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Stereochemistry of Reduction of D-Glyceraldehyde Catalyzed by a Nicotinamide Adenine Dinucleotide Phosphate Dependent Dehydrogenase from Skeletal Muscle†

D. J. Walton

ABSTRACT: Methods have been developed for determining the stereochemistry of the dehydrogenase-catalyzed reduction of D-glyceraldehyde by NADPH. To decide upon whether the A (*pro*-4*R*) or B (*pro*-4*S*) hydrogen of the dihydronicotinamide ring of NADPH is transferred to the substrate, D-[^{14}C]glyceraldehyde is reduced by A- and B-labeled [^{3}H]NADPH, and the ^{3}H:^{14}C ratios of the resulting samples of glycerol are measured. The direction of hydride attack at the carbonyl carbon is determined by examining the configuration of the tritiated carbon of the [^{3}H,^{14}C]glycerol resulting from the reduction involving tritium transfer. The [^{3}H,^{14}C]glycerol is successively phosphorylated (ATP, glycerokinase) and oxidized (NAD^+, glycerol-3-phosphate dehydrogenase) to form [1-^{3}H,^{14}C]dihydroxyacetone 1-phosphate. Hydrolysis of the latter (acid phosphatase) and phosphorylation (ATP, triokinase) of the resulting [^{3}H,^{14}C]dihydroxyacetone gave a mixture of [1-^{3}H,^{14}C]- and [3-^{3}H,^{14}C]dihydroxyacetone 1-phosphate. The configuration of the tritiated carbon of the latter, which is the same as that of the original [^{3}H,^{14}C]glycerol, is decided by determining which of the two enzymes, triosephosphate isomerase or muscle aldolase, labilizes the tritium atom. These procedures have been used to show that in the reduction catalyzed by the muscle glycerol dehydrogenase described by Kormann, A. W., Hurst, R. O., and Flynn, T. G. [(1972), *Biochim. Biophys. Acta 258*, 40] the A (*pro*-4*R*) hydrogen of NADPH attacks the *re* face of the carbonyl of D-glyceraldehyde.

A number of $NADP^+$-linked dehydrogenases which catalyze the reduction of aldehydes have been reported to occur in mammalian tissues (Kormann *et al.*, 1972; Bosron and Prairie, 1972). Since some of the enzymes are poorly characterized, and their substrate specificities overlap, the separate existence of each enzyme has been queried by the latter authors.

The preferred physiological substrate for the majority of these enzymes is D-glyceraldehyde, which is reduced to glycerol. It was assumed, *a priori*, that like most dehydrogenase-catalyzed reductions of carbonyl compounds (Levy *et al.*, 1962), the reduction of D-glyceraldehyde effected by each dehydrogenase is stereospecific. The overall objective of the present work is to explore the possibility of using the stereospecificity of reduction of D-glyceraldehyde by NADPH as an aid to the characterization of each dehydrogenase. This approach makes use of the ability of each enzyme to discrimi-

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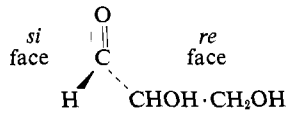
nate between diasterotopic[1] atoms of the coenzyme molecule (NADPH) and between diastereotopic faces of a single substrate molecule (D-glyceraldehyde). This contrasts with previous work, in which reaction rates of several substrates were compared for each dehydrogenase.

With this objective in mind, a method has been devised which provides answers to the following questions: (1) in the course of dehydrogenase-catalyzed reduction, which of the two hydrogen atoms, A (*pro*-4*R*)[1] or B (*pro*-4*S*), of the dihydronicotinamide ring of NADPH is transferred to D-glyceraldehyde, and (2) which of the two faces, *re* or *si*, of the trigonal (carbonyl) carbon atom is attacked by a hydride ion?



The first question is answered by performing separate reductions of D-[^{14}C]glyceraldehyde with A- and B-labeled [^{3}H]-NADPH. The degree of transfer of the labeled hydrogen atom is then determined by measurement of the ^{3}H:^{14}C ratios of the resulting samples of [^{3}H,^{14}C]glycerol.

The following strategy is used to answer the second question. Stereospecific attack at the *si* or *re* face of C-1 of D-glyceraldehyde by a tritium anion from [^{3}H]NADPH would give (3*S*)-*sn*-[3-^{3}H]glycerol[2] (I) or the 3*R* diastereomer (II), respectively (Figure 1, reaction a). In order to determine the configuration of the enzymically formed *sn*-[3-^{3}H]glycerol it is converted into [3-^{3}H]dihydroxyacetone phosphate ([3-^{3}H]-DHAP,[3] VII or VIII) without inversion or racemization at the tritiated carbon, using the reaction sequence of Figure 1. (Figure 1 also includes a similar reaction sequence starting with L-glyceraldehyde, which should be ignored for the present.) The determination of configuration of the [3-^{3}H]-DHAP (*S* for VII or *R* for VII) is based on the ability of aldolase and triosephosphate isomerase to specifically labilize the tritium of VII and VIII, respectively (Johnson *et al.*, 1965; Rieder and Rose, 1959).

The *sn*-[3-^{3}H]glycerol is first subjected (step b) to coupled phosphorylation (ATP, glycerokinase) and oxidation (NAD^{+}, glycerol-3-phosphate dehydrogenase) to form (*S*)- or (*R*)-[^{3}H]DHAP (V or VI). Owing to the stereospecificity of the glycerokinase reaction (Karnovsky *et al.*, 1957), only the tritiated carbinol (C-3) of *sn*-[3-^{3}H]glycerol is phosphorylated. The [^{3}H]DHAP (V or VI) resulting from step b is therefore tritiated only at the phosphorylated carbinol (C-1) and is unsuitable for configurational assignment by the test enzymes. To overcome this problem V or VI is hydrolyzed (step c) with

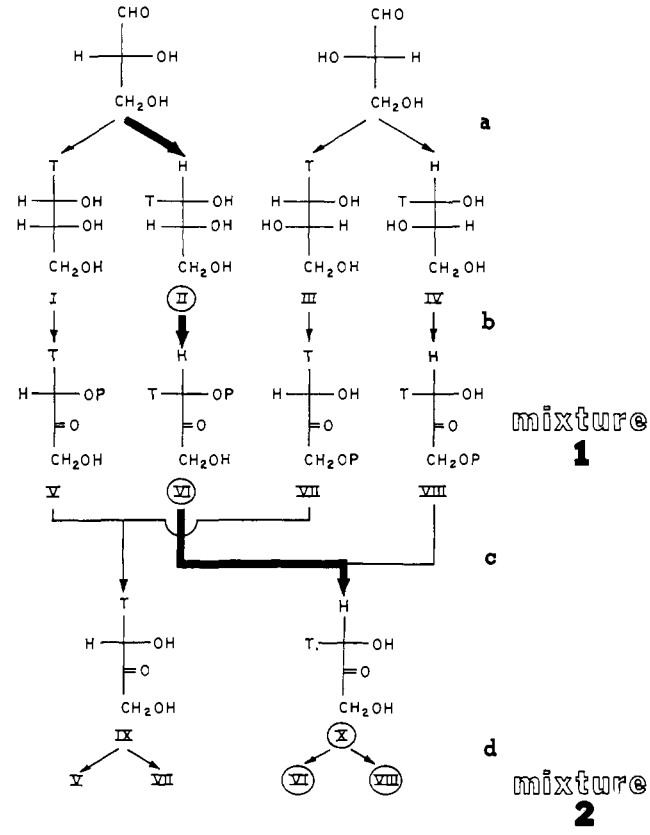


FIGURE 1: Conversion of glyceraldehyde into [1,3-^{3}H$_{1}$,^{14}C]DHAP. For the conversion of D-[U-^{14}C]glyceraldehyde, reagent a was A-labeled [^{3}H]NADPH and glycerol dehydrogenase. For the conversion of DL-glyceraldehyde, reagent a was [^{3}H]NaBH$_{4}$, and the resulting *rac*-[1-^{3}H]glycerol was supplemented with [U-^{14}C]glycerol. Other reagents were (b) ATP, glycerokinase, NAD^{+}, and glycerol-3-phosphate dehydrogenase; (c) acid phosphatase; (d) ATP and triokinase. The heavy arrows indicate the reaction pathway commencing with the enzymic reduction of D-[U-^{14}C]glyceraldehyde. Isomers resulting from this reaction are identified by encircled Roman numerals.

acid phosphatase to afford (*S*)- or (*R*)-[^{3}H]dihydroxyacetone (IX or X). Since the two carbinol groups of dihydroxyacetone are homotopic,[1] phosphorylation (step d) by ATP (triokinase) occurs at either the tritiated or nontritiated carbinol group, giving a mixture of [1-^{3}H]- and [3-^{3}H]DHAP (*S*, V + VII; or *R*, VI + VIII). The configuration of the [3-^{3}H]DHAP (VII or VIII) in the pair of isomers obtained is determined by checking for labilization of tritium by aldolase or triosephosphate isomerase.

In the work described below these reactions are applied to D-[U-^{14}C]glyceraldehyde to take advantage of double-isotope labeling methods. The procedures have been used successfully for defining the stereochemistry of the reduction catalyzed by the rabbit muscle glycerol dehydrogenase described by Kormann *et al.* (1972) and should be applicable to other dehydrogenases which effect the reduction of D-glyceraldehyde by NADPH.

Experimental Section

Materials

Glycerol dehydroganese from rabbit muscle, purified according to Kormann *et al.* (1972), exhibited similar electrophoretic behavior to that described by these authors, and has a specific activity of 0.25 unit/mg. Bovine triokinase (0.15 unit/

[1] In describing the stereochemistry of individual atoms or groups in a molecule the terminology summarized by Hirschmann and Hanson (1971) is used. The *R*–*S* nomenclature follows the Tentative Rules, Section E, of Fundamental Stereochemistry (IUPAC Commission on Nomenclature of Organic Chemistry, 1971).

[2] Stereochemical numbering of glycerol (IUPAC–IUB Commission, 1967) is used throughout. On reduction of D-glyceraldehyde the carbonyl carbon (C-1) becomes C-3 of glycerol. Glycerokinase phosphorylation of glycerol takes place at the hydroxyl of C-3, which therefore corresponds to the phosphorylated carbinol (C-1) of dihydroxyacetone phosphate. According to the stereochemical numbering rules a 1:1 mixture of 1- and 3-substituted glycerol is named as a 1-substituted derivative of *rac*-glycerol.

[3] Abbreviations used are: DHAP, dihydroxyacetone phosphate; A_{340}, absorbance at 340 nm with a path length of 1 cm; NAD^{+} and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADP^{+} and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate.

mg) was isolated as described by Charlton and van Heyningen (1969). Pig heart isocitrate dehydrogenase (Sigma "type IV"; 7 units/mg) was dialyzed against several changes of 0.02 M Tris-HCl (pH 7.5), thus reducing the concentration of glycerol (used in storage) to 50 mM. Sigma "type I" isocitrate dehydrogenase was supplied as a solid of 0.05 unit/mg.

rac-[1-^{3}H]Glycerol[2] (I–IV) prepared by reduction of DL-glyceraldehyde with [^{3}H]$NaBH_4$ and [U-^{14}C]glycerol were obtained from the Amersham-Searle Corp. An aqueous solution containing both these compounds was made with specific activities of 9.90×10^6 and 105×10^6 dpm/μmol for ^{14}C and ^{3}H, respectively. The mixture was termed *rac*-[1-^{3}H,U-^{14}C]-glycerol. All other radioactive compounds were products of New England Nuclear. D-[U-^{14}C]Glyceraldehyde was purified by paper chromatography in 1-butanol–pyridine–water (6:4:3, v/v). Its specific activity (3.88×10^6 dpm/μmol) was determined after enzymic assay by the procedure of Charlton and van Heyningen (1969). The supplier's samples of D-[1-^{3}H]-glucose (5.1 Ci/mmol) and potassium [2-^{3}H]isocitrate (8.1 Ci/mmol) did not contain significant amounts of impurities. It was assumed (Lowenstein, 1963) that one-quarter of the radioactivity of the 2-[^{3}H]isocitrate was attributable to the 2*R*,3*S* isomer.

The potassium salt of DHAP was prepared from the dimethyl ketal according to the directions of the manufacturer (Sigma). A solution of the disodium salt of ATP (60 mM) was brought to pH 8 with NaOH before use. To prepared glycine–hydrazine buffer (pH 9.8), glycine (1.5 g) and hydrazine hydrate (41.6 g) were dissolved in 30 ml of water. The solution was adjusted to pH 9.8 with 6 M HCl and water was added to a total volume of 100 ml.

Methods

Radioactive samples were dissolved in 10 ml of Aquasol scintillator solution (New England Nuclear). The total volume of water in each counting vial (including that introduced in aqueous samples) was adjusted to 1.0 ml, and the resulting solution was counted in a Unilux II machine (Nuclear-Chicago Corp.).

DHAP was estimated by measurement of the change of A_{340}[3] resulting from oxidation of NADH in the presence of glycerol-3-phosphate dehydrogenase (Bergmeyer, 1963).

[U-^{14}C]DHAP was isolated from enzyme incubation solutions by a modification of the procedure of Bartlett (1959). Each solution was adjusted to pH 7.5 with HCl or NaOH and applied to a column (1 × 18 cm) of Dowex 1-X8 chloride (50–100 mesh). The column was washed with 100 ml of water and eluted (4-ml fractions) with a linear gradient made from 250 ml of water (mixer) and 250 ml of 0.02 M HCl (reservoir). [U-^{14}C]DHAP appeared in fractions 50–80 (approximately) and elution curves of concentration and radioactivity coincided. When present, NAD^+ was eluted before DHAP, but NADH, $NADP^+$, NADPH, ADP, and ATP remained on the column. The combined DHAP fractions were evaporated to half-volume and lyophilized.

Prior to paper electrophoresis, which was performed at 4000 V in 8% formic acid, samples of [^{14}C]DHAP or *sn*-[^{14}C]glycerol 3-phosphate were passed through small columns of Dowex 50-W (H^+). The papers were scanned for radioactivity and then sprayed with alkaline $AgNO_3$ (Trevelyan *et al.*, 1950) to locate DHAP and dihydroxyacetone.

To determine the tritium distribution in [^{3}H,U-^{14}C]DHAP a known weight of the latter (Tables II and III, column 2) together with 5 μmol of unlabeled DHAP and 18 μmol of formaldehyde was treated with 12 μmol of $NaIO_4$ as described by Friedberg *et al.* (1971). After oxidation the resulting [^{14}C]-formaldehyde (from C-3 of DHAP) was converted into the dimedon derivative, which was extracted with *n*-hexane. The *n*-hexane solution (C-3) and the remaining aqueous solution (C-1 and C-2) were each concentrated to dryness, and the residues were counted in Aquasol–water (10:1, v/v).

All enzymic reaction mixtures were incubated at 25°. Before incubation of DHAP solutions with enzymes it was sometimes necessary to adjust the pH to that of the buffer employed.

Enzymic Conversions

[1,3-$^3H_1$,U-^{14}C]DHAP (V–VIII, Mixture 1) from rac-[1-^{3}H,-U-^{14}C]Glycerol (I–IV). An aqueous solution (0.2 ml) of *rac*-[1-^{3}H,U-^{14}C]glycerol (I–IV; 0.114 μmol) was mixed with 0.5 ml of glycine–hydrazine buffer (pH 9.8), 25 μl of ATP (1.5 μmol of sodium salt), 0.1 ml of NAD^+ solution (2.5 μmol), 25 μl of glycerol-3-phosphate dehydrogenase (3 units), 75 μl of glycerokinase (8 units), and 0.1 ml of $MgCl_2$ solution (2.5 μmol). The reaction was followed spectrophotometrically using a control solution containing no glycerol. An increase in A_{340} was observed over 20 min, corresponding to the formation of 0.09 μmol of NADH. The incubation mixture was shaken twice with benzaldehyde (0.5 ml) and the resulting hydrazone was removed by filtration. On shaking with benzaldehyde a third time no precipitate was formed, and the aqueous layer was separated and extracted three times with ether. The ether and recovered benzaldehyde were combined and back extracted with water. Carrier DHAP (1.0 μmol) in 0.17 ml of water was added to the combined aqueous solutions. The resulting solution was adjusted to pH 7.5 with 1 M NaOH. [1,3-$^3H_1$,U-^{14}C]DHAP (0.76 μmol) was isolated by Dowex 1 chromatography and dissolved in 2 ml of water. It contained four isomers (V–VIII) and was termed mixture 1. The ^{14}C specific activity (9.54×10^5 dpm/μmol) indicated that 0.106 μmol of labeled DHAP had been formed in the reaction mixture. Mixture 1 was electrophoretically pure (Figure 2), except for contamination with 2–3% of glycerophosphate (presumably the *sn*-3 isomer, formed by the glycerokinase reaction). Electrophoresis of a sample of mixture 1 which had been reduced by NADH in the presence of glycerol-3-phosphate dehydrogenase gave a single band corresponding to *sn*-[^{14}C]-glycerol 3-phosphate. Mixture 1 migrated as a single spot ($AgNO_3$ spray and radioscan) when chromatographed on paper in 1-butanol–acetic acid–water (74:19:50, v/v).

[^{3}H,U-^{14}C]Dihydroxyacetone (IX and X). A solution (5 ml) containing 0.60 μmol of [1,3-$^3H_1$,U-^{14}C]DHAP (V–VIII, mixture 1; 5.67×10^5 dpm of ^{14}C) and wheat germ acid phosphatase (2 mg, 1 unit) in 0.05 M sodium citrate buffer (pH 5.6) was incubated for 30 min. More acid phosphatase (2 mg, 1 unit) was added, and the incubation was continued for a further period of 30 min. The solution was adjusted to pH 7.5, deionized by passage through small columns of Dowex 1 acetate and Dowex 50-W (H^+), and lyophilized. On electrophoresis (Figure 2) the product migrated as a neutral, radioactive compound, which reduced $AgNO_3$. This behavior, together with its subsequent conversion into DHAP in the presence of triokinase (below), indicated that it was [^{3}H,U-^{14}C]dihydroxyacetone (0.51 μmol, based on disintegrations per minute of ^{14}C).

[1,3-$^3H_1$,U-^{14}C]DHAP (V–VIII, Mixture 2) from [^{3}H,U-^{14}C]-Dihydroxyacetone (IX and X). [^{3}H,U-^{14}C]Dihydroxyacetone (0.43 μmol) (IX and X), 40 μmol of $MgCl_2$, and 8.3 μmol of ATP were incubated for 20 min with triokinase (0.2 unit) and 0.05 M triethanolamine–HCl buffer (pH 7.5), in a total volume of 3 ml. Carrier DHAP (6.0 μmol) was added in

1.02 ml of water and 4.29 μmol of [1,3-$^3H_1$,U-^{14}C]DHAP (4.05×10^4 dpm of ^{14}C/μmol) was isolated by ion-exchange chromatography (0.27 μmol of product had therefore been formed during the incubation). The product, termed mixture 2, was chromatographically pure and free of *sn*-[^{14}C]glycerol 3-phosphate (Figure 2). Mixture 2 was completely reducible to the latter compound in the presence of glycerol-3-phosphate dehydrogenase and NADH.

Incubation of Mixture 2 with Aldolase and Triosephosphate Isomerase. V–VIII (1.43 μmol) (mixture 2, 5.79×10^4 dpm of ^{14}C) was incubated for 2 hr with rabbit muscle aldolase (1 unit) and 0.05 M triethanolamine buffer (pH 7.5) in a total volume of 2.5 ml. [^{3}H,U-^{14}C]DHAP (1.02 μmol) was recovered from the incubation mixture, using a Dowex 1 column, and dissolved in 3 ml of water. Another portion (1.43 μmol) of mixture 2 was subjected to the same procedure, except that triosephosphate isomerase (24 units) was used instead of aldolase.

A-Labeled [^{3}H]NADPH. An incubation mixture (1.2 ml) was made by dissolving the following in 0.1 M Tris-HCl buffer (pH 7.5): 0.8 μmol of $NADP^+$, 1 μmol of (2*R*,3*S*)-isocitric acid, 8 nmol of racemic potassium [2-^{3}H]isocitrate containing *ca.* 35×10^6 dpm of the 2*R*,3*S* isomer, 0.25 μmol of $MnCl_2$, and 1 unit of dialyzed, type IV, isocitrate dehydrogenase. The change of A_{340} indicated that 0.66 μmol of NADPH was formed. The solution was heated on a boiling water bath for 1.5 min to denature the enzyme, and the precipitate was removed by centrifugation. To obtain the specific activity of the product, the [^{3}H]NADPH of one-half of the supernatant was purified with a column of Whatman DE-52 DEAE-cellulose (Davies *et al.*, 1972). A value of 39.2×10^6 dpm/mol was obtained, based on A_{340}. The remainder of the supernatant solution was set aside for glyceraldehyde reduction.

B-Labeled [^{3}H]NADPH. In this case the incubation mixture (3.0 ml, in 0.1 M Tris-HCl buffer, pH 7.5) contained 0.9 μmol of $NADP^+$, 17 μmol of ATP, 20 μmol of $MgCl_2$, 1.0 μmol of D-glucose, 3 nmol of D-[1-^{3}H]glucose (*ca.* 35×10^6 dpm), 5 units of glucose-6-phosphate dehydrogenase, and 2 units of hexokinase. Formation of 0.8 μmol of [^{3}H]NADPH was indicated by the increase in A_{340}. Enzymes were denatured and the specific activity (32.1×10^6 dpm/μmol) was measured as for the A diastereomer. One-half of the supernatant was set aside for glyceraldehyde reduction.

Reduction of D-[U-^{14}C]Glyceraldehyde by A- and B-Labeled [^{3}H]NADPH. Each of the supernatant solutions of A- or B-labeled [^{3}H]NADPH (*ca.* 0.3 μmol) was incubated with 0.05 M triethanolamine buffer (pH 7.5), 0.15 μmol of D-[U-^{14}C]-glyceraldehyde (5.83×10^5 dpm), and glycerol dehydrogenase (0.23 unit) in a total volume of 3 ml. When the reactions were complete (20 min) water (5 ml) and glycerol (10 mmol) were added and each solution was kept at 100° for 4 min and centrifuged. The supernatants were deionized by successive passage through columns of Dowex 1 acetate and Dowex 50-W (H^+) and evaporated to dryness. Each sample of [U-^{14}C]-glycerol was benzoylated as described by Rose *et al.* (1962). Radioactivities of the tribenzoates derived from A- and B-labeled [^{3}H]NADPH were measured after successive recrystallization from methanol and *n*-hexane (Table I). The [^{3}H,U-^{14}C]-glycerol formed by reduction with A-labeled [^{3}H]NADPH was later shown to be compound II (see Results).

(3R)-sn-[3-^{3}H,U-^{14}C]Glycerol (II), for Determination of Configuration at C-3. A-Labeled [^{3}H]NADPH, of specific activity 137×10^6 dpm/μmol, was prepared as described above, except that 32 nmol of [2-^{3}H]isocitrate was used, and Sigma type I isocitrate dehydrogenase was employed to obviate complications arising from addition of unlabeled glycerol associated with type IV enzyme. D-[U-^{14}C]Glyceraldehyde (0.3 μmol) (1.16×10^6 dpm) was reduced by incubation with 0.6 μmol of A-labeled [^{3}H]NADPH (82.2×10^6 dpm) in the presence of glycerol dehydrogenase, as described above. The incubation mixture was then kept at 100° for 4 min, centrifuged, deionized, and concentrated to dryness. The residue of (3*R*)-*sn*-[3-^{3}H,U-^{14}C]glycerol was dissolved in 0.8 ml of water. [U-^{14}C]Glycerol (0.05 μmol) (2.0×10^6 dpm) in 0.2 ml of water was then added, to give *solution A* (subsequently used for configurational assignment) which contained 0.25 μmol of II (27.5×10^6 dpm of ^3H and 2.35×10^6 dpm of ^{14}C).

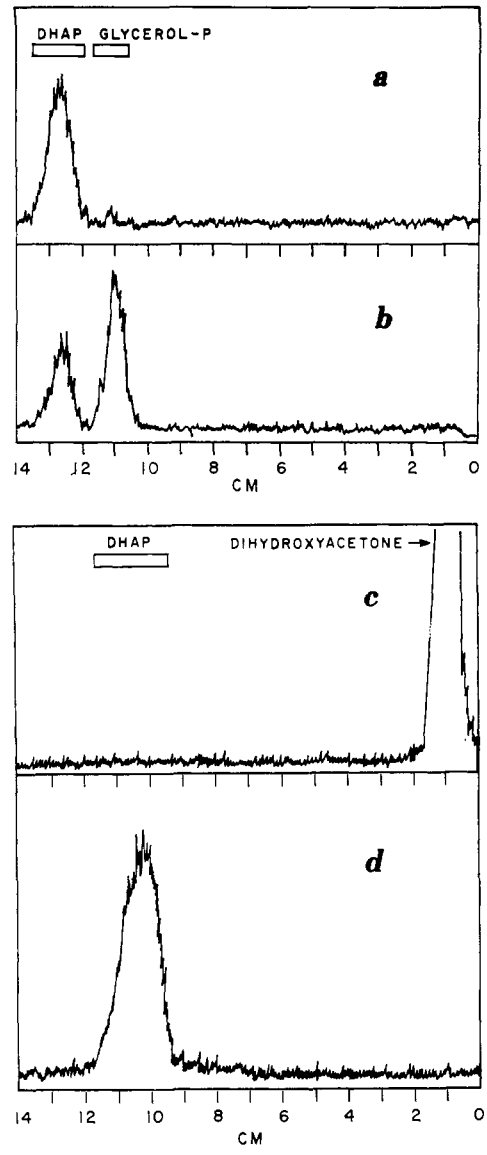


FIGURE 2: Scans of radioactivity, following high voltage electrophoresis, of compounds formed from chemically prepared *rac*-[1-^{3}H,U-^{14}C]glycerol. Radioactivity was detected by a Berthold Model LB 2722 radioscanner under the following conditions: time constant, 10 sec; range, 10 cpm; scanning speed, 2 in./hr; (a) [1,3-$^3H_1$,U-^{14}C]DHAP, mixture 1 (6,300 dpm of ^{14}C); (b) mixture 1 (5100 dpm of ^{14}C), supplemented with *sn*-[U-^{14}C]glycerol 3-phosphate (6100 dpm of ^{14}C, 0.02 nmol) and carrier *rac*-glycerol 1-phosphate (65 nmol), run for 15 min on same paper as a; (c) [^{3}H,^{14}C]-dihydroxyacetone (16,100 dpm of ^{14}C); (d) [1,3-$^3H_1$,U-^{14}C]DHAP, mixture 2 (14,000 dpm), run for 12 min on same paper as c. Bands attributed to DHAP and dihydroxyacetone were visualized with alkaline $AgNO_3$ spray.

TABLE I: Incorporation of Tritium into [U-^{14}C]Glycerol during Reduction of D-[U-^{14}C]Glyceraldehyde by A and B Forms of [^{3}H]NADPH.

Position of Label in [^{3}H]-NADPH	Data for [U-^{14}C]Glycerol Tribenzoate					g-atoms of Labeled Hydrogen Transferred to D-Glyceraldehyde	μmol of [U-^{14}C]Glycerol Formed in Incubation Mixture[b]
	Recrystzn Solvent	dpm/100 mg		^{3}H:^{14}C			
		^{3}H	^{14}C	Found	Calcd[a]		
A	Methanol	123,000	12,100	10.2			
A	*n*-Hexane	124,000	12,000	10.3	10.1	1.02	0.125
B	Methanol	1,420	13,000	0.11			
B	*n*-Hexane	1,310	12,800	0.10	8.3	0.01	0.13

[a] Value expected for complete transference of labeled hydrogen (specific activity of [^{3}H]NADPH:specific activity of D-[U-^{14}C]glyceraldehyde). [b] Calculation based on reverse isotope dilution of [^{14}C]glycerol. Approximately 0.15 mol of [^{3}H]NADPH was oxidized in each incubation, based on the observed decrease of A_{340}.

(R)-[1-^{3}H,U-^{14}C]DHAP (VI) from II. For the remainder of the conversions and incubations described in this section, the quantities of reactants and enzymes, experimental conditions, and yields were similar to those described for the conversion of I–IV into V–VIII (mixture 2). Radioactivities are shown in Table III.

II (0.12 μmol) (0.5 ml of *solution A*) was converted into VI. After carrier dilution and ion-exchange purification the specific activity of the resulting (*R*)-[1-^{3}H,U-^{14}C]DHAP (VI) was 8.83×10^{5} dpm/μmol (^{14}C). The electrophoretic behavior of VI was similar to that of mixture 1.

Hydrolysis of VI to Form X and Conversion of X into (R)-[1,3-$^{3}H_1$,U-^{14}C]DHAP (VI and VIII). (*R*)-[1-^{3}H,U-^{14}C]DHAP (VI) was converted into (*R*)-[^{3}H,U-^{14}C]dihydroxyacetone (X), which, in turn, was phosphorylated to give an electrophoretically pure mixture of VI and VII (4.30×10^{4} dpm/μmol of ^{14}C).

Incubation of a Mixture of VI and VIII with Aldolase- and Triosephosphate Isomerase. Samples (1.4 μmol each) of the mixture of VI and VIII were incubated separately with aldolase- and triosephosphate isomerase, and the [^{3}H,U-^{14}C]-DHAP was reisolated and counted in each case. Finally, [1,3-$^{3}H_1$,U-^{14}C]DHAP (V–VIII, mixture 2 of Table II; 0.94 μmol, 3.81×10^{4} dpm of ^{14}C) and (*R*)-[1,3-$^{3}H_1$,U-^{14}C]DHAP (VI and VIII, already incubated once with aldolase; 0.214 μmol, 9.20×10^{3} dpm of ^{14}C) were mixed and incubated with aldolase. The [^{3}H,U-^{14}C]DHAP was isolated and counted.

Results

Measurement of the ratio of ^{3}H:^{14}C in [^{14}C]glycerol formed by reduction of D-[^{14}C]glyceraldehyde with 4-labeled [^{3}H]-NADPH (in the presence of glycerol dehydrogenase) indicated that 1.02 and 0.01 g-atoms of hydrogen were transferred to 1 mol of glyceraldehyde from the A (*pro*-4*R*) and B (*pro*-4*S*) positions of NADPH, respectively (Table I). Use of ^{14}C-labeled D-glyceraldehyde of known specific activity permitted the quantity of [^{14}C]glycerol produced in each reduction to be estimated by the reverse isotope dilution principle. A reasonable correlation was obtained between the number of micro

TABLE II: Radioactivity of Chemically Prepared [^{3}H,^{14}C]Glycerol and of Derived [^{3}H,^{14}C]DHAP.

Sample[a]	nmol Counted	Whole Molecule			^{3}H/^{14}C			Distribution of ^{3}H,[b] % ^{3}H on	
		dpm × 10^{-3}			Dimedon Adduct (C-3)	Aqueous Solution (C-1 + C-2)	% Loss of ^{3}H from C-3	C-3	C-1
		^{14}C	^{3}H	^{3}H/^{14}C					
[1,3-$^{3}H_1$]Glycerol (I–IV)	5.35	53.0	563	10.6[e]					
[1,3-H_2]DHAP (V–VIII; mixture 1)	43.4	41.4	430	10.4	15.3	7.5		49	48
[1,3-H_2]DHAP (V–VIII; mixture 2)	154	6.23	65.4	10.5	16.1	7.4		51	47
Mixture 2, incubated with aldolase	202	8.18	66.8	8.2	8.4	7.7	48	34	63
Mixture 2, incubated with triosephosphate isomerase	210	8.52	69.0	8.1	8.5	7.6	47	35	63

[a] All compounds were uniformly labeled with ^{14}C. [b] Calculated thus: per cent ^{3}H on C-1 = $(200 \times (^{3}H/^{14}C)_{C\text{-}1+C\text{-}2})/(3 \times (^{3}H/^{14}C)_{\text{whole molecule}})$, and per cent ^{3}H on C-3 = $(100 \times (^{3}H/^{14}C)_{C\text{-}3})/(3 \times (^{3}H/^{14}C)_{\text{whole molecule}})$. [e] Isotope ratio was checked by dilution with carrier glycerol and measurement of radioactivity of benzoyl derivative.

TABLE III:[a] Radioactivity of Enzymically Prepared [^{3}H,^{14}C]Glycerol and of Derived [^{3}H,^{14}C]DHAP.

No.	Sample	nmol Counted	Whole Molecule dpm × 10^{-3} ^{14}C	Whole Molecule dpm × 10^{-3} ^{3}H	Whole Molecule ^{3}H/^{14}C	^{3}H/^{14}C Dimedon Adduct (C-3)	^{3}H/^{14}C Aqueous Solution (C-1 + C-2)	% Loss of ^{3}H from C-3	Distribution of ^{3}H, % ^{3}H on C-3	Distribution of ^{3}H, % ^{3}H on C-1
1	(3*R*)-*sn*-[3-^{3}H]Glycerol (II)[b]	7.25	68.2	798	11.7					
2	(*R*)-[1-^{3}H]DHAP (VI)	30.2	26.7	310	11.6	0.4	17.5		1	101
3	(*R*)-[1-^{3}H]DHAP (VI) and (*R*)-[3-^{3}H]DHAP (VIII)	224	9.65	113	11.7	17.1	9.3		49	53
4	Sample 3, after incubation with aldolase	171	7.34	86.6	11.8	17.0	9.1	1	48	51
5	Sample 3, after incubation with triosephosphate isomerase	213	9.18	58.2	6.3	0.6	9.3	97	3	98
6	Sample 4 + mixture 2 of Table II (1:4.39, w/w)	220	9.02	96.5	10.7					
7	Sample 6, after incubation with aldolase	179	7.35	66.6	9.1					

[a] Footnotes to Table II apply to this table also. [b] *Solution A* (see text). The configurational assignment is included for clarity, but was obviously not known initially.

moles of glycerol formed and the number of micromoles of B-labeled [^{3}H]NADPH oxidized (estimated spectrophotometrically). It was therefore safe to attribute the low tritium specific activity of the [^{3}H,^{14}C]glycerol formed by reduction with B-labeled [^{3}H]NADPH to an insignificant degree of tritium transfer and not to a low yield of glycerol. Glycerol dehydrogenase is therefore an A-specific enzyme which effects the transference of the *pro*-4*R* hydrogen of NADPH to D-glyceraldehyde.

In order to test the feasibility of the proposed method of configurational assignment, the reaction sequence of Figure 1 was applied to a mixture of [U-^{14}C]glycerol and *rac*-[1-^{3}H]-glycerol prepared by reduction of DL-glyceraldehyde with [^{3}H]$NaBH_4$. Whether or not the borohydride reduction was stereoselective, the resulting labeled glycerol would be expected to consist of a racemic mixture of I–IV containing equal weights of I + III [(*S*)-CHTOH] and II + IV [(*R*)-CHTOH]. Since the tritium of the final [^{3}H,U-^{14}C]DHAP (mixture 2) was almost equally distributed between C-1 and -3 (Table II), the ratio of (V + VI) to (VII + VIII) was approximately unity. About one-half of the tritium of C-3 was lost on incubation of mixture 2 with aldolase or triosephosphate isomerase, from which it was deduced that mixture 2 contained approximately equal weights of (*S*)- and (*R*)-[3-^{3}H,U-^{14}C]DHAP (VII and VIII, respectively), reflecting the 1:1 ratio of *S* to *R* isomers in the original [^{3}H,U-^{14}C]glycerol. Judging from ^{3}H:^{14}C ratios, no tritium was lost to the medium in the course of reactions b, c, and d, thus eliminating the possibility of formation of enolate intermediates, with possible inversion or racemization involving the loss of a *pro-S* or *pro-R* methylene tritium atom. The effectiveness of this method of determining the configuration of the [^{3}H,^{14}C]glycerol depends upon the fact that a constant ratio of (*S*)- to (*R*)-CHTOH groups is maintained throughout the reaction sequence, presumably because enzymic conversions b, c, and d do not involve cleavage or formation of the carbon–tritium bond. Apparently, similar degrees of conversion must have been achieved for isomers differing only in the location of a tritium atom or in the configuration of a tritiated carbon atom (*e.g.*, V and VII or IX and X), and for tritiated and nontritiated groups in the same molecule (*e.g.*, CHTOH and CH_2OH of IX).

It follows that the *sn*-[^{3}H,U-^{14}C]glycerol 3-phosphate which contaminated the [^{3}H,U-^{14}C]DHAP of mixture 1 would have had a similar ^{3}H:^{14}C ratio and tritium distribution to that of the [^{3}H,U-^{14}C]DHAP. This impurity was probably hydrolyzed by acid phosphatase, but the resulting [^{3}H,U-^{14}C]-glycerol would not have been phosphorylated by triokinase (Heinz and Lamprecht, 1961), thus accounting for the absence of labeled glycerophosphate in mixture 2.

The procedure was used to determine the configuration of the tritiated carbon atom of *sn*-[3-^{3}H,U-^{14}C]glycerol prepared by reduction of D-[U-^{14}C]glyceraldehyde with A-labeled [^{3}H]NADPH in the presence of glycerol dehydrogenase. As expected, [^{3}H,U-^{14}C]DHAP formed in step b was tritiated almost entirely at C-1, whereas the tritium of the final [^{3}H,U-^{14}C)DHAP formed in step d was almost equally distributed between C-1 and -3 (Table III). Incubation of the final [^{3}H,-U-^{14}C]DHAP with triosephosphate isomerase resulted in the loss of 97% of the tritium attached to C-3, and a 1% loss was incurred on incubation with aldolase.

Although every effort was made to ensure that the aldolase incubation conditions were the same as those used for the equivalent incubation of [^{3}H,U-^{14}C]DHAP formed from DL-glyceraldehyde, it was conceivable that the failure of aldolase to labilize the tritium of dehydrogenase-derived [^{3}H,U-^{14}C]-DHAP was due to deactivation of aldolase by an unknown impurity. To investigate this possibility, some of the aldolase-treated [^{3}H,U-^{14}C]DHAP from enzymically prepared [^{3}H,U-^{14}C]glycerol (Table III, entry 4) was mixed with a sample of mixture 2, which contained approximately equal proportions of V, VI, VII, and VIII. On incubation with aldolase the ^{3}H:^{14}C ratio fell from 10.7 to 9.1. Assuming that the dehydrogenase-formed [^{3}H,U-^{14}C]DHAP had not lost any tritium, the calculated loss of tritium from mixture 2 was 20%. Since 22% of the tritium of mixture 2 had already been shown to be aldolase labile (Table II), it was concluded that the aldolase

employed in the incubations of Table III was capable of labilizing the *pro-S* tritium atom of VII, but that the [^{3}H,U-^{14}C]-DHAP derived from enzymically prepared [^{3}H,U-^{14}C]glycerol did not contain a significant proportion of this isomer.

It was concluded that the final [^{3}H,U-^{14}C]DHAP contained *ca.* 50% (*R*)-[3-^{3}H,U-^{14}C]DHAP (VIII) which lost the majority of its tritium on incubation with triosephosphate isomerase. It was infrerred that the other constituent was (*R*)-[1-^{3}H,U-^{14}C)DHAP (VI). Assuming that the configuration of the tritiated carbon atom was retained throughout steps b–d, the glycerol dehydrogenase effected reduction must have afforded (3*R*)-*sn*-[3-^{3}H,U-^{14}C]glycerol (II). Therefore, during the enzymic reduction the hydride of NADPH attacked the *re* face of the carbonyl of D-glyceraldehyde, to become the *pro*-3*R* hydrogen atom of glycerol.

Discussion

(a) This work illustrates two advantages of using ^{14}C-labeled substrates in stereochemical studies of tritium transfer. (i) The degree of conversion of substrate into product may be estimated by reverse ^{14}C isotope dilution of the latter, regardless of its tritium content. If the specific activity is sufficiently high, such an estimate requires the use of only a small fraction of the product, leaving the remainder for further conversion. (ii) Following the preparation of a double-labeled compound, tritium retention can be estimated more accurately by comparisons of ^{3}H:^{14}C ratios (<2% error) than by measurements of specific activities of single-labeled ^{3}H compounds, which are often based on enzymic assays in which errors of 5% can be incurred.

(b) The observed stereospecificity of D-glyceraldehyde reduction is the same as that of the reduction of acetaldehyde by yeast and liver alcohol dehydrogenases (Popják, 1970), in that a *pro*-4*R* hydrogen is transferred from the coenzyme to the *re* face of the carbonyl group.

(c) Bentley (1970) has made the generalization that "the stereospecificity of a particular reaction is fixed and does not depend on the source of the enzyme." If this applies to the reduction of D-glyceraldehyde by different NADP$^+$-linked dehydrogenases, it would be impossible to characterize each enzyme according to the stereospecificity of D-glyceraldehyde reduction which it catalyzes. However, the proposed comparative study seems worthwhile in view of the fact that several examples have been cited (Popják, 1970) of dehydrogenases from different sources which exhibit different stereospecificities with respect to the direction of hydride attack on a single substrate. Such enzymes are exceptions to Bentley's generalization, which is based largely on data concerning the A or B stereospecificity of hydride transfer to or from NAD$^+$.

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