Lyase Activity of Nucleoside 2-Deoxyribosyltransferase: Transient Generation of Ribal and Its Use in the Synthesis of 2'-Deoxynucleosides

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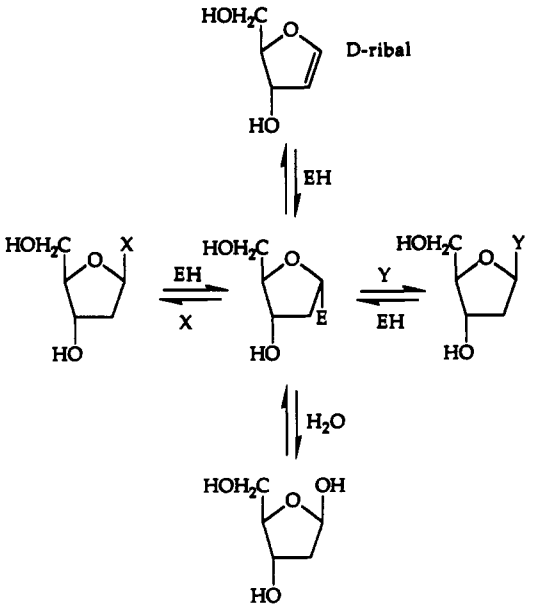
ABSTRACT: In the absence of acceptors nucleoside 2-deoxyribosyltransferase catalyzes the slow hydrolysis of 2'-deoxynucleosides. During this hydrolytic reaction, D-ribal (1,4-anhydro-2-deoxy-D-*erythro*-pent-1-enitol), a glycal of ribose hitherto encountered only as a reagent in organic synthesis, is generated spontaneously, disappearing later as 2'-deoxynucleoside hydrolysis approaches completion. Nucleoside 2-deoxyribosyltransferase is found to catalyze the hydration of D-ribal in the absence of nucleic acid bases and the synthesis of deoxyribonucleosides from ribal in their presence, affording a new method for the preparation of 2'-deoxyribonucleosides. The stereochemistry of nucleoside formation from ribal supports the intervention of a deoxyribosyl-enzyme intermediate. The equilibrium constant for the covalent hydration of ribal is found to be approximately 65.

β-Galactosidase and β-glucosidase are strongly inhibited by glycals, 1,2-unsaturated analogues of substrates that gradually undergo covalent hydration in the presence of enzyme to yield 2-deoxygalactose and 2-deoxyglucose, respectively (Lehmann & Schröter, 1972; Wentworth & Wolfenden, 1974; Legler et al., 1975; Viratelle & Yon, 1980). Slow onset of inhibition and release from inhibition of β-galactosidase by D-galactal are observed, and the K_i value of this pseudocompetitive inhibitor is equivalent to the rate constant for hydrolytic breakdown of the EI complex to 2-deoxygalactose divided by the rate constant for formation of the EI complex (Wentworth & Wolfenden, 1974). In the case of β-glucosidase, hydrolysis of a common 2-deoxyglycosyl-enzyme intermediate determines the rates of both the hydration of D-glucal and the hydrolysis of D-glucosides. The glycosyl-enzyme intermediate has been identified as an acylal involving a carboxylate group at the active site (Roeser & Legler, 1981).

The present paper deals with some novel reactions of nucleoside 2-deoxyribosyltransferase, an enzyme not previously known to have much in common with β-galactosidase or β-glucosidase. Nucleoside 2-deoxyribosyltransferase II [EC 2.4.2.6] (Holguin & Cardinaud, 1975) differs from these glycosidases in that it acts on pentose rather that hexose derivatives, is specific for substrates lacking a hydroxyl group at the 2' position, and is normally not considered a hydrolase. Instead, the usual function of nucleoside 2-deoxyribosyltransferase II is to catalyze the transfer of deoxyribose between nucleic acid bases, shown as X and Y in Scheme I. This enzyme, a hexamer containing six identical subunits of MW 16000, was recently crystallized with the use of protein purified from a strain of *Escherichia coli* that expresses the *ntd* gene of *L. leishmanii* (Cook et al., 1990).

In the absence of acceptors, nucleoside 2-deoxyribosyltransferase catalyzes the slow hydrolysis of 2'-deoxynucleosides. We find that during this latter hydrolytic reaction D-ribal (1,4-anhydro-2-deoxy-D-*erythro*-pent-1-enitol, a glycal of ribose hitherto encountered only as a reagent in organic synthesis (Ireland et al., 1980; Cheng et al., 1985)), is generated spontaneously, disappearing later as 2'-deoxynucleoside hydrolysis approaches completion. Nucleoside 2-deoxyribosyltransferase is also found to catalyze the hydrolysis of D-ribal in the absence of nucleic acid bases and the synthesis of deoxyribonucleosides from ribal in their presence, completing the equilibration process shown in Scheme I. The equilibrium constant for covalent hydration of ribal has been determined, and the stereochemistry with which nucleosides are synthesized from ribal supports the intervention of a deoxyribosyl-enzyme intermediate as shown in Scheme I. This latter reaction offers a new method for the preparation of 2'-deoxyribonucleosides.

Scheme I: Reactions Catalyzed by Nucleoside Deoxyribosyltransferase II[a]



[a] X and Y represent purine or pyrimidine bases, and EH represents a nucleophilic group at the enzyme's active site.

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Experimental Procedures

Materials

Crystalline nucleoside 2-deoxyribosyltransferase II from *Lactobacillus leishmanii* (Cook et al., 1990) exhibited a specific activity of 12.3 units/mg, where 1 unit of activity was defined as the number of micromoles of 2′-deoxycytidine produced per minute at 28 °C in sodium phosphate buffer (0.1 M, pH 6.0), with 1 mM 2′-deoxyinosine and 1 mM cytosine as substrates. Samples of D-ribal used in our initial studies were a generous gift from Prof. Doyle Daves of Rensselaer Polytechnic Institute. Uniformly labeled [^{14}C]thymidine (500 mCi/mmol) was obtained from Amersham Corp. Nucleic acid bases and 2′-deoxynucleosides were obtained from Sigma Chemical Co. Plastic-backed silica gel TLC plates (20 × 20 cm) and cellulose TLC plates (20 × 20 cm), both containing fluorescent indicator, were obtained from Kodak Co. Eluants used for the development of silica gel plates were (solvent A) ethyl acetate saturated with 0.1 M sodium phosphate buffer, pH 6.0; (solvent B) solvent A with 7% methanol or ethanol added; (solvent C) ethyl acetate/acetone/methanol 8:8:1. Radioactivity associated with the products of the action of the enzyme on uniformly labeled [^{14}C]thymidine was measured after chromatography with use of an Ambis autoradiographic detector and by liquid scintillation counting of samples removed from plates by scraping. All kinetic experiments were carried out at 25 °C in the presence of sodium phosphate buffer (0.1 M, pH 6.0).

Methods

D-Ribal as a Substrate. Hydration of D-ribal was followed by observing the appearance of 2-deoxyribose by TLC, with use of silica gel plates and solvent C. A solution containing D-ribal (0.1 mmol) and enzyme (0.94 mg) in 100 mL of buffer was incubated at 22 °C, and samples were removed at intervals and dried on TLC plates along with authentic D-ribal and 2-deoxyribose standards. After development, D-ribal and 2-deoxyribose were visualized by spraying with a mixture of 10% H_2SO_4 in 60% aqueous ethanol and heating at 100 °C for a few minutes. Alternatively, D-ribal and 2-deoxyribose were visualized by spraying with a freshly prepared aqueous solution containing equal volumes of a 2% solution of $FeCl_3$ and a 1% solution of $K_3Fe(CN)_6$, heating at 100 °C for 5 min, followed by spraying with 2 N HCl. The enzyme dependence of the hydration of D-ribal was demonstrated by incubating a 1 mM solution of D-ribal in buffer under similar conditions in the absence of enzyme.

Enzymatic synthesis of 2′-deoxyadenosine from D-ribal and adenine was observed by incubating enzyme (0.09 mg) in buffer (100 mL) containing adenine (0.2 mmol) and D-ribal (2 mmol), removing samples at intervals for TLC analysis with use of cellulose plates and water as eluant. After chromatography as described above, deoxynucleosides and nucleic acid bases were visualized with a short-wavelength UV lamp. The production of other deoxynucleosides, including thymidine and deoxyuridine, was followed in a similar manner except that TLC analysis was performed with silica gel TLC plates and solvent A followed by solvent B.

Stereochemistry of the Reaction of Ribal with Adenine. In these experiments, an aqueous buffer containing 0.1 M potassium phosphate, pH 5.7, was used, and to obtain the deuterated product, the same buffer was taken to dryness in a vacuum oven and dissolved in D_2O to yield pD = 6.1. HPLC, using a Whatman Partisil 10 M9 ODS-2 semipreparative column (9.4 mm × 50 cm), eluting with 30% methanol in water at a flow rate of 4 mL/min, gave a retention time of 9.5 min for 2′-deoxyadenosine. ^{1}H NMR spectra were observed in D_2O, with a Varian 400-MHz spectrometer. To prepare the protonated product, D-ribal (18 mg, 0.155 mmol) was incubated with adenine (12.5 mg, 0.093 mmol) and enzyme (2.84 mg) in buffer (2.9 mL) at 37 °C for 3 h. The reaction was stopped by the addition of absolute ethanol (5 mL). After centrifugation to remove precipitated protein, the supernatant was evaporated to dryness under reduced pressure and dissolved in water (3 mL). The entire 3 mL was purified in the following manner. Approximately 300 mL was dried in a band near the bottom of a cellulose plate and developed with distilled water to a distance of 15–16 cm. The band corresponding to 2′-deoxyadenosine was removed by scraping and was eluted twice with water (3 mL), and the eluate was dried under vacuum to yield a slightly brown solid. This material was dissolved in distilled water (1 mL) and purified by HPLC as described above. Fractions containing purified 2′-deoxyadenosine were combined, evaporated to dryness, and dissolved in a small amount of D_2O for analysis by NMR. Deuterated 2′-deoxyadenosine was generated in a similar manner except that this slower reaction was allowed to proceed for 32 h.

Production of D-Ribal during the Hydrolysis of Thymidine and the Reversibility of Glycal Hydration. Hydrolysis of uniformly labeled [^{14}C]thymidine, with concomitant formation of [^{14}C]-D-ribal, was followed by TLC using an initial substrate concentration of 20 mM and varying amounts of enzyme. At intervals ranging from 10 s to 23 h, 5-mL aliquots were removed from the incubation mixture and added to 5 mL of methanol. From this methanolic mixture, 5 mL was withdrawn and applied to a silica gel TLC plate, which had been divided into lanes measuring 1 cm wide. The assay procedure was designed in such a way that 5 mL of the methanolic mixture spotted onto the TLC plate contained approximately 50 000 cpm. The first lane of each plate contained authentic D-ribal, thymidine, thymine, and 2-deoxyribose, and the second lane contained [^{14}C]thymidine (50 000 cpm), as standards. With use of solvent A, each plate was developed to a height of 15 cm, which proved sufficient to provide separation of the desired compounds. In most cases, a second development with solvent B was used to enhance resolution.

Two methods were used to measure the radioactivity associated with the products of the hydrolysis reaction. The first method involved the use of an Ambis analytical autoradiographic detector, with a typical scan time of 1 h; in this procedure, background counts were determined by selecting a large area of the TLC plate that had been eluted with solvent but was beyond any part of the plate that contained radioactivity. Alternatively, radioactive spots were removed from the TLC plate by scraping, at positions corresponding to those of authentic D-ribal, thymine, thymidine, 2-deoxyribose, and the origin and analyzed with use of a liquid scintillation counter. In this procedure, background counts were determined by scraping an area of the plate, of a size similar to those of the other spots removed, from a portion of the plate that had been eluted with solvent but was beyond any point that had been reached by radioactive material. Results obtained by these methods were in satisfactory agreement with one another. Because uniformly labeled [^{14}C]thymidine contained twice the number of radioactive carbon atoms as each of the products of its hydrolysis, its specific activity was assumed to be twice that of D-ribal, thymine, or 2-deoxyribose. The counts associated with thymidine were therefore divided by 2 before addition to the total, and the radioactivity associated with each spot, after this correction, is reported as the percentage of the total counts per minute.

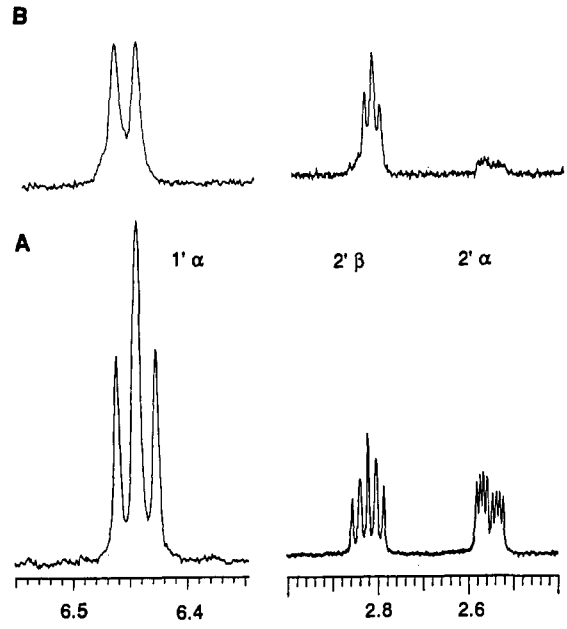

FIGURE 1: Simplified ^1H NMR spectra of 2'-deoxyadenosine produced via the enzyme-catalyzed condensation of D-ribal with adenine, performed in H_2O buffer (A) or D_2O buffer (B).

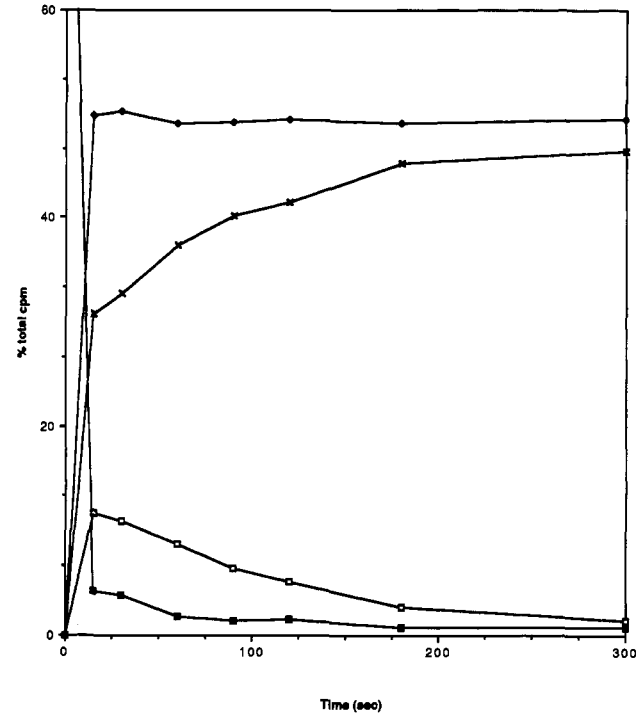

FIGURE 2: Time course showing the decrease in thymidine (■), the increase in thymine (◆) and 2-deoxyribose (×), and the transient increase and subsequent decrease of D-ribal (□) during the enzyme-catalyzed hydrolysis of uniformly labeled [^{14}C]thymidine at a substrate concentration of 2×10^{-5} M and an enzyme concentration of 8×10^{-6} M. Time points assayed were 15, 30, 60, 90, 120, 180, 300, 900, 1800, and 4500 s. The last three time points are not shown.

RESULTS

D-Ribal as a Substrate. D-Ribal was found to serve as a substrate for nucleoside 2'-deoxyribosyltransferase in either the presence or the absence of nucleic acid bases. Incubation with enzyme in the absence of acceptors led to the formation of 2-deoxyribose. This hydration reaction was enzyme-catalyzed, since incubation of ribal in buffer without enzyme led to negligible formation of 2-deoxyribose over a period of 12 h. Incubation with adenine or other purines or pyrimidines led to formation of the corresponding 2'-deoxynucleoside. In either the presence or absence of enzyme, we detected small quantities of an unidentified compound that migrated just behind D-ribal on silica gel plates, with the use of either solvent system A or B. D-Ribal undergoes slow decomposition in protic solvents (D. Daves, personal communication). This side reaction was found to occur much more slowly than the enzymatic hydration of D-ribal and did not appear to be enzyme-catalyzed.

Stereochemistry of Enzyme-Catalyzed Formation of 2'-Deoxyadenosine from D-Ribal and Adenine. The α and β anomeric protons of deoxynucleosides have distinctive coupling patterns (Robins & Robins, 1965), and if the enzyme-catalyzed condensation of ribal and adenine is performed in deuterated buffer, then the resulting 2'-deoxyadenosine isolated will be deuterated at the 2' position. Comparison of the ^1H NMR spectra of the deuterated deoxynucleoside with that of authentic ribal should indicate whether ribal was deuterated from the *re* or *si* face. Thus, the enzyme-catalyzed formation of 2'-deoxyadenosine was performed in buffered aqueous solution in order to determine its stereochemistry at the anomeric position and in buffered D_2O solution in order to determine the stereochemistry of deuterium addition during the formation of 2'-deoxyadenosine. The partial ^1H NMR spectra of the isolated 2'-deoxyadenosine and deuterated 2'-deoxyadenosine are shown in Figure 1. Spectrum A was observed for 2'-deoxyadenosine isolated from the reaction carried out in H_2O, and spectrum B was observed for 2'-deoxyadenosine isolated from the reaction carried out in D_2O.

The 1' proton of the β anomers of deoxynucleosides is observed as an apparent triplet, whereas that of the α anomers is observed as a doublet of doublets (Robins & Robins, 1965). Spectrum A indicates that the β anomer of 2'-deoxyadenosine was produced by DRT II from ribal and adenine. In addition, decoupling experiments showed that the resonance of the α 2' proton of deoxynucleosides occurs at approximately 2.56 ppm, whereas the resonance of the β 2' proton occurs at 2.82 ppm, in agreement with that of 2'-deoxyadenosine produced by the enzyme reaction. In spectrum B, the signal from the anomeric proton (6.45 ppm) was simplified, indicating that deuterium substitution had occurred at the 2' position. In addition, the signal from the β 2' proton (2.85 ppm) was simplified and the signal corresponding to the α 2' proton (2.56 ppm) was greatly diminished. These results indicate that ribal is protonated from the bottom or *si* face of the glycal ring during the formation of 2'-deoxynucleosides. The small signal at 2.56 ppm, corresponding to the α 2' proton, arises from the fact that the enzyme was dialyzed against H_2O and then diluted approximately 33-fold into a reaction mixture containing D_2O, so that a small amount of ^1H was incorporated at the 2' position.

With use of similar substrate and enzyme concentrations, condensation of ribal with adenine was found to proceed significantly more rapidly in H_2O than in D_2O buffer. Thus, within 3 h of initiation, a quantity of 2'-deoxyadenosine was generated that was sufficient for NMR analysis, whereas under similar conditions in D_2O, deuterated 2'-deoxyadenosine was barely detectable by TLC. These results are consistent with the possibility that protonation of ribal determines the rate of enzymatic synthesis of 2'-deoxyadenosine, at least in part.

Production of D-Ribal during the Hydrolysis of Uniformly Labeled [^{14}C]Thymidine. Table I and Figures 2 and 3 show the results of a series of experiments designed to test the reversibility of glycal hydration. When uniformly labeled [^{14}C]thymidine (2 mM) was exposed to high concentrations

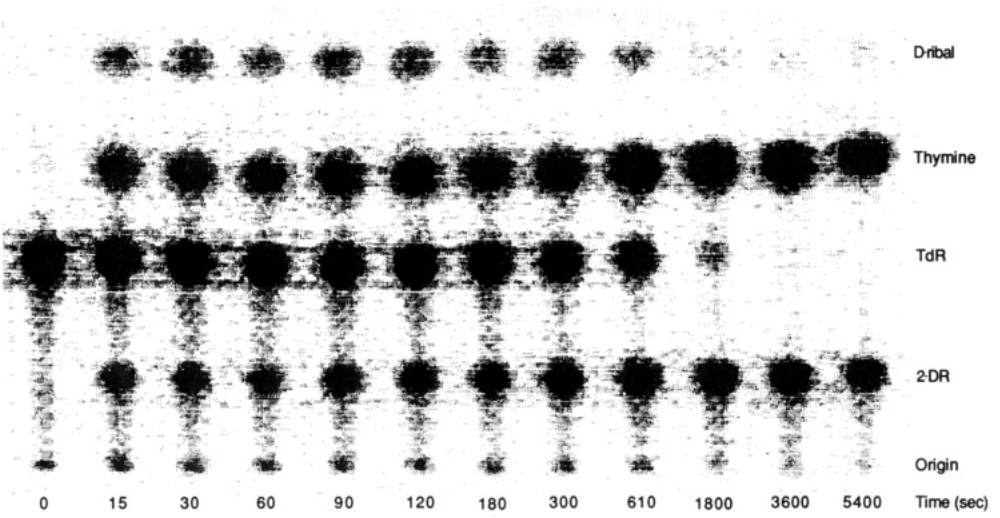

FIGURE 3: Results of a 2-h Ambis scan of the enzyme-catalyzed hydrolysis of [U-^{14}C]thymidine at a substrate concentration of 20 mM and an enzyme concentration of 0.52 mM. A 20 × 20 cm silica gel TLC plate was developed first with solvent A and then with solvent B, to a height of approximately 15 cm.

Table I: Selected Data from the Generation of D-Ribal during the Hydrolysis of Thymidine (2×10^{-5} M) at 25 °C in the Presence of Nucleoside 2-Deoxyribosyltransferase

[enzyme][a] (μM)	$[ribal]_{max}$[b] (% total cpm)	$[ribal]_{max}$ (μM)	$[ribal]_{equil}$[c] (% total cpm)	$[ribal]_{equil}$ (μM)	[2-dR)/ $[ribal]_{equil}$	$t_{1/2}$ (s[d])
8	11.7	4.7	0.61	0.24	70	70
8	13.9	5.6	0.73	0.29	66	50
8.8	15.7	6.3	0.60	0.24	70	60
0.53	5.2	2.1	0.60	0.24	70	340
0.53	5.6	2.2	0.64	0.25	63	390
0.18	2.0	0.64	0.70	0.28	59	>1000

[a] Based on a molecular mass of 86 kDa (Cook et al., 1990). [b] The maximum concentration of D-ribal attained during thymidine hydrolysis (see Figures 2 and 3). [c] The concentration of D-ribal remaining after very long periods of incubation (see Figures 2 and 3). [d] Estimated from a semi-logarithmic plot of the percentage of total counts per minute vs time.

of enzyme (8×10^{-6} M), a "burst" of D-ribal was formed within the first 15 s of the reaction, the earliest time at which the products were isolated for analysis. As time progressed, the concentration of D-ribal declined until an apparent equilibrium between D-ribal, thymidine, thymine, and 2-deoxyribose had been established after approximately 15 min. Under these conditions, the half-time for the disappearance of D-ribal ws estimated as roughly 60 s, and the overall hydrolysis of [^{14}C]thymidine was nearly complete after 15 s. From these data, the concentration ratio of D-ribal to 2-deoxyribose was estimated as 1:2.5 after 15 s, whereas at longer times it approached a value of 1:70. These initial experiments suggested that glycal hydration might be a reversible process but did not establish whether the concentration of D-ribal was still increasing after 15 s or had already begun to decline. In a second series of experiments, conducted at a lower enzyme concentration (5×10^{-7} M), hydrolysis was found to proceed more slowly but the reaction was again essentially complete after 30 min. The concentration ratio of D-ribal to 2-deoxyribose was estimated as 1:3 after 15 s, whereas at longer times it approached a value of 1:65. In this second set of experiments, the half-time for the disappearance of D-ribal was estimated as 345 s. Thus, the maximum amount of D-ribal formed was found to have decreased by only a factor of 2, and the half-time for its disappearance had increased by only a factor of 6, although the concentration of enzyme had been lowered by a factor of 15.

In a third set of experiments, in which the enzyme concentration (1.8×10^{-7} M) was reduced by an additional factor of 3, a further reduction was observed in the overall rate of hydrolysis and hydrolysis was not complete until after more than 1 h. Table I shows that the concentration ratio of D-ribal to 2-deoxyribose at early times attained a somewhat lower maximum value (1:4.1) than in previous experiments. These results also differed from those of the previous experiments in that D-ribal was not produced in a burst but remained at a similar level throughout the first hour of the experiment, approching a final value of approximately 1:65 as in the previous experiments.

Discussion

Is Ribal Normally Released into Free Solution? The appearance of D-ribal during enzymatic hydrolysis of thymidine, reminiscent of the appearance of *cis*-aconitate as an intermediate during the action of aconitase on citric acid, leads one to question whether this glycal is generated in free solution or only as an enzyme-bound intermediate. Speyer and Dickman (1956) showed that during the action of aconitase, *cis*-aconitic acid remained largely enzyme-bound. In our experiments, the extent and time course of formation of D-ribal were found to be dependent on the concentration of enzyme that was present. Except at the lowest concentrations of enzyme, D-ribal was produced in a burst, reaching a maximum concentration at the earliest time points examined. D-Ribal was produced in a burst at two different enzyme concentrations, 8×10^{-6} and 5.3×10^{-7} M, from 2×10^{-5} M thymidine. The enzyme appears to be a hexamer (Cook et al., 1990), and if one assumes that a single active site is present per monomer, then the concentrations of active sites would be 3.2×10^{-5} and 2.2×10^{-6} M, respectively. Reduction of the enzyme concentration by a factor of 15 resulted in a 2-fold decrease in the amount of D-ribal formed initially, and its rate of disap-

Table II: Equilibrium Constants for the Enzymatic Hydration of C=C Double Bonds[a]

reaction	$K_{hydration}$[a]
phosphoenolpyruvate → 2-phosphoglycerate[b]	0.16
crotonyl-CoA → 2-hydroxybutyryl-CoA[c]	3.4
fumarate → malate[d]	4.4
stearate → 2-hydroxystearate[e]	30
cis-aconitate → citrate[f]	31
ribal → 2-deoxyribose[g]	65
NADH → NADH 5,6-hydrate ("NADHX")[h]	92

[a] Based on the overall concentration of the adduct divided by the overall concentration of the addend. [b] From Wold and Ballou (1957). [c] From Stern and del Campillo (1956). [d] From Bock and Alberty (1953). [e] From Niehaus and Schroepfer (1965). [f] From Krebs (1953). [g] From the present work. [h] From Acheson et al. (1990).

pearance was reduced by a factor of 6. These disparities would be difficult to understand if D-ribal remained enzyme-bound throughout the course of the reaction because events following deoxynucleoside binding should then appear to be a single unimolecular process. If, on the other hand, ribal were released to some extent into free solution, then one would expect to observe a higher proportion released at lower concentrations of enzyme, since dissociation produces two molecules from one. In a qualitative sense, the present findings suggest that D-ribal may be released, at least to some extent, into free solution during the course of nucleoside hydrolysis by nucleoside deoxyribosyltransferase II. During deoxyribosyl-transfer reactions to better acceptors than water, however, it is entirely possible that D-ribal is never released in appreciable quantities.

Position of Equilibrium of Hydration of D-*Ribal.* Lehmann and Schröter (1972) were unable to detect the formation of galactal during the hydrolysis of a 2-deoxygalactoside by β-galactosidase and concluded that glycal hydration was irreversible. Our results confirm that equilibrium lies strongly in favor of 2-deoxyribose and indicate an apparent equilibrium constant of 65 for hydration of D-ribal. This is not far removed from values observed for the hydration of *cis*-aconitate and for the 5,6-hydration of NADH (Acheson et al., 1990). Like D-ribal, these compounds contain isolated double bonds. Table II compares these values with equilibrium constants that have been reported for the hydration of other compounds of biological importance.

Stereochemistry and Implications for the Mechanism of Deoxyribosyl Transfer. The present results indicate that nucleoside deoxyribosyltransferase II shares with certain glycosidases the ability to catalyze hydration and hydrolysis reactions as well the glycosyl-transfer reactions that it normally catalyzes. The stereochemical changes observed in the present experiments extend this similarity. Thus, glycosidases that retain, as well as those that invert, the configuration of the sugar at C-1 have been found to catalyze the hydration of glycals (Chiba et al., 1988; Hehre et al., 1977; Lehmann & Ziegler, 1977; Kanda et al., 1986). Glycosidases that act with overall retention of configuration protonate the glycal on the side of the ring opposite the side from which the aglycone leaving group would depart in the normal hydrolytic reaction. In contrast, glycosidases that act with overall inversion of configuration protonate D-glucal on the same side as that from which the leaving group departs in the normal hydrolytic reaction (Hehre et al., 1986; Chiba et al., 1988). If nucleoside 2-deoxyribosyltransferase, a configuration-retaining glycosyltransferase, acted on D-ribal by a mechanism similar to the action of the configuration-retaining glycosidases mentioned above, then the protonation of D-ribal during the formation of 2′-deoxyadenosine would be expected to occur from the bottom (or *si*) face of ribal and the β anomer of 2′-deoxyadenosine would be expected to result from the enzymatic condensation of ribal with adenine. The present findings show that this is the case, supporting the analogy to configuration-retaining glycosidases.

Practical Implications for the Chemical Synthesis of 2-Deoxynucleosides. Nucleoside 2-deoxyribosyltransferase II lacks specificity with respect to donors and acceptors, rendering this enzyme potentially useful for chemical synthesis of novel 2-deoxyribonucleosides from simple and abundant precursors: a nucleoside and an acceptor base. After the reaction is complete, it becomes necessary to separate the product nucleoside from any substrate nucleoside that remains at the end of the reaction. The present reactions, illustrated by the synthesis of 2′-deoxyadenosine from ribal and adenine, circumvent this difficulty by eliminating the substrate nucleoside from the reaction mixture.

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