Stereochemical Analysis of the Elimination Reaction Catalyzed by D-Amino-Acid Oxidase†

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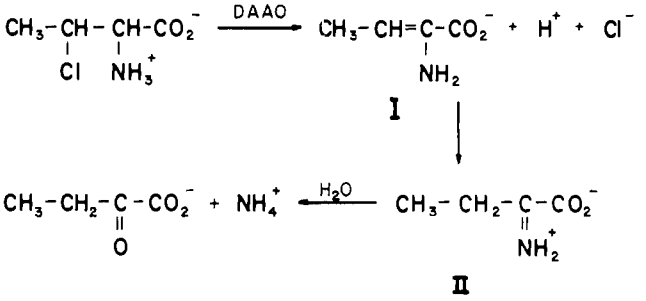
ABSTRACT: The stereochemistry of the intramolecular proton transfer catalyzed by the flavoenzyme, D-amino-acid oxidase, during the elimination reaction of β-chloro-α-amino acid substrates (Walsh et al. (1973), *J. Biol. Chem. 248*, 1946) has been established. Both D-*erythro*- and D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate have been shown to yield (3*R*)-2-keto[3-^{3}H]butyrate predominantly. Tritium kinetic isotope effects on the rate of the reaction (4.7 for the D-erythro, and 3.8 for the D-threo compound) and percentages of intramolecular triton transfer (7.2% for the D-erythro- and 2.6% for the D-threo compound) have been measured. Their implications on the mechanism of this unusual elimination reaction are discussed.

Of the various flavoenzymes that catalyze the oxidation of α-hydroxy acids or α-amino acids, three have recently been shown to carry out the α,β elimination of the elements of HCl from β-chloro substrates. Thus the *Mycobacterium smegmatis* L-lactate oxidase catalyzes the formation of pyruvate from 3-chloro-L-lactate (Walsh et al., 1973a), while hog kidney D-amino-acid oxidase and snake venom L-amino-acid oxidase produce the same compound from the appropriate enantiomer of 3-chloroalanine (Walsh et al., 1971). In each case, these β-chloro substrates partition between this oxygen-independent elimination pathway and the normal oxygen-utilizing conversion to the β-chloro-α-keto acid product. Evidently, the eliminations proceeded via some carbanion intermediates and, by analogy, it was suggested that the normal oxidations did the same.

Extension of the investigations with 3-chloroalanine to the four carbon homologue, namely the two diastereomers of 2-amino-3-chlorobutyrate, revealed the unexpected result that D-amino-acid oxidase carried out exclusively the oxygen-independent elimination reaction, the only products being NH_4^+, Cl^-, and 2-ketobutyrate. No oxygen uptake could be detected (Walsh et al., 1973b). The most likely initial product from the HCl elimination is the enamine intermediate I which would subsequently tautomerize to the imine II, followed by hydrolysis to the keto acid (as shown in Scheme I). When the elimination was carried out in ^2H_2O, deuterium was incorporated into C3 of the product 2-ketobutyrate, but only to the extent of 0.5 atom per molecule. Furthermore, the deuterated product was achiral at C3 (Walsh et al., 1973b). By analogy with the intramolecular tritium transfer observed in the case of 3-chloro[2-^{3}H]alanine (Walsh et al., 1973b), we suspected that a similar intramolecular proton transfer might have been responsible for this incomplete deuterium incorporation. Indeed, we were able to show that, when D-*erythro*-2-amino-3-chloro[2-^{2}H]butyrate was the substrate, the product, 2-ketobutyrate, did incorporate 0.2 atom of deuterium per molecule (Walsh et al., 1973b).

Scheme I



To further our investigation on the nature of this unusual elimination reaction, we have now prepared samples of α-tritiated 2-amino-3-chlorobutyrate, which only undergoes the nonoxidative elimination. Noting that the substrate has two asymmetric centers (i.e., C2 and C3), we have synthesized and characterized the (2*R*,3*R*) (i.e., D-erythro) and (2*R*,3*S*) (i.e., D-threo) diastereomers labeled with tritium at C2. Separate incubations with D-amino-acid oxidase produced samples of 2-keto[3-^{3}H]butyrate. The absolute configuration of each sample has now been established. The results reported in this paper have thus elucidated certain stereochemical aspects of this D-amino-acid oxidase catalyzed elimination reaction.

Experimental Procedure

Materials

Coenzyme A (lithium salt), NADH, D-threonine, avidin, L-threonine, and DL-threonine were purchased from Sigma Chemical Company; DL-allothreonine was from Matheson Scientific. All other chemicals and solvents were of commercial reagent grade.

Pig heart propionyl-CoA carboxylase (EC 6.4.1.3) was purified according to the procedures of Kaziro et al. (1961), omitting the alumina gel treatment. The enzyme had a specific activity of 0.7 unit/mg at 20 °C and was free of ATPase and methylmalonyl-CoA isomerase activities. D-Amino-acid oxidase (EC 1.4.3.3) was isolated and purified from pig kidneys using Brumby and Massey's method (1968). The enzyme after the calcium phosphate-cellulose column was essentially homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had specific activities ranging from 5 to

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10 μmol of D-alanine min^{-1} mg^{-1} at ambient temperatures. Lactic dehydrogenase (chicken heart) and catalase were from Sigma, and pyruvate kinase was from Boehringer Mannheim.

The sodium salt of [2-^{14}C]propionic acid (14 Ci/mg), DL-2-amino[1-^{14}C]butyrate (100 μCi/1.4 mg), and tritiated water (1 Ci/ml) were purchased from New England Nuclear.

(*R,S*)-[2-^{3}H]Propionic acid was prepared as reported (Cheung, et al., 1975) using a modified procedure of Murray and Williams (1958).

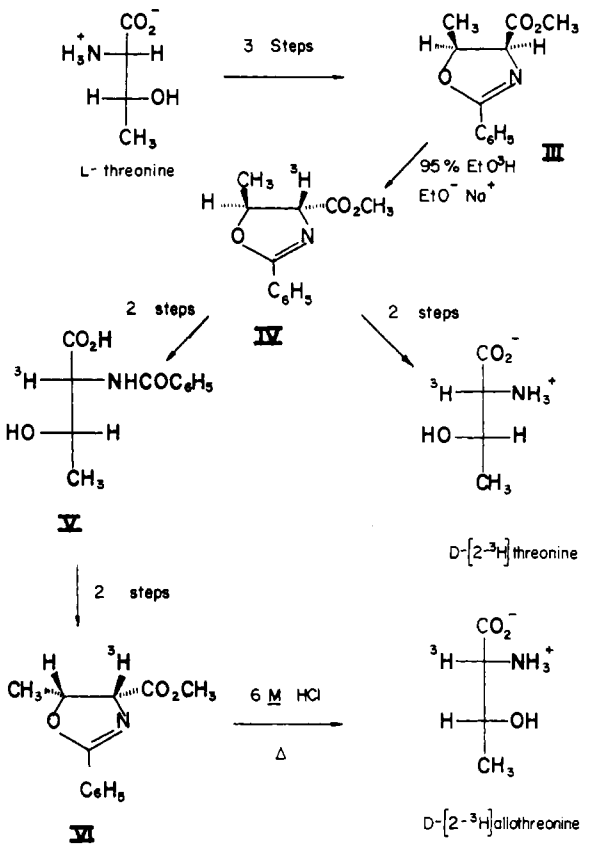
Radiochemical Syntheses

D-[2-^{3}H]Threonine (2R,3S). D-[2-^{3}H]Threonine was synthesized by modifying the elegant procedures of Elliot (1950) for interconverting the four isomers of threonine as shown in Scheme II. Thus 2.0 g of methyl *cis*-L-2-phenyl-5-methyl-Δ^2-oxazoline-4-carboxylate (III) (Elliot, 1950) was dissolved in 6.0 ml of absolute ethanol. With stirring and cooling in an ice bath, 3.5 ml of absolute ethanol containing 0.23 g of dissolved clean sodium metal and 0.5 ml (0.5 Ci) of $^{3}H_2O$ were added. The color of the solution instantly changed from yellow to reddish brown. After stirring for 30 min, the solution solidified to a pale yellow mass. Five milliliters of H_2O was added and the solution refluxed for 15 min. The tritiated solvent was removed by bulb-to-bulb distillation. The solid residue was repeatedly dissolved in H_2O and lyophilized. The residue was refluxed for 4 h in 20 ml of 6 M HCl. Workup of the D-[2-^{3}H]threonine was identical with the procedure of Elliot (1950) and Walsh et al. (1973b). Typically, 700 mg of recrystallized D-[2-^{3}H]threonine (specific radioactivity of 3.2 × 10^5 cpm/μmol) was obtained, representing a yield of about 60% from the *cis*-oxazolinecarboxylate (III).

D-[2-^{3}H]Allothreonine (2R,3R). In the case of D-[2-^{3}H]allothreonine (erythro, 2*R*,3*S*), again 2 g of methyl *cis*-L-2-phenyl-5-methyl-Δ^2-oxazoline-4-carboxylate (III) was the starting material. After the epimerization in tritiated ethanol, and removal of excess tritiated solvent as described, the residue was taken up in 10 ml of cold 2.0 N HCl. After standing at room temperature for 4 h, the solution was cooled in an ice bath and titrated with concentrated NaOH to pH 8–9 (pH paper). The solution was stirred for another 15 min. The solution, still cooled in the ice bath, was reacidified to pH 2 with concentrated HCl. Now the solution was extracted five times with 10 ml of ethyl acetate. After drying over anhydrous Na_2SO_4, the volume of the combined ethyl acetate solution was reduced to about 5 ml by boiling off the solvent. After filtration, crystals began to form on cooling; 1.5 g of a white solid, presumably *N*-benzoyl-D-[2-^{3}H]allothreonine (V; mp 145–146 °C (lit. 146–148 °C)), was collected. The methylation of this compound and cyclization to methyl *cis*-D-2-phenyl-5-methyl-Δ^2-oxazoline-4-carboxylate (VI) were as reported (Elliot, 1950). A reddish oil (VI; 1.34 g) was obtained. Eight milliliters of 6 N HCl was added; a white emulsion formed immediately. On swirling the flask the emulsion changed to a clear solution. The insoluble brown oily residue was discarded. The solution was refluxed for 3 h. Workup, as usual, yielded 0.60 g of D-[2-^{3}H]allothreonine (specific radioactivity, 6.0 × 10^5 cpm/μmol), representing a 50% yield from the *cis*-oxazoline intermediate (III).

The Two Diastereomers of 2-Amino-3-chloro[2-^{3}H]butyrate (See Scheme IV): D-Erythro-2-amino-3-chloro[2-^{3}H]butyric acid (2R,3R) was prepared from D-[2-^{3}H]threonine (2*R*,3*S*) as previously reported by Walsh et al. (1973b); *D-threo-2-amino-3-chloro[2-^{3}H]butyric acid (2R,3S)* was prepared with a minor modification from D-[2-^{3}H]allothreonine by the same procedure. Owing to the specific insolubility of the ethyl ester hydrochloride of D-allothreonine in chloroform, the *n*-propyl ester was employed instead in the chlorination step. 1-Propanol was substituted for ethanol in the preparation of the ester. The yield was 50% from D-[2-^{3}H]allothreonine.

Scheme II

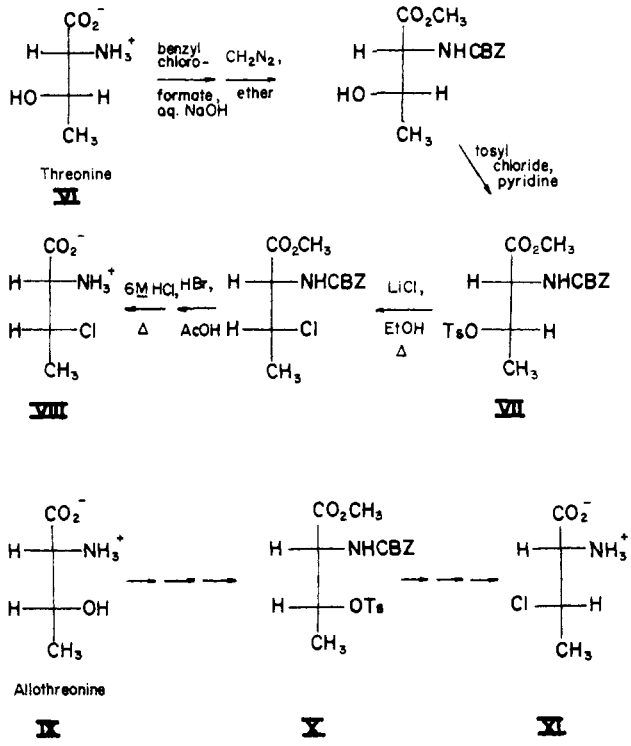


The structures of the four above-mentioned compounds were confirmed by NMR. In the case of the [2-^{3}H]threonines, purity was determined by paper chromatography (Shaw and Fox, 1955). Each was free from the other diastereomer. Each of the four compounds showed only one radioactive spot on the same paper chromatograph system, corresponding exactly to the ninhydrin spot.

In order to confirm the structures of the two diastereomers of 2-amino-3-chloro[2-^{3}H]butyrate prepared as described, the two compounds were alternatively synthesized from DL-allothreonine and DL-threonine through independent chemical transformations as shown in Scheme III. Standard procedures for protecting and subsequent deblocking of the functional groups involved were employed (Greenstein and Winitz, 1961; Elliot, 1950; Plattner et al., 1957). Chlorination was accomplished by refluxing the tosylates (VII and X in Scheme III) in absolute ethanol with excess LiCl for 17 h.

2-Hydroxy[1-^{14}C]butyrate was synthesized enzymatically from DL-2-amino[1-^{14}C]butyrate by the action of D-amino-acid oxidase followed by the lactic dehydrogenase–NADH reduction of the 2-keto[1-^{14}C]butyrate produced. The incubation contained 15 μmol of $NaPP_i$ (pH 8.5), traces of catalase, 0.6 mg of D-amino-acid oxidase, and 0.7 mg (50 μCi) of DL-2-amino[1-^{14}C]butyrate in a total volume of 0.25 ml. At the end of 1 h at room temperature, the solution was transferred to a cuvette. $NaPP_i$ (0.5 ml, 0.1 M, pH 7) was added, followed by 0.5 μmol of NADH. Fifty units of lactic dehydrogenase was added and the disappearence of NADH followed spectrophotometrically by the decrease in A_{340}. More NADH was

Scheme III



added, 0.5 μmol at a time until no further reaction. About 2 μmol of L-2-hydroxy[1-^{14}C]butyrate were formed. The solution, after dilution by threefold with H_2O, was passed through a Dowex 50 (H^+ form) column (4 ml of resin). The column was washed with 10 ml of H_2O. The combined solution was continuously extracted with ether overnight. An aliquot was analyzed by paper chromatography (Whatman no. 1 paper, developed in 1-butanol–acetic acid–H_2O (12:5:2); (R_f values 2-aminobutyrate, 0.48; 2-hydroxybutyrate, 0.86) and shown to be free of the starting material.

Methods

Enzymatic Elimination Reaction and Assay of Chirality in the 2-Keto[3-^{3}H]butyrate Products. Two separate samples of [2-^{3}H]propionate were prepared from D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*R*) and D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*S*) by the nonoxidative elimination reaction catalyzed by D-amino-acid oxidase (Walsh et al., 1973b), followed by oxidative decarboxylation with H_2O_2 of the 2-ketobutyrate thus formed. Typically, the incubation contained (in μmol) $NaPP_i$ (400, pH 8.5), the appropriate 2-amino-3-chloro[2-^{3}H]butyrate (100), and 5–8 mg of D-amino-acid oxidase in a total volume of 5 ml. The reaction was allowed to proceed at room temperature for 100 min. The progress of the reaction was followed by assaying aliquots for 2-ketobutyrate with lactic dehydrogenase. The solution was passed through a small Dowex 50 column to get rid of unreacted substrate. The column was washed with 10 ml of H_2O. To the neutralized combined elute was added 1.5 ml of 30% H_2O_2. After standing at room temperature for 1 h, excess H_2O_2 was destroyed by catalase. The volume of the solution was reduced to 5 ml by lyophilization, acidified with concentrated HCl to pH 1, and continuously extracted with ether overnight. Typically, 20–30 and 30–40 μmol of [2-^{3}H]propionate could be obtained through one operation with D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*S*) and D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*R*) as the starting material, respectively. Thus, the operation had to be repeated two to three times with each diastereomer to accumulate enough [2-^{3}H]propionate for the preparation of the CoA derivatives. Two milliliters of H_2O was added to the combined ether extract. Ether was then removed carefully on a rotavaporator. The aqueous solution was passed through a Dowex 1 column (Cl^- form, 0.8 × 3 cm) and eluted with 20 mM HCl. The radioactive fractions were pooled and again continuously extracted with ether overnight. The ether extract was thoroughly dried with anhydrous Na_2SO_4 and then filtered. Two milliliters of H_2O was added to the filtrate. Ether was again carefully removed on a rotavaporator. The aqueous solution remaining was titrated with 40 mM NaOH from a microburette on a pH meter, generating a titration curve with a sharp inflection point. The specific radioactivity of the [2-^{3}H]propionate could thus be estimated.

Another sample of [2-^{3}H]propionate was prepared by running the D-amino-acid oxidase incubation in ^3H_2O, D-*erythro*-2-amino-3-chlorobutyrate being the substrate. The incubation contained (in μmol) $NaPP_i$ (100, pH 8.5), substrate (20), 200 mCi of ^3H_2O, and 2 mg of D-amino-acid oxidase in a total volume of 1 ml. At the end of 1 h, 12 μmol of 2-ketobutyrate was formed as shown by lactic dehydrogenase assay. Excess ^3H_2O was removed by lyophilization. Forty micromoles of sodium 2-ketobutyrate were added. Subsequent decarboxylation and workup of the [2-^{3}H]propionate were identical with the procedures described above.

The various samples of [2-^{3}H]propionyl CoA were prepared from the corresponding [2-^{3}H]propionate using the mixed anhydride method of Arigoni et al. (1966). Excess unreacted [2-^{3}H]propionic acid was thoroughly removed by continuous extraction with ether for 3 h, after which the aqueous solution was lyophilized. The [2-^{3}H]propionyl CoA was taken up in dilute HCl (pH 2.5) and stored frozen at −20 °C.

The concentrations of the various propionyl CoA samples and their specific radioactivities were determined as previously reported (Cheung, et al., 1975). The values agreed with those of their corresponding precursor [2-^{3}H]propionates.

Radioactivity was determined by scintillation counting in 10 ml of Beckman Cocktail D (100 g of naphthalene and 5 g of diphenyloxazole per l. of solution in dioxane). The scintillation samples were all adjusted such that 1 ml of water was present, in order to correct for the quenching effect.

Results

Structural Proofs of the Diastereomeric 2-Amino-3-chlorobutyrates. Our syntheses as detailed in the Materials section (see Scheme II) represent the first reported preparations of [2-^{3}H]threonine and [2-^{3}H]allothreonine of both high specific radioactivity and stereoisomeric purity. The two diastereomers of [2-^{3}H]threonine prepared as described can be easily distinguished by NMR (as compared with the spectra of authentic compounds) and paper chromatography (Shaw and Fox, 1955).

The two diastereomers of 2-amino-3-chlorobutyrate derived from threonine and allothreonine also display distinctively different NMR spectra as shown in Figure 1. They do not separate as well in the same paper chromatographic system, but do separate on cellulose plates developed in the same solvent system (R_f values: the diastereomer derived from threonine, 0.39; that from allothreonine, 0.35; threonine, 0.30; allothreonine, 0.24).

In order to interpret the nature of the D-amino-acid oxidase catalyzed elimination reaction with each diastereomer, i.e., if it is a syn or anti process, it is imperative that we unambigu-

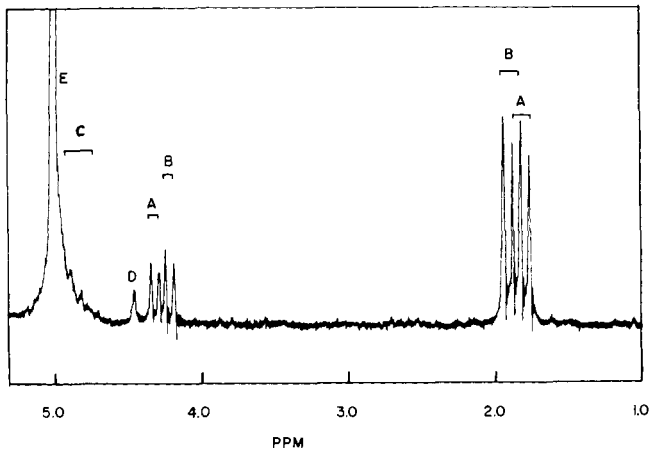
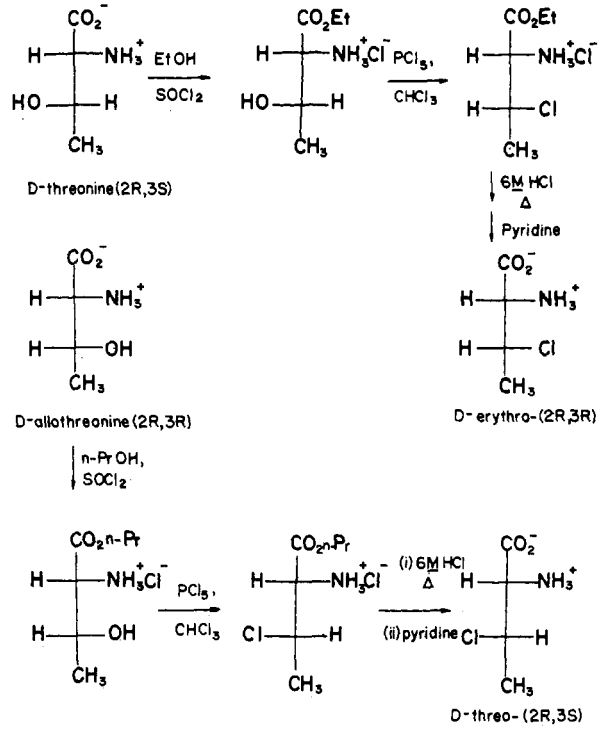


FIGURE 1: NMR spectrum of a mixture of *erythro*- and *threo*-2-amino-3-chlorobutyrate in ^2H_2O. (A) Signals corresponding to the erythro isomer, (B) the threo isomer, (C) signals derived from the β protons of both compounds, (D) spinning side band of the H_2O peak, and (E) H_2O peak. The spectrum was taken on a Varian T-60 NMR spectrometer.

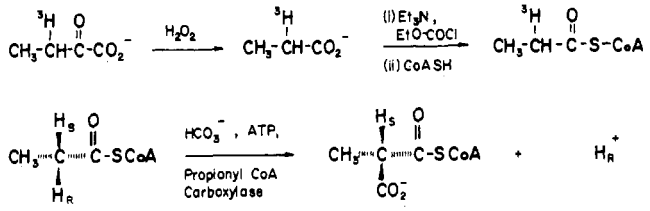
ously determine the absolute configurations at both C2 and C3 of the two diastereomers of D-2-amino-3-chlorobutyrate. It is obvious that the configuration at C2 remained unchanged in the conversions of D-threonine (2*R*,3*S*) and D-allothreonine (2*R*,3*R*) to the 2-amino-3-chlorobutyrates since the latter were quantitative substrates for D-amino-acid oxidase. As far as the configuration at C3 is concerned, while it had previously been shown that the PCl_5 chlorination probably involved inversion of the configuration (Plattner et al., 1957), we proceeded further to confirm this observation by synthesizing the two diastereomers of 2-amino-3-chlorobutyrate independently through synthetic transformations of well-established stereochemistry. The analytical preparations of the two authentic diastereomers are outlined in Scheme III. Since the chlorination of the tosylates (VII and X) clearly involved an SN2 inversion of the configurations at C3, the 2-amino-3-chlorobutyrate derived from D-threonine (VI, 2*R*,3*S*) had to be of the erythro configuration (i.e., 2*R*,3*R*), while that (XI) from D-allothreonine (IX, 2*R*,3*R*) must possess the threo configuration (i.e., 2*R*,3*S*). The 2-amino-3-chlorobutyrate (VIII) thus obtained from threonine displayed identical NMR and ir spectra with that derived from threonine through the PCl_5 chlorination. On the other hand, the thus-derived 2-amino-3-chlorobutyrate (X) from allothreonine was identical with the diastereomer obtained from allothreonine through the PCl_5 chlorination. These compounds were also identified with the aforementioned TLC system. Thus we conclude that the PCl_5 chlorination does involve inversion of configuration at C3, as shown in Scheme IV.

Stereochemistry of the D-Amino-Acid Oxidase Catalyzed Elimination Reaction with Each Diastereomer of D-2-Amino-3-chlorobutyrate. To probe the stereochemistry of the elimination reaction of Scheme I, one requires that the product of the reaction, 2-ketobutyrate, be labeled at C3 with either deuterium or tritium. The chirality of a sample of 2-keto[3-^{2}H]butyrate was determined by Krongelb et al. (1968). It involved initial decarboxylation of the compound to [2-^{2}H]propionate. Its ORD spectrum was recorded and compared with that of chemically synthesized (2*R*)-[2-^{2}H]propionate. We chose to use tritium as the label instead since it requires much less material (thus offers greater sensitivity) than does the ORD method. However, this choice excluded us from using any physical technique, and we had to use a chiral chemical reagent instead.

Scheme IV



Scheme V



The absolute configuration of 2-keto[3-^{3}H]butyrate can be determined as outlined in Scheme V. As described in the Materials section, the 2-keto[3-^{3}H]butyrate can be oxidatively decarboxylated to [2-^{3}H]propionate, which was chemically converted to [2-^{3}H]propionyl CoA. It was shown by Arigoni et al. (1966) and Prescott and Rabinowitz (1968) that in the ATP-dependent carboxylation of propionyl CoA catalyzed by propionyl-CoA carboxylase, only the pro-*R* proton at C2 of the substrate was released into the medium (see Scheme V), and at a rate equal to the carboxylation rate. Thus the relative amount of tritium released as ^3H_2O to that retained in the product methylmalonyl CoA during the propionyl-CoA carboxylase catalyzed carboxylation of a given sample of [2-^{3}H]propionyl CoA will indicate its chiral purity.

If the events of Scheme I occur, theoretically, the 2-ketobutyrate will be expected to incorporate deuterium into C3 from the medium if ^2H_2O is used as the solvent since the enamine intermediate (I) is obligated to pick up a proton at C3 from some source (most likely the solvent). This was indeed observed, as reported by Walsh et al. (1973b). When the elimination reaction was carried out in ^2H_2O, using as substrate *erythro*-D-2-amino-3-chlorobutyrate (2*R*,3*R*), 50% of the 2-ketobutyrate was labeled with deuterium at C3, as shown by NMR analysis. However, the 2-keto[3-^{2}H]butyrate thus formed appeared by ORD to be achiral (Walsh et al., 1973b). We have now confirmed this observation, using ^3H_2O instead of ^2H_2O, and determined the chirality of the 2-keto[3-^{3}H]-

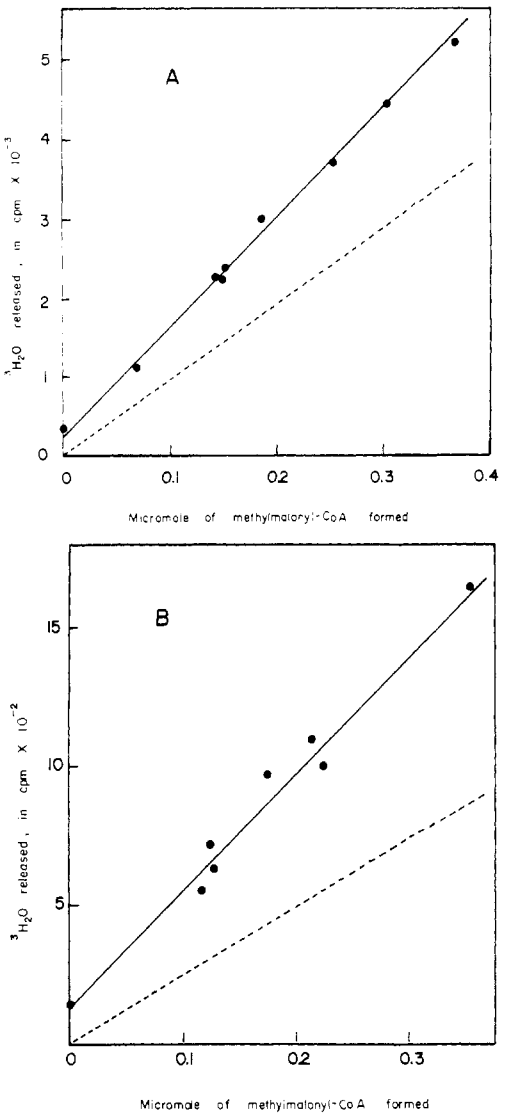


FIGURE 2: Tritium released from [2-^{3}H]propionyl CoA (A) derived from D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate, (B) derived from D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate, upon carboxylation by propionyl-CoA carboxylase. (- - -) Theoretical 50% tritium release (i.e., if the samples were achiral (*R,S*)-[2-^{3}H]propionyl CoA) based on (A) specific radioactivity of 17 000 cpm/μmol, and (B) 5000 cpm/μmol. The incubations contained (in μmol) Tris buffer (100, pH 8.0), $KHCO_3$ (100), $MgCl_2$ (2), phosphoenolpyruvate (2.5), ATP (2.5), dithiothreitol (2.5), NADH (0.6), 50 units of lactic dehydrogenase, 2 units of pyruvate kinase, and [2-^{3}H]propionyl CoA (0.37 in A, and 0.25 in B, except one incubation in B, which contained 0.38 μmol instead) in a total volume of 0.75 ml. The reaction was started by the addition of 0.16 mg of propionyl-CoA carboxylase, and followed on a Gilford spectrophotometer by the decrease in A_{340}. The temperature was maintained at 30 °C by a thermostat. The reaction was stopped by the addition of 0.5 unit of avidin. The solution was then pipetted into a 50-ml round-bottomed flask and immediately frozen in liquid nitrogen. The ^3H_2O was collected by lyophilization of the sample.

butyrate formed from D-*erythro*-2-amino-3-chlorobutyrate (2*R*,3*R*), as described.

The [2-^{3}H]propionate recovered from decarboxylation of the 2-keto[3-^{3}H]butyrate had a specific radioactivity of 9 × 10^5 cpm/μmol, which was about 10% that of the ^3H_2O. This lowered specific radioactivity could be due to two factors: (i) a partial internal transfer of the α proton of the substrate (elaborated below); and (ii) a possible tritium kinetic isotope effect against the picking up of a solvent triton by the released enamine.

The thus-derived [2-^{3}H]propionyl CoA released 50% of the tritium label at a rate equal to the rate of carboxylation catalyzed by propionyl-CoA carboxylase (data not shown), indicating that the sample was indeed achiral. We interpret this fact as indicating that during the elimination process some fraction of an enamine species was released by D-amino-acid oxidase into the medium, and subsequently tautomerized nonenzymatically (and achirally) to the imine, followed by hydrolysis to the keto acid.

An alternate way of generating [3-^{3}H]- or [3-^{2}H]2-ketobutyrate during the elimination reaction on 2-amino-3-chlorobutyrate would be to use substrate labeled with deuterium or tritium at carbon 2, because D-amino-acid oxidase catalyzes the unexpected partial retention and transfer of the C2 hydrogen of the substrate to C3 in the keto acid product (Walsh et al., 1973b). The species protonated is presumed to be the bound enamine I of Scheme I. While we previously reported that 20% of the ketobutyrate molecules formed from D-*erythro*-2-amino-3-chloro[2-^{2}H]butyrate contained deuterium at carbon 3, insufficient material was available for chirality determination by the ORD method.

Thus, we have now prepared the β-chloroamino acid diastereomers labeled specifically with ^{3}H at carbon 2, and examined for tritium incorporation at C3 of the 2-ketobutyrate enzymatic product. The [2-^{3}H]-D-threo substrate (2*R*,3*S*) yields [3-^{3}H]-2-ketobutyrate at 0.7% the specific radioactivity of the amino acid. The D-erythro diastereomer (2*R*,3*R*) transfers 1.5% of its tritium to the ketobutyrate formed. These low specific activities were predictable on the basis of kinetic isotope selections and only partial intramolecular hydrogen transfer. The tritiated 2-ketobutyrates were separately converted to [2-^{3}H]propionyl CoA samples. We have previously shown that 2-amino[2-^{3}H]butyrate oxidation catalyzed by D-amino-acid oxidase yielded no tritiated 2-ketobutyrate.

Figure 2A shows the rate of tritium release from [2-^{3}H]propionyl CoA, ultimately derived from D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*R*), upon carboxylation to methylmalonyl CoA. The dashed line shows the theoretical line of 50% tritium release, to be expected if the sample of [2-^{3}H]propionyl CoA is an achiral *R,S* mixture. However, the actual experimental result indicates that 80% of the tritium label was released indicating the sample to be 80% (2*R*)-[2-^{3}H]propionyl CoA. Therefore, D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*R*) gave rise to (3*R*)-2-keto[3-^{3}H]butyrate in the D-amino-acid oxidase catalyzed elimination reaction.

The tritium release from the [2-^{3}H]propionyl CoA that was derived from D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate is similarly shown in Figure 2B. The results indicate 87% of the tritium label was released as ^3H_2O upon carboxylation, again showing the sample to be (2*R*)-[2-^{3}H]propionyl CoA. Thus D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*S*) also gave rise to (3*R*)-2-keto[3-^{3}H]butyrate.

In order to rule out the possibility that the more than 50% tritium release rates with the [2-^{3}H]propionyl CoA samples may be due to nonenzymatic exchange of tritium out of the product, methylmalonyl CoA, the proper control experiments utilizing known achiral (*R,S*)-[2-^{3}H]propionyl CoA were performed. As reported earlier (Cheung et al., 1975), such samples released only 50% of the tritium label upon carboxylation, as expected.

Tritium Kinetic Isotope Effect and Percentage Tritium Retention in the Products during the Elimination Reaction. A deuterium kinetic isotope effect of 1.8 on the V_{max} of the D-amino-acid oxidase catalyzed elimination reaction on D-*erythro*-2-amino-3-chloro[2-^{2}H]butyrate has been reported

Table I: Tritium Kinetic Isotope Effects and Partition Percent of Tritium Label in the Products.[a]

Substrates	Tritium Label in Products: Tritium Kinetic Isotope Effects	% in H_2O	% in 2-Ketobutyrate
D-*erythro*-2-Amino-3-chloro-[2-^{3}H]butyrate (2*R*,3*R*)	4.7	89[b]	7.2[b]
D-*threo*-2-Amino-3-chloro-[2-^{3}H]butyrate (2*R*,3*S*)	3.8	*c*	2.6
D-[2-^{3}H]Threonine (2*R*,3*S*)	6.2	100	0
D-[2-^{3}H]Allothreonine (2*R*,3*R*)[d]	2.0	100	0

[a] These values were determined as described in the legend of Figure 3. [b] These two numbers only add up to 96%, due to experimental uncertainties. [c] Again due to experimental uncertainties, this number could not be accurately determined. [d] The amount of (3*R*)-3-hydroxy-2-ketobutyrate formed in each time point was determined by standard 2,4-dinitrophenylhydrazone assay instead of the lactic dehydrogenase–NADH coupled assay.

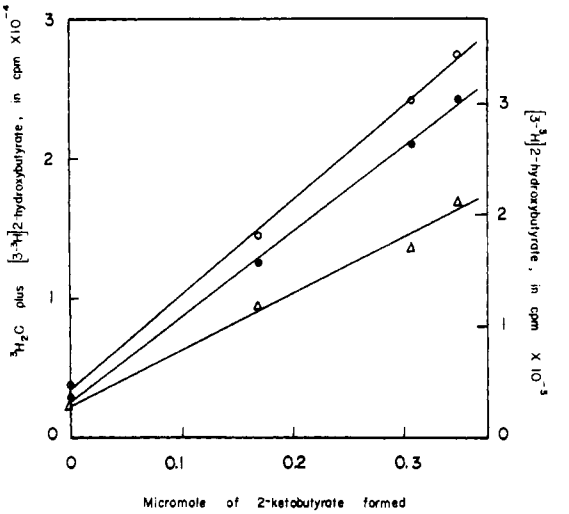


FIGURE 3: Tritium kinetic isotope effect and tritium partitioning in the products of the D-amino acid oxidase catalyzed elimination reaction of D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate. (○) Radioactivity corresponding to ^3H_2O and L-2-hydroxy[3-^{3}H]butyrate combined; (●) radioactivity corresponding to ^3H_2O alone; (△) radioactivity corresponding to L-2-hydroxy[3-^{3}H]butyrate. The incubations contained (in μmol) KPP_i buffer (30, pH 8.5), NADH (0.6), 50 units of lactic dehydrogenase, and D-*erythro*-2-amino-3-chloro[2-^{3}H]butyrate (7.5; specific radioactivity, 3.2×10^5 cpm/μmol) in a total volume of 0.73 ml. The reaction was started by the addition of 0.5 mg of D-amino acid oxidase, and followed spectrophotometrically on a Gilford spectrophotometer by the decrease in A_{340}. Temperature was maintained at 30 °C by a thermostat. The reaction was stopped by the addition of 50 μl of 2 M HCl. An aliquot of L-2-hydroxy[1-^{14}C]butyrate (2200 cpm) was added. The solution was titrated back to neutrality with 2 M NaOH, and passed through a Dowex 50 (H^+ form) column (0.5 × 3.5 cm). The column was washed with 3 ml of water. The combined eluate was titrated with 2 M NaOH to neutrality and diluted to a total volume of 5.0 ml. This solution (0.5 ml) was pipetted out and its tritium content determined by liquid scintillation counting. From this, the tritium content in the combined ^3H_2O and L-2-hydroxy[3-^{3}H]butyrate was calculated. The remaining 4.5 ml of solution was passed through a Dowex 1 (Cl^- form) column (0.5 × 3.5 cm) and washed with water, bringing the total volume of the eluate up to 10.0 ml. One milliliter of this solution was again counted for tritium (no detectable ^{14}C radioactivity), corresponding to the tritium content in ^3H_2O only. The column was now eluted with 20 mM HCl, 1.0 ml at a time, the eluate being collected directly into a scintillation vial. The samples were each titrated with 2 M NaOH to neutrality, and both the ^{3}H and ^{14}C content determined again by scintillation counting, from which the amount of L-2-hydroxy[3-^{3}H]butyrate was calculated.

(Walsh et al., 1973b), along with a stopped-flow kinetic study and deuterium kinetic isotope effect on the rate of pre-steady-state Cl^- release of the same compound using rapid quench techniques (Massey et al., 1975). We have now examined the tritium kinetic isotope effect on both diastereomers of D-2-amino-3-chloro[2-^{3}H]butyrate under initial velocity conditions. Reaction of the bulk protio substrate was monitored by lactic dehydrogenase coupled spectrophotometric assay. The amount of tritiated substrate turned over in the same time interval was determined by summing the tritium label in the solvent and the product 2-hydroxy[3-^{3}H]butyrate (formed by lactic dehydrogenase–NADH reduction, the tritium label was no longer labile). The ratio of the specific radioactivity of these products to that of the starting material indicated the tritium kinetic isotope effect. The amount of tritium label specifically in the 2-hydroxy[3-^{3}H]butyrate was determined by isolating the compound on a small Dowex 1, Cl^- column. In order to correct for possible incomplete recovery during the workup, a double-label technique using 2-hydroxy[1-^{14}C]butyrate was employed. Results for D-*erythro*-2-amino-3-chloro[2-^{3}H]-butyrate (2*R*,3*R*) are shown in Figure 3. The same kinetic parameters for D-*threo*-2-amino-3-chloro[2-^{3}H]butyrate (2*R*,3*S*) were similarly determined. Both results are summarized in Table I.

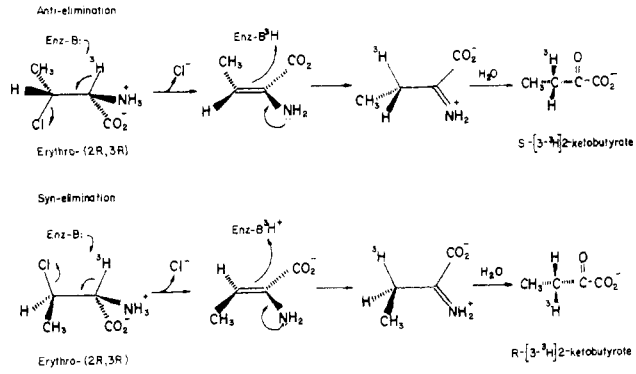
Also included in Table I for comparison are the tritium kinetic isotope effects for D-[2-^{3}H]threonine (2*R*,3*S*) and D-[2-^{3}H]allothreonine (2*R*,3*R*). Each of these substrates undergoes only the normal D-amino-acid oxidase catalyzed oxidation producing (3*S*)-3-hydroxy-2-ketobutyrate and (3*R*)-3-hydroxy-2-ketobutyrate, respectively, at V_{max} values very similar to the rates of enzymatic processing of the respective 2-amino-3-chlorobutyrate diastereomers.

Discussion

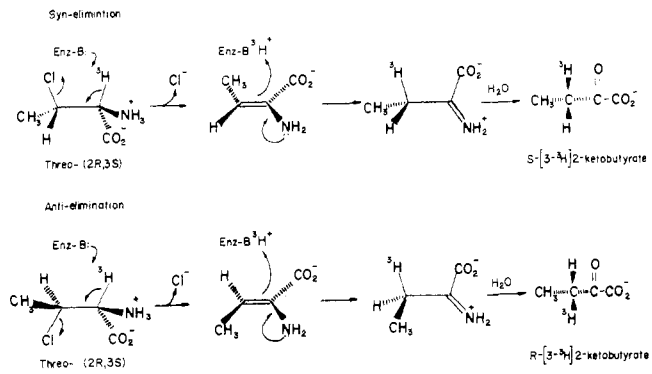
The determination of the stereochemical path for the D-amino-acid oxidase catalyzed elimination of HCl from the diastereomeric D-2-amino-3-chloro[2-^{3}H]butyrates described here depends on the establishment of the chirality of the 2-keto[3-^{3}H]butyrate produced enzymatically. The absolute configuration at C3 of this presumably chiral compound can, in principle, be determined by decarboxylation to [2-^{3}H]propionate and esterification to the CoA thiol ester, and subjected to the carboxylation catalyzed by propionyl-CoA carboxylase, provided three conditions are met. The first is that there is no kinetic isotope selection against the removal of tritium from the 2*R* position of propionyl CoA.[1] This fact was established by Prescott and Rabinowitz (1968). Secondly, we had to show that a sample of known chiral [2-^{3}H]propionyl CoA would indeed be carboxylated with the expected stereospecificity. We have previously prepared a sample of (2*S*)-[2-^{3}H]propionyl CoA (Cheung et al., 1975) and found 74% retention of tritium

[1] One can use this assay system even with a tritium kinetic isotope effect provided: (i) the tritium kinetic isotope effect for the carboxylation reaction is accurately known, and (ii) the reaction is not allowed to proceed past initial conditions. Another solution is to let the reaction proceed to *completion*. However, both of these methods can be technically difficult.

Scheme VI



Scheme VII



in the product methylmalonyl CoA.[2] The third requirement is that there is no substantial loss of tritium from the product methylmalonyl CoA molecules due to nonenzymatic enolization.[3] Overath et al. (1962) reported that such exchange was indeed slow at pH 8.1 (half-life of 2500 min). We have further shown that, in our hands, (*R,S*)-[2-^{3}H]propionyl CoA released only 50% of the tritium upon carboxylation, as expected (Cheung et al., 1975).[4]

Use of the carboxylase in this way constitutes a general assay system then not only for chiral [2-^{3}H]propionyl CoA samples, but also for any chiral 2-keto[3-^{3}H]butyrate. In turn, any precursor of 2-ketobutyrate (or propionate) is also amenable to this analysis. For instance, cystathionine γ-synthase has been reported to exchange one of the two methylene protons at C3 of L-2-aminobutyrate (Posner and Flavin, 1972) with solvent protons. Performing this exchange reaction in ^3H_2O followed by oxidation of the amino acid with L-amino-acid oxidase would generate the tritiated 2-ketobutyrate.

The most surprising result reported in this paper is that both D-erythro-(2*R*,3*R*) and D-threo-(2*R*,3*S*) diastereomers of 2-amino-3-chlorobutyrate yield the same chiral (3*R*)-2-keto[3-^{3}H]butyrate. The detection of the chirally labeled products demonstrates that the incorporation of tritium at C3 must be enzyme catalyzed. In our hands the D-erythro-2-^{3}H-labeled substrate yielded a sample of 2-keto[3-^{3}H]butyrate which was subsequently assayed to be of 80% *R* configuration, while the D-threo-2-^{3}H-labeled substrate produced 87% *R* product. This difference in apparent optical purity could be due to experimental uncertainties. One possible means for loss of chirality was some nonenzymatic enolization of the product, 2-keto[3-^{3}H]butyrate during the D-amino-acid oxidase incubation and subsequent chromatography on a Dowex 50 column prior to the H_2O_2-induced decarboxylation. In this regard, the sample of (2*S*)-[2-^{2}H]propionate which Krongelb et al. (1968) prepared with this methodology might have been only 90% optically pure and Yang et al. recently reported 80% optical purity in a sample of (3*S*)-2-keto[3-^{2}H]butyrate produced by the action of D-serine dehydrase on D-threonine (Yang et al., 1975).

We can make an analysis of the stereochemical course of this D-amino-acid oxidase catalyzed elimination reaction for each diastereomer provided two assumptions are made. Firstly, the α proton of both diastereomers (and presumably all substrates that undergo normal oxidation) is abstracted by the same base at the enzyme active site. The second requirement is that, with each diastereomer, once the elimination has proceeded to form the bound enamine and the protonated active site base, there is no reorientation of these components prior to transfer of the sequested triton back to the enamine to generate the chiral 2-imino[3-^{3}H]butyrate (i.e., the protonated enzyme base delivers the triton back to the enamine intermediate on the same face that the proton had been abstracted, see Schemes VI and VII).[5] This seems a reasonable assumption, although the single example of aconitase (Rose and O'Connell, 1967) reminds one that such reorientation is not without precedent.

With these provisos in mind, Scheme VI shows the predicted stereochemical outcome for loss of the elements of HCl from the tritiated D-erythro substrate by either an *overall* syn or anti process. The anti process would produce an enamine initially in the cis configuration. Return of the triton to the same face on ketonization would yield (3*S*)-2-imino[3-^{3}H]butyrate. By contrast, an overall syn elimination involves the formation of bound enamine at the active site in a trans conformation; reprotonation at the same face produces (3*R*)-2-imino[3-^{3}H]butyrate. Since the latter is in fact the observed product, elimination from the D-erythro (2*R*,3*R*) substrate is *overall* syn. Scheme VII shows the corresponding predictions for the anti or syn process with the D-threo (2*R*,3*S*) substrate. Now, the observed (3*R*)-2-keto[3-^{3}H]butyrate was formed by an *overall* anti pathway instead. Note also that the same trans enamine is formed from the threo diastereomer as was formed from the erythro diastereomer, in one case by an overall anti and in the other by an overall syn process, respectively. Thus, we see the highly unusual situation that the flavoenzyme processes the two diastereomers by different routes to give the same initial bound enamine and subsequently the same chiral 2-keto[3-^{3}H]butyrate. Indeed, this appears to be only the

[2] We previously reported (Cheung et al., 1975) that propionyl-CoA carboxylase released 23%, and transcarboxylase 17% of the tritium label from our sample of (2*S*)-[2-^{3}H]propionyl CoA upon carboxylation. However, we subsequently established by degradation (Phares, E. F. (1951), *Arch. Biochem. Biophys. 33*, 173) of the sample of (2*S*)-[2-^{3}H]propionate to acetate that actually 17% of the tritium label was on C3. Thus the values should be corrected to 26 and 20%, respectively.

[3] If nonenzymatic enolization of the product methyl[2-^{3}H]malonyl CoA causing excess loss of tritium is to occur, it will be more serious in the case of (2*S*)-[2-^{3}H]propionyl CoA since most of the tritium will be retained in the product. With (2*R*)-[2-^{3}H]propionyl CoA, this will not be much of a problem since most of the tritium has already been released into the medium during the carboxylation.

[4] Obviously, this control experiment only contributed to establish the validity of the propionyl-CoA carboxylase assay system; it did not rule out the possibility of nonenzymatic scrambling of the tritium label during workup of the 2-keto[3-^{3}H]butyrate, and handling of the [2-^{3}H]propionyl CoA.

[5] If, on the other hand, such orientation does occur, our conclusions to be given below will not be changed, i.e., effectively syn elimination for one diastereomer, and anti for the other, but only the assignment of *which* process is overall syn and *which* anti. We think the reorientation of the catalytic components at the active site in one case but not the other is too perverse to occur. Presently, we have not determined how to resolve these ambiguities.

second enzyme known that catalyzes a net elimination on two diastereomers through different stereochemical courses (the first being β-methylaspartate ammonia-lyase acting on *threo*- and *erythro*-β-methylaspartate (Hansen and Havir, 1973)).

Of the various enzymes known which eliminate HX (X = O, N; there has been no other report on HCl elimination), the large majority proceed by anti pathways. There are, however, a few examples of syn eliminations, such as the production of (*E*)-3-methylglutaconyl CoA from (3*S*)-hydroxymethylglutaconylCoA catalyzed by 3-methylglutaconyl-CoA dehydrase (Messner et al., 1975) and the interconversion of 5-dehydroshikimate and 5-dehydroquinate catalyzed by dehydroquinase (Butler et al., 1974).

One question that now arises is whether the stereochemical outcomes can be used to provide information on whether the loss of HCl is concerted (as in nonenzymatic E_2) or nonconcerted, i.e., by initial carbanion formation (as in the corresponding nonenzymatic E_1cB). Theoretically, both anti and syn eliminations are possible in a concerted reaction (Saunders and Cockerell, 1972; Sicher, 1972), although in the majority of nonenzymatic elimination reactions, the anti process is the preferred one. Presumably, for synchronous eliminations, steric crowding in the transition state due to eclipsing groups favors the anti process compared with the syn.

Rose and O'Connell (1967) speculated that the few known enzymatic syn eliminations were most likely two step process. Experimental evidence has recently been obtained by Alworth's group for a discrete enamine intermediate following proton abstraction but preceding OH^- ejection in the dehydroquinase catalysis (Butler et al., 1974). Similarly, as Eggerer has noted, the enoyl CoA syn hydration–dehydration sequence could utilize a two-step carbanion mechanism. In this connection the α proton of acyl thiol ester is reasonably acidic (Bruice and Benkovic, 1966), and the resulting carbanion can be stabilized. Thus, stereochemical outcome in enzymatic reactions is not likely to have obvious mechanistic conclusions. In fact, even in the more straightforward nonenzymatic eliminations stereochemistry does not necessarily establish one or the other mechanism (Saunders and Cockerell, 1972).

In the D-amino-acid oxidase catalyzed elimination reaction, a nonconcerted alternative would also involve an α-carbanion intermediate. Direct evidence that D-amino-acid oxidase (and glucose oxidase) can oxidize substrate carbanions comes from the work of Porter et al. (1973) which showed that nitroalkane anions were oxidized to nitrite and the appropriate aldehydes. Additionally, the acetylenic hydroxy acid, 2-hydroxy-3-butynoate, is a suicide substrate for all flavin-dependent α-hydroxy-acid oxidases examined (Cromartie and Walsh, 1975; Schonbrunn et al., 1975; Walsh et al., 1972a,b). One possible mechanism for the formation of the covalent adducts with the flavin coenzymes is by rearrangement of an acetylenic carbanion to an alkylating allenic anion. With these suggestive precedents, together with our earlier results that 3-chloroalanine actually partitioned itself between the normal oxidation and the elimination reactions, it can be inferred that carbanions are formed from normal amino acids and 3-chloroalanine during oxidation catalyzed by D-amino-acid oxidase. They are likely formed in the case of 2-amino-3-chlorobutyrates during the elimination reaction, which would of course be nonconcerted by definition.

Certainly the substitution of either β hydrogen of 3-chloroalanine with a methyl group, i.e., the 2-amino-3-chlorobutyrates, reveals that the methyl group has a profound influence on both the partitioning between elimination and oxidation (Walsh et al., 1973b) and in determining the stereochemical path of the elimination itself. Still unanswered is why the 2-amino-3-chlorobutyrates undergo exclusively elimination (turnover 5–10 min^{-1}) but 3-chloroalanine undergoes both oxidation and elimination (turnover $\simeq$500 min^{-1}). If, in addition to its electronic assistance to the release of Cl^- (now the compound being a secondary halide), the methyl group in 2-amino-3-chlorobutyrate imposes an orientation on the substrate at the active site such that carbanion formation can occur but subsequent electron transfer to the flavin coenzyme is now geometrically or spatially inhibited, one might have an explanation for the exclusive elimination reaction.

The C4 methyl group of the diastereomeric 2-amino-3-chlorobutyrates could control the stereochemical path of the elimination in different manners depending on whether the elimination is concerted or nonconcerted. In the case of a concerted mechanism, binding of the erythro substrate must occur to provide a dihedral angle between C2–H and C3–Cl of close to 0°. On the other hand, with the methyl group occupying the same active site region when the threo diastereomer binds, the dihedral angle between C2–H and C3–Cl would now be close to 180° instead. These dihedral angles dictate syn and anti eliminations, respectively.

On the other hand, the methyl group may not be all important in determining the conformations of bound substrates. Then, in a two-step nonconcerted mechanism, carbanion formation would occur first (analogous to an E_1cB nonenzymatic case). Although free rotation could occur around the C2–C3 single bond of the carbanion intermediate, the geometry of the active site could impose specificity such that elimination of Cl^- to the trans enamine involves lower activation energy than the formation of the corresponding cis enamine. In this connection, we have noted that the enzyme shows a high degree of discrimination in reactions with some β-substituted amino acids. For instance, D-*erythro*-phenylserine (2*R*,3*R*) undergoes oxidation at 100 nmol/min^{-1} mg^{-1}, while the D-threo isomer (2*R*,3*S*) binds at the active site but does not undergo detectable oxidation.[6]

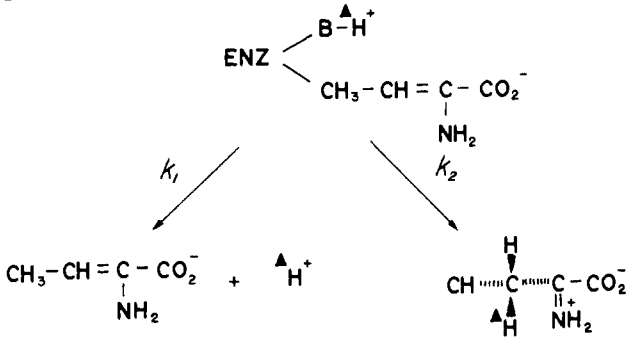
The experimental result that an achiral (*R*,*S*)-2-keto[3-^{3}H]butyrate is derived from D-*erythro*-2-amino-3-chlorobutyrate in ^3H_2O confirms our earlier suggestion (Walsh et al., 1973b) that two competing pathways exist during the product release. The preceding discussion has indicated that some molecules can be chirally reprotonated at the active site with tritium derived from the substrate. The ^3H_2O data indicate that any enamine proceeding to iminobutyrate by acquiring a solvent proton does so in an achiral manner, thus off the enzyme, i.e., after the enamine has been released into the medium. We emphasize two points: (i) enamine release into solvent must be competitive with reprotonation at the active site; (ii) to the limit of our experimental detection (about 5%), the protonated enzymatic base does not exchange its proton with those of the solvent while the enamine remains bound. If it did exchange, then in ^3H_2O, enrichment of tritium in the 3*R* position of the product 2-keto[3-^{3}H]butyrate should have been seen. None was detected. Thus, substrate-derived C2 protons are fully (at least 95%) sequested during catalysis. This phenomenon occurs as well during normal oxidation of amino acids by the same enzyme (Porter and Bright, 1975).

The partial retention and transfer of the substrate C2 proton to C3 of the product 2-ketobutyrate coupled to the fact that the per cent transfer decreases in the order of increasing mass of the isotopic proton involved, i.e., 20% for deuteron (Walsh et al., 1973b) and 7.2% for triton, seems to indicate the exis-

[6] C. Walsh, unpublished results.

tence of two competing pathways for the release of the product as shown in Scheme VIII.

Scheme VIII



A simple analysis of this phenomenon can be made if one assumes that only the rate of reprotonation of the enamine, i.e., k_2 of Scheme VIII, is decreased by isotopic substitution. Simple algebraic treatment yields the following relationship:

$$\frac{k_2(^2\mathrm{H})}{k_2(^3\mathrm{H})} = \frac{a(^2\mathrm{H})}{[1 - a(^2\mathrm{H})]} \frac{[1 - a(^3\mathrm{H})]}{a(^3\mathrm{H})} \quad (1)$$

where $a(^2\mathrm{H})$ and $a(^3\mathrm{H})$ are the per cent transfer for deuteron and triton, respectively. Thus $k_2(^2\mathrm{H})/k_2(^3\mathrm{H})$ can be estimated to be 3. Using the expected relationship of isotope effects (Swain et al., 1958), $k_2(^1\mathrm{H})/k_2(^2\mathrm{H})$ can in turn be computed to be about 7. Substitution of this value into eq 1 (with the appropriate changes of the notations) would yield a value of 65% for the transfer of a proton. This is in close agreement with the reported 50% transfer in the experiment with D-*erythro*-2-amino-3-chloro[2-^{1}H]butyrate in ^2H_2O (Walsh et al., 1973b). In the latter case, some kinetic properties of the enzyme were probably changed due to solvent isotope effect.

Indirect evidence has been presented arguing for the fact that product release (i.e., the steps with rate constants k_1 and k_2 in Scheme VIII) in this elimination reaction is the rate-determining step (Massey et al., 1975). As shown earlier in this section, it can be estimated that for the D-erythro 2-^{1}H-labeled substrate, $k_1:k_2 \simeq 35:65$. Using the estimated deuterium kinetic isotope effect of 7, $k_1:k_2 \simeq 35:9$ for the corresponding 2-^{2}H-labeled substrate. If these two parallel steps are indeed rate determining, a deuterium kinetic isotope effect of 2.3 (i.e., the ratio of [$k_1 + k_2$, protio substrate] to [$k_1 + k_2$, deuterio substrate]) on the overall catalysis should be expected. Indeed, Walsh et al. (1973b) observed a deuterium kinetic isotope effect of 1.8. This, in our view, serves as another indication of product release being the slow step in the overall reaction.

Using the same analysis, one would predict a tritium kinetic isotope effect of about 2.6, apparently substantially lower than our reported value of 4.7. However, as pointed out by Cleland (1975), with tritium as the tracer, one does not in general obtain the kinetic isotope effect on V_{max} of an enzymatic reaction. One must be cautioned that all the above estimates are only approximate. The real situation could be more complicated since a kinetic isotope effect also shows up in an earlier fast step, namely the formation of a spectrophotometrically detectable intermediate (Walsh et al., 1973b; Massey et al., 1975) which is believed to also involve the Cl^- release step.

The last result to be explained is the difference in percentage tritium transfer from C2 of the D-*erythro*- and D-*threo*-2-amino-3-chlorobutyrate to C3 of the 2-iminobutyrate product. After correction for kinetic isotope selection, the erythro isomer yields 7.3% intramolecular triton transfer and the threo isomer only 2.6%. Although the D-erythro diastereomer has been examined intensively, less quantitative data are available for the D-threo substrate. While the threo substrate yields the same spectrophotometrically detectable intermediates (Walsh et al., 1973b), it undergoes the elimination reaction with a twofold lower V_{max}, and the data of Table I show a somewhat lower tritium kinetic isotope effect. It is as yet unclear whether product release (k_1 and k_2 of Scheme VIII) is the sole rate-determining step for the reaction of the D-threo substrate. Given that a bound transoid enamine intermediate is formed initially from both diastereomeric substrates, it is likely that k_1 and k_2 of Scheme VIII are not changed and that some earlier steps must be slower to account for the twofold reduction in V_{max} observed when the threo diastereomer is the substrate. This could certainly account for the observed difference in the percentage of intramolecular tritium transfer.

Acknowledgment

We thank Mr. Alex Y. P. Cheung for accomplishing most of the structural proof syntheses, as shown in Scheme III, and Professor J. Knowles and Mr. Jed Fisher for making helpful suggestions.

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A Calorimetric Study of the Thermotropic Behavior of Aqueous Dispersions of Natural and Synthetic Sphingomyelins†

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ABSTRACT: A recently developed differential scanning calorimeter has been used to characterize the thermotropic behavior of aqueous dispersions of liposomes containing sphingomyelin. Liposomes derived from sheep brain sphingomyelin exhibit a broad gel–liquid crystalline phase transition in the temperature range of 20–45 °C. The transition is characterized by maxima in the heat capacity function at 31.2 and 37.1 °C and a total enthalpy change of 7.2 ± 0.4 kcal/mol. Beef brain sphingomyelin liposomes behave similarly but exhibit heat capacity maxima at 30, 32, and 38 °C and a total enthalpy change of 6.9 kcal/mol. The thermotropic behavior of four pure synthetic sphingomyelins is reminiscent of multilamellar lecithin liposomes in that a single, sharp, main transition is observed. Results obtained for liposomes containing mixtures of different sphingomyelins are complex. A colyophilized mixture of *N*-palmitoylsphingosinephosphorylcholine, *N*-stearoylsphingosinephosphorylcholine, and *N*-lignocerylsphingosinephosphorylcholine in a 1:1:1 mol ratio exhibits a single transition with a T_m below that observed for the individual components. On the other hand a 1:1 mixture of *N*-stearoylsphingosinephosphorylcholine and 1-palmitoyl-2-oleylphosphatidylcholine exhibits three maxima in the heat capacity function. It is clear from these results that the thermotropic behavior of sphingomyelin-containing liposomes is a complex function of the exact composition. Furthermore, it appears that the behavior of the liposomes derived from natural sphingomyelins cannot be explained in terms of phase separation of the individual components.

Considerable information relating to the physical properties and molecular organization of glycerophospholipids in bilayer vesicles (Huang, 1969; Suurkuusk et al., 1976; Lee, 1975) or multilamellar liposomes (Bangham et al., 1967; Hinz and Sturtevant, 1972; Chapman, 1968; Ladbrooke and Chapman, 1969) has been obtained from calorimetric studies of their thermotropic behavior. However, little similar information about sphingomyelin liposomes is available. This latter type of liposome is particularly intriguing in that the sphingomyelins undergo thermotropic phase transitions in the physiological temperature range (Shinitzky and Barenholz, 1974; Shipley et al., 1974).

Shipley et al. (1974) have recently reported that an aqueous dispersion of mixed sphingomyelins derived from bovine brain undergoes a complex series of thermally induced transitions, exhibiting several maxima in the heat capacity function in the temperature range of 30–45 °C. Those workers suggested that the distinguishable transitions may be the result of phase separation of the components, although strong evidence for this proposal was lacking.

In this communication calorimetric results on the thermotropic behavior of aqueous dispersions of sphingomyelins derived from sheep and bovine brain, of four essentially pure synthetic sphingomyelins, of a 1:1:1 colyophilized mixture of three of the synthetic sphingomyelins, and of mixtures of a pure sphingomyelin with 1-palmitoyl-2-oleyl-L-α-phosphatidylcholine are reported. The results obtained with the naturally occurring sphingomyelins are in essential agreement with those of Shipley et al. (1974). However, the results obtained with the pure sphingomyelins demonstrate that the special "rules" governing the thermotropic behavior of glycerophospholipids do not apply to the sphingomyelins; that the phase transition characteristics of sphingomyelin-containing liposomes are a complex function of the exact composition; and that it is unlikely that the thermotropic behavior of natural sphingomyelin liposomes is the result of phase separation of the components.

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