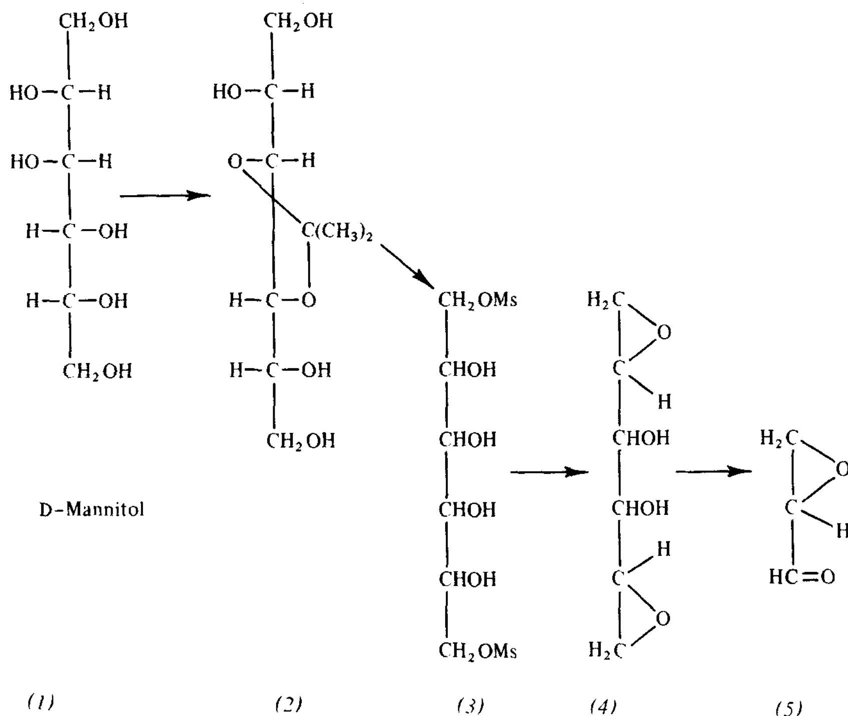
HCl, pH 7.5, and 2500 μmoles of glycidaldehyde are incubated at 25° in a final volume of 5.0 ml with 10 units of aldolase that is free of triosephosphate isomerase.[7] After 30 min, less than 3% of DHAP remains as assayed by glycerol-P dehydrogenase.[5] Aldolase is inactivated and precipitated by the addition of an equal volume of absolute alcohol. After 10 min at 25°, denaturated protein is removed by centrifugation. The supernatant liquid is concentrated to its original volume and chromatographed on a Sephadex G-10 column (2.5 × 92 cm) equilibrated with 0.05 *M* sodium acetate. The condensation product appears at 1.1 times the column void volume in approximately 20 ml. The condensation product is detected by the formation of dihydroxyacetone-P with adolase and its coupled reduction with glycerol-P dehydrogenase and DPNH. Attempts to avoid the Sephadex step and to purify the condensation product directly by barium precipitation led to an impure product.

The condensation product is adjusted to pH 9.0 and maintained at that pH during the stepwise addition of $NaBH_4$. If the hexitols are to be isotopically labeled, 5 μmoles of pyruvate are added as an internal standard for determination of tritium specific activity. An appropriate amount of carrier-free $NaBT_4$ is added and, after 10 min at 20°, is followed by 100 μmoles of $NaBH_4$. The loss of condensation product was followed by the coupled assay system described above. The reaction is terminated by dropping the pH to 4.0 with glacial acetic acid to destroy excess $NaBH_4$. After pH readjustment to 7.5, the volume is reduced to 4.0 ml in a rotary flash evaporator at 25° or less, and 1 ml of 1 *M* barium acetate and 16 ml of absolute ethanol are added. A precipitate forms slowly in an ice bath and is collected by centrifugation. The supernatant liquid is used for determination of specific activity of the lactate. The precipitate is dissolved in 2 ml of water; insoluble material is removed, and the precipitate is reformed by the addition of an equal volume of absolute ethanol. The precipitate is dried by washing with ethanol and ether and is weighed (34 mg; 75% yield containing 75 μmoles of organic phosphate). The specific activity that is used for further experiments is based on phosphorus content. The product is stored over desiccant at —70° to prevent decomposition.

Preparation of L-Glycidaldehyde[2] (for Synthesis of L-Glycidol-P and 2*S* Epoxihexitol-6-P's)

D-Mannitol to 1,6-di-*O*-methane sulfonyl-D-mannitol, (*1*) → (*3*) consists of three steps: (*i*) mannitol to the 1,2:3,4:5,6-tri-*O*-isopropylidine,[8]

[7] C. Richards and W. J. Rutter, *J. Biol. Chem.* **236**, 3185 (1961).
[8] L. J. Wiggins, *J. Chem. Soc. London* **1946**, 13.



(*ii*) partial hydrolysis to 3:4-isopropylidine mannitol,[9] and (*iii*) mesylation and hydrolysis to give (*3*).[10,11]

(*i*) Dry powdered mannitol (*1*), 160 g, is suspended in 2 liters of dry acetone with magnetic stirring. Concentrated H_2SO_4, 16 ml, is added, and the suspension is stirred for 48 hr; all the solid dissolves and the solution turns yellow. The stirred solution is neutralized by adding solid Na_2CO_3 until a sample is neutral to moist pH paper. After filtration, the residue is washed with acetone, and the combined filtrate plus wash is concentrated under reduced pressure with a flash evaporator. The solid residue is dissolved in acetone and dried twice to remove residual water. The solid is dissolved in absolute ethanol and redried twice to remove acetone. The solid is dissolved in 500 ml of 100% ethanol, filtered, and added slowly with stirring to 3 liters of water. The mixture is allowed to remain in the cold overnight, filtered, washed with H_2O until free of alcohol, and air dried. Yield, 140 g (53%); m.p. 69°.

(*ii*) The triacetone, 120 g, is selectively hydrolyzed according to Stern and Wasserman[9] to the 3,4-acetonyl mannitol in 72% yield.

[9] R. Stern and H. H. Wasserman, *J. Org. Chem.* **24,** 1689 (1959).
[10] L. Vargha, J. Kuszman, and B. Dumbovich, *Chem. Abstr.* **55,** 15365b (1959).
[11] L. Vargha and J. Kuszman, *Naturwissenschaften* **46,** 84 (1959).

(*iii*) To 55 g of 3,4-acetone-D-mannitol (0.25 mole) (*2*), dissolved in 300 ml of anhydrous pyridine, is added dropwise with stirring at −10°, 57.5 g (0.51 mole) of methanesulfonyl chloride over a period of 60 min. The mixture is stirred at room temperature for 4 hr and then poured into 500 ml of ice water. After three extractions with 400 ml of chloroform each, the combined chloroform extracts are washed with 400 ml of ice-cold H_2O, twice with 400 ml each of ice-cold 5 *N* H_2SO_4, twice with 400 ml of ice-cold saturated $NaHCO_3$, and twice with 400 ml of ice-cold H_2O. The chloroform is dried over $MgSO_4$ and evaporated under reduced pressure to yield a syrup. The syrup is dissolved in 100 ml of anhydrous dioxane, and 8.9 ml of 50% aqueous methanesulfonic acid are added. The reaction is maintained at room temperature for 24 hr, after which it is heated to 60° for 2 hr, cooled, and allowed to crystallize. The product(s) are recrystallized from warm absolute ethanol and have yielded 16.8 g (18%) of 1,6-di-*O*-methanesulfonyl-D-mannitol, m.p. 129°–131°.

(3) → (5) Epoxidation of (3)[12]

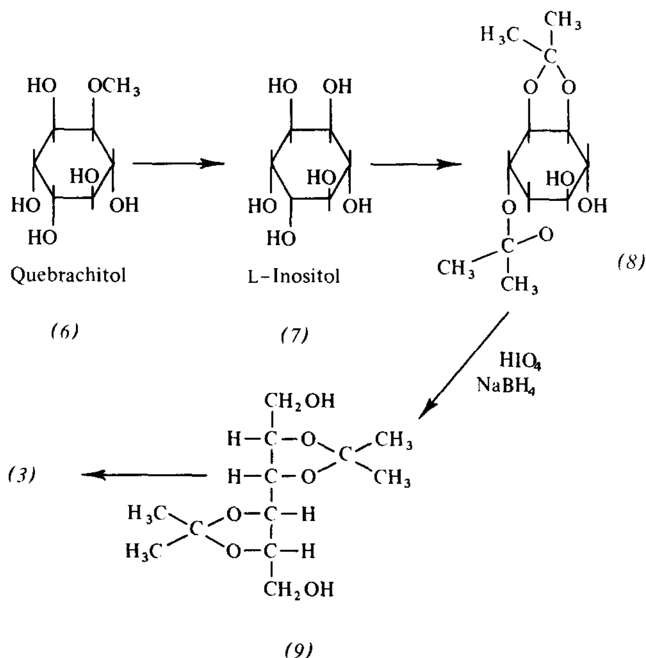
Compound (3), 6.8 g (20 mmoles), is suspended in 5 ml of H_2O with stirring, warmed to 37°, and carefully titrated to the phenolphthalein end point with 5 *N* NaOH until no additional alkali is consumed (40 mmoles NaOH are used). This solution is added dropwise to a stirred mixture of 150 ml of ethyl acetate and 30 g of anhydrous Na_2CO_3 and more Na_2CO_3 is added until the solid returns to an easily suspended solid. The residue is washed on the filter with ethyl acetate, and the filtrate is concentrated under reduced pressure to about 25 ml at a bath temperature of 30°. This material is redried with $MgSO_4$ and concentrated to a volume of 5 ml. A gummy solid is obtained upon cooling in an ice bath. The solid is washed with a small amount of ethyl acetate, and 1 g is dissolved in 4 ml of warm ethyl acetate. The insoluble material is removed by centrifugation and the product is allowed to crystallize and is dried under reduced pressure. The 0.7 g (4.8 mmoles) of slightly gummy solid (m.p. 59°–63°) is dissolved in 4 ml of water. Methyl red is added as indicator and the solution is adjusted with NaOH to the acid side of the indicator end point. Add 5 mmoles $NaIO_4$ (1.07 g) in small portions, adjusting the pH with NaOH to the indicator end point until no additional periodate is consumed (by starch potassium iodide paper indicator). The pH of the mixture is readjusted to the indicator end point. The solution is transferred to a small stopcock-sealable distillation apparatus, frozen in a Dry Ice–acetone bath, and evacuated under high vacuum; the distillate is

[12] M. Jarman and W. C. J. Ross, *Carbohydr. Res.* 9, 139 (1969).

collected in a receiver kept in a Dry Ice–acetone bath while the reaction solution is allowed to warm to 25°. The condensate contained 6 mmoles (62%) of glycidaldehyde as determined by the thiosulfate titration method of Ross.[13] The glycidaldehyde distillate is neutralized and redistilled. The distillate is stored at —70°.

Preparation of D-Glycidaldehyde[2] (for Synthesis of D-Glycidol-P and 2*R* Epoxihexitol-6-P's)

The procedure differs from that for L-glycidaldehyde because L-mannitol is not available commercially. Instead, 1,6-di-*O*-methanesulfonyl-L-mannitol is prepared from quebrachitol (*6*) by the following steps:



A crude sample of quebrachitol (Uniroyal Rubber Company, Plantation Division) was purified as reported.[2] The compound, 161 g, was converted to 76 g of (*9*) following the procedure of Gillett and Ballou.[14] The method for preparation of 1,6-di-*O*-methanesulfonyl-L-mannitol in 43% yield is given by Schray *et al.*[2]

[13] W. C. J. Ross, *J. Chem. Soc. London* **1950,** **2257.**

[14] J. W. Gillett and C. E. Ballou, *Biochemistry* **2,** 547 (1963).

Acknowledgments

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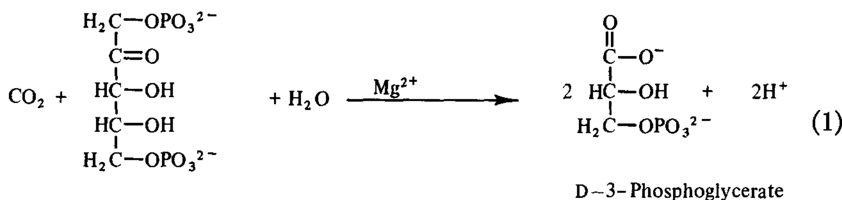
[42] Ribulosebisphosphate Carboxylase[1]

By I. LUCILE NORTON and FRED C. HARTMAN

Ribulosebisphosphate carboxylase[2-4] (EC 4.1.1.39), synonymous with fraction I protein,[5] is responsible for fixation of atmospheric CO_2 by plants and photosynthetic microorganisms.[6] The reaction catalyzed by this component of the reductive pentosephosphate cycle is shown in Reaction (1).



The divalent cation requirement can be served by Mn^{2+}, Co^{2+}, Fe^{2+}, or Ni^{2+} as well as Mg^{2+}.[2,7] Carbon dioxide, not bicarbonate, is the carbon species active in carboxylation.[8] Ribulosebisphosphate carboxylase also catalyzes the oxygenation of ribulosebisphosphate to yield phosphoglycolate and phosphoglycerate.[9-14] Thus, a single enzyme (termed ribu-

[1] Research from the authors' laboratory was sponsored by the Energy Research and Development Administration under contract with the Union Carbide Corporation.
[2] A. Weissbach, B. L. Horecker, and J. Hurwitz, *J. Biol. Chem.* **218**, 795 (1956).
[3] W. B. Jakoby, D. O. Brummond, and S. Ochoa, *J. Biol. Chem.* **218**, 811 (1956).
[4] E. Racker, *Arch. Biochem. Biophys.* **69**, 300 (1957).
[5] S. G. Wildman and J. Bonner, *Arch. Biochem. Biophys.* **14**, 381 (1947).
[6] M. Calvin, *Science* **135**, 879 (1962).
[7] J. M. Paulsen and M. D. Lane, *Biochemistry* **5**, 2350 (1966).
[8] T. G. Cooper, D. Filmer, M. Wishnick, and M. D. Lane, *J. Biol. Chem.* **244**, 1081 (1969).
[9] G. Bowes, W. L. Ogren, and R. H. Hageman, *Biochem. Biophys. Res. Commun.* **45**, 716 (1971).
[10] T. J. Andrews, G. H. Lorimer, and N. E. Tolbert, *Biochemistry* **12**, 11 (1973).
[11] G. H. Lorimer, T. J. Andrews, and N. E. Tolbert, *Biochemistry* **12**, 18 (1973).
[12] T. Takabe and T. Akazawa, *Biochem. Biophys. Res. Commun.* **53**, 1173 (1973).
[13] F. J. Ryan and N. E. Tolbert, *J. Biol. Chem.* **250**, 4229 (1975).
[14] F. J. Ryan and N. E. Tolbert, *J. Biol. Chem.* **250**, 4234 (1975).