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Differentiation of Diastereomeric Salts of Hydroxyethyl(thiamin) and Analysis of Stereochemical Requirements in Pyruvate Decarboxylase

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Pyruvate undergoes enzyme-catalyzed decarboxylation through formation of an adduct with thiamin diphosphate. The adduct is chiral and loss of carbon dioxide produces a second chiral intermediate, 2-(1-hydroxyethyl)thiamin diphosphate (HETDP). It has been shown previously that 2-(1-hydroxyethyl)thiamin (HET) can be resolved as a salt of (+)- or (−)-2,3-dibenzoyltartaric acid. The resulting optically active samples of HET have been converted to HETDP and used for enzyme studies. Both (+) and (−) HETDP were converted by the apoenzyme of pyruvate decarboxylase. This apparent lack of stereospecificity prompted us to establish independently the extent of the resolution accomplished by fractional crystallization. The diastereomeric dibenzoyltartrate salts have now been shown to be distinguishable by ^{1}H NMR spectroscopy of solutions in methanol-d_4. Calculated spectra permit an estimation of the relative concentrations of the enantiomers of HET in any sample under these conditions. On the basis of this information, it is concluded that the enzyme can react with both enantiomers of HETDP, suggesting that the active site of wheat germ pyruvate decarboxylase can accept the interchange of the hydrogen and methyl groups of the $C_{2\alpha}$ position of HETDP.

The conversion of pyruvic acid to acetaldehyde and carbon dioxide through catalysis by pyruvate decarboxylase involves covalent intermediates derived from enzyme-bound thiamin diphosphate (TDP) (*1, 2*). The reactants, products, and coenzyme are achiral while intermediates, 2-(2-lactyl)thiamin diphosphate (LTDP) and 2-(1-hydroxyethyl)thiamin diphosphate (HETDP) derived from the addition of C-2′ of thiamin diphosphate to the carbonyl group of the substrate, are chiral (See Scheme I). Stereochemical analysis can therefore be used as a probe of the mechanism if the intermediates can be studied.

Critical support for the steps of the mechanism, which was proposed on the basis of chemical studies by Breslow (*3*), came from the observation reported in 1961 by Krampitz and co-workers that wheat germ pyruvate decarboxylase which had been freed of thiamin diphosphate by dialysis will convert exogenous racemic HETDP to acetaldehyde and enzyme-bound TDP (*4*), as in the final step of the mechanism in Scheme I. Later, Ullrich and Mannschreck isolated HETDP from an inactivated subunit of pyruvate dehydrogenase. The isolated HETDP was optically active ([α] = 10 ± 2°) (*5*), showing that this enzyme can produce (+)-HETDP stereospecifically from LTDP.

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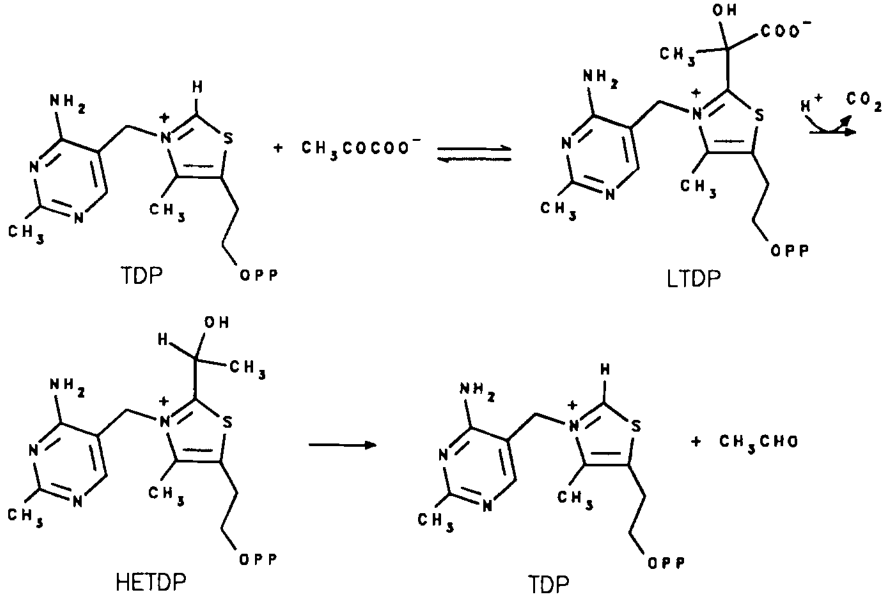


SCHEME I

Extensions of the Krampitz experiments were conducted in our laboratory using resolved samples of HETDP to activate the apoenzyme of wheat germ pyruvate decarboxylase (*6*). These samples had molar optical rotations equivalent to those reported by Ullrich and Mannschreck (*5*) for the material isolated from pyruvate dehydrogenase as well as equal and opposite circular dichroism Cotton effects (*6*). Crystallographic analysis of a derivative established that the stereocenter of (+)-HETDP has the *R* configuration (*6*). It was found that both optical isomers bind to the apoenzyme and are converted to TDP and acetaldehyde (K_m [(+)-HETDP] = 4.5 μM; K_m [(−)-HETDP] = 12 μM). We interpreted this to mean that the enzymic site recognizes the hydroxyl group of the side chain of HETDP and can accommodate the hydrogen and methyl groups of the side chain nonspecifically. However, an alternative explanation is that the samples which we believed to be resolved were actually partially racemic mixtures and the enzyme-catalyzed reaction involves only a single enantiomer which is present in both the (+) and the (−) samples. In order to interpret the enzyme activation experiment without the uncertainty due to the interpretation of the relationship of optical rotation to enantiomeric purity, it is necessary to have an independent and unambiguous measure of the stereochemical content of the samples. (Since optical activity is the net result of rotations due to all the species present in solution, its measurement without an independent standard does not establish the enantiomeric excess in a sample.)

NMR spectra of the diastereomeric derivatives of HET and dibenzoyltartrate (dissolved in dimethyl sulfoxide-d_6) which serve as precursors did not show any distinction between the salts of the *R*-(+) and *S*-(−) samples. A recent paper by

Zingg *et al.* (*7*) provided us with the insight necessary to develop a method for the determination of enantiomeric excess in samples of HETDP. They described studies of NMR spectra of solutions of diastereomeric chiral salts. Differentiated signals are most likely to be observed when the solvent does not dissolve the individual ions but rather solvates the intact ion pair and maintains the diastereomeric contacts. Thus, the use of a poor solvent is the key to obtaining diastereomeric resolution in the spectrum. We now report that application of the Zingg–Arnett principle to dibenzoyltartrate salts of HET permits observation of the enantiomeric purity of HETDP. The resulting data permit a more confident interpretation of previous enzyme kinetic experiments with the enantiomeric forms of HETDP.

EXPERIMENTAL SECTION

Materials. Thiamin chloride hydrochloride (USP) was from Novopharm, Ltd. (Scarborough, Ontario). (+)- and (−)-2,3-Dibenzoyltartaric acid monohydrates were purchased from the Aldrich Chemical Co. Solvents were distilled prior to use. Dimethyl sulfoxide-d_6 (99.9%) and methanol-d_4 (99.5% D) were from the Aldrich Chemical Co. 2-(1-Hydroxyethyl)thiamin (HET) was synthesized from thiamin and acetaldehyde, purified, and resolved as the 1 : 1 (+)-2,3-dibenzoyltartrate salt or as the (−)-2,3-dibenzoyltartrate salt (*8*). The samples were freed from the resolving agent by reaction with acid and extraction after three consecutive crystallizations. The crystals were separated from the mother liquor, washed with ice-cold ethanol, and dried *in vacuo*. The polarimetric rotations ($[\alpha]_D$) were $+12.6 \pm 0.1°$ (water, $c = 0.1$) and $-12.6 \pm 0.1°$ (water, $c = 0.1$), respectively. Salts of unresolved HET with (+)-2,3-dibenzoyltartrate and (−)-2,3-dibenzoyltartrate were obtained by freeze-drying the solutions formed by direct mixing.

Methods. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Proton NMR spectra were recorded on a Varian XL-400 NMR spectrometer with samples (0.01 M) in methanol-d_4. Computer-calculated ^{1}H NMR spectra were produced using the program "NMRCALC" (by M. D. Johnston, Jr., University of South Florida) with a PC-AT compatible microcomputer. The program uses the standard Bloch equations to calculate patterns based on input chemical shifts, coupling, and machine parameters.

RESULTS AND DISCUSSION

The enantiomers of HET form diastereomeric salts with (−)-2,3-dibenzoyltartaric acid (and with its enantiomer). The salts are of limited and differing solubility in alcohols, providing the basis for the resolution procedure we have used (*8, 9*). However, both diastereomers dissolve readily in dimethyl sulfoxide and ^{1}H NMR spectra of solutions in dimethyl sulfoxide-d_6 show no differences in peaks for the diastereomeric salts. This is consistent with the dimethyl sulfoxide solutions containing the individual ions rather than cosolvated ion pairs.

Application of the Zingg–Arnett principle (7) suggested that the use of a poorer solvent for the salt would improve our ability to observe different signals arising from each diastereomer. Since alcohols provided the limited and differential solubility for optical resolution, we tried these as solvents for NMR analysis. We were able to observe diastereomeric distinctions in the spectra of the pair of chiral salts which result from the combination of racemic HET and (−)-2,3-dibenzoyltartaric acid (DBT) in methanol-d_4 at 400 MHz. We observed an upfield shift of all signals compared to free HET (Table 1). In addition, the magnetic nonequivalence of protons directly attached to the carbon atom at the stereocenter of HET ($C_1\alpha$) is 0.005 ppm or 2.0 Hz in a 400-MHz spectrum (Table 1, Fig. 1). Parallel results were obtained when (+)-DBT was combined with racemic HET under the same conditions.

Figure 2 shows the same details of the spectrum for the resolved (*R*)-(+)-HET × (−)-DBT. (The spectra of racemic HET × (+)-DBT and resolved (−)-HET × (+)-DBT are identical, as expected, to those of the enantiomeric systems described above.) The calculated spectra test the limits of the resolution of the spectra in assessing the stereoisomeric purity. It is clear that the resolved salt is diastereomerically pure to within the limits of detection of the system and the resulting HET will be released with the same enantiomeric purity.

The use of this method for the determination of the diastereomeric excess is limited by the fact that the NMR signals are not baseline separated. By calculating and matching of the spectra we established the limits of detection at about 90% diastereomeric excess. Attempts to increase the magnetic nonequivalence by mix-

TABLE 1

Proton NMR Spectra (400 MHz, Methanol-d_4) of HET and Its Salt with (−)-DBT (Only HET Signals Are Listed)

	HET		HET × (−)-DBT		
	δ	J(Hz)	δ	J(Hz)	$\Delta\delta$[a]
$C_{6'}$-*H*	7.57 s		7.257 s		0.0
N_3-CH_2	5.6 d	17.6	5.512 d	17.6	0.0
	5.54 d	17.6	5.458 d	17.6	0.0
HO-C_αH-CH_3	5.39 q	6.4	5.353 q	6.5	0.005
CH_2-OH	3.85 t	5.6	3.809 t	5.6	0.0
C_5-CH_2	3.13 t	5.6	3.076 t	5.6	0.0
$C_{2'}$-CH_3	2.58 s		2.418 s		0.0
C_4-CH_3	2.41 s		2.328 s		0.0
HO-C_αH-CH_3	1.69 d	6.4	1.604 d	6.5	0.005

[a] The magnetic nonequivalence ($\Delta\delta$) is defined as the chemical shift of the salt in which both the acid and the base rotate light in the same direction, i.e., the (+)-HET × (+)-DBT or the (−)-HET × (−)-DBT salt, less the chemical shift of the salt in which the signs of the optical rotation of the base and the acid are different, i.e., the (+)-HET × (−)-DBT or the (−)-HET × (+)-DBT salt.

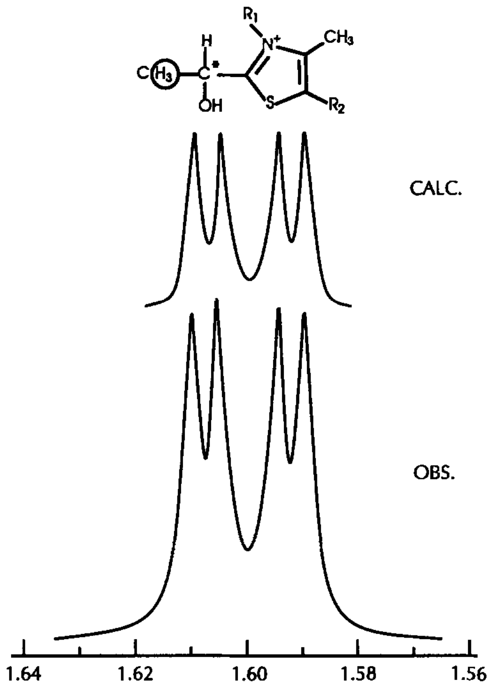


FIG. 1. Proton NMR spectrum (400 MHz) corresponding to protons of the methyl group connected to the carbon atom at the stereocenter of HET ($C_{2\alpha}$) in the (−)-DBT salt of racemic HET. In the absence of DBT, the signal is a doublet. The calculated spectrum is shown for comparison. The absolute chemical shifts vary between samples due to changing lock frequencies of the NMR spectrometer on the various days on which each spectrum was recorded.

ing methanol-d_4 with 0.1 vol of chloroform-d_3 decreased the diastereomeric discrimination. Mixing with 0.1 vol of deuterium oxide led to a loss of spectral resolution.

In order to calibrate the use of this method for analysis of samples of HET, a series of combinations of resolved and racemic HET were prepared and analyzed. A mixture of 2 eq of racemic HET × (−)-DBT with 1 eq of the resolved (*R*)(+)HET × (−)-DBT showed a normalized integral ratio of 64 : 36 (±2%) (Fig. 3). A mixture of 1 eq of racemic HET × (−)-DBT with 2 eq of (+)-HET × (−)-DBT gave an integral ratio of 77 : 23 (±2%) (Fig. 3). Using this method of analysis, we determined the diastereomeric excess of the salt obtained after three consecutive crystallizations to be 89 ± 7%.

In a previous report from our laboratory, samples of (−)-HETDP and (+)-HETDP were used to activate the apoenzyme of wheat germ pyruvate decarboxylase. In this process, HETDP is converted to enzyme-bound TDP and acetaldehyde. Thus, after HETDP associates with the enzyme either HETDP or TDP dissociates, K_m measures the net affinity adjusted by the rates of conversion. K_m for (*S*)(−)-HETDP is 12 μM while K_m for (*R*)-(+)-HETDP is 4.5 μM. The present results show that the assays of binding of (+)- and (−)-HETDP to pyruvate

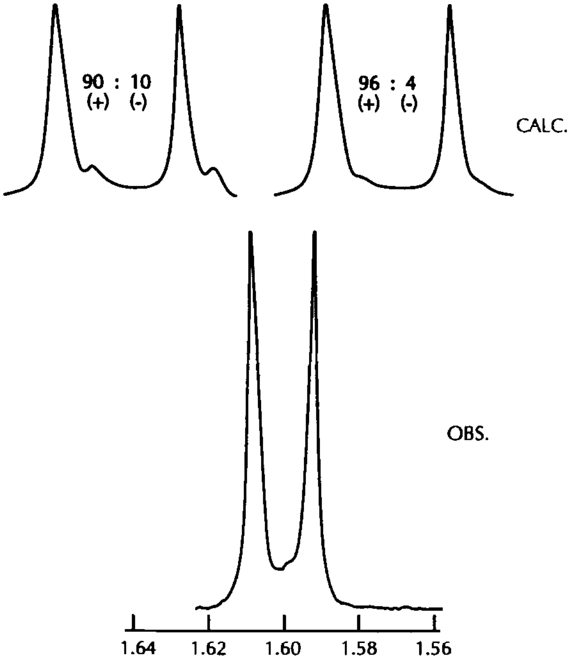


FIG. 2. Proton NMR spectrum (400 MHz) corresponding to protons of the methyl group connected to the carbon atom at the stereocenter of HET ($C_{2\alpha}$) in the (−)-DBT salt of (+)-HET. The calculated spectrum is shown for comparison.

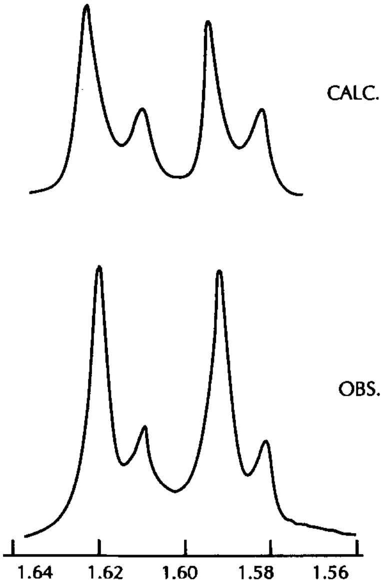


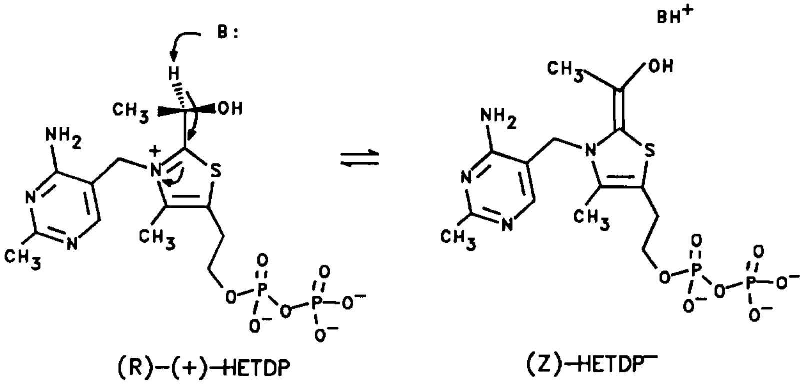
FIG. 3. Observed and calculated spectra as in Fig. 2 for a mixture of the (−)-DBT salts of (+)-HET and (−)-HET.

decarboxylase were carried out with material that contained no detectable amount of contaminating enantiomer. Since the sensitivity of the NMR analysis is limited by the resolution of the instrument, the upper limit of certainty of purity is also limited. Could the activity be due only to the presence of the higher-affinity (*R*)-(+) isomer in the (−) sample? The uncertainty in our measurements (<18%) places an upper limit on the amount of the (*R*) enantiomer in the sample we designate as (*S*)-(−). At the upper limit of possible contamination, we would expect that the apparent K_m for the (−) enantiomer would be about four times higher than that for the pure (+) enantiomer (since the apparent K_m for the (+) sample would be higher due to an equivalent contamination by the (−) enantiomer). However, the difference in K_m is only a factor of 2.5 and therefore contamination cannot account for the effect within these limits although the difference in affinities may be greater than the apparent values indicate. These results are thus consistent with a mechanism in which both enantiomers bind and are converted by the enzyme.

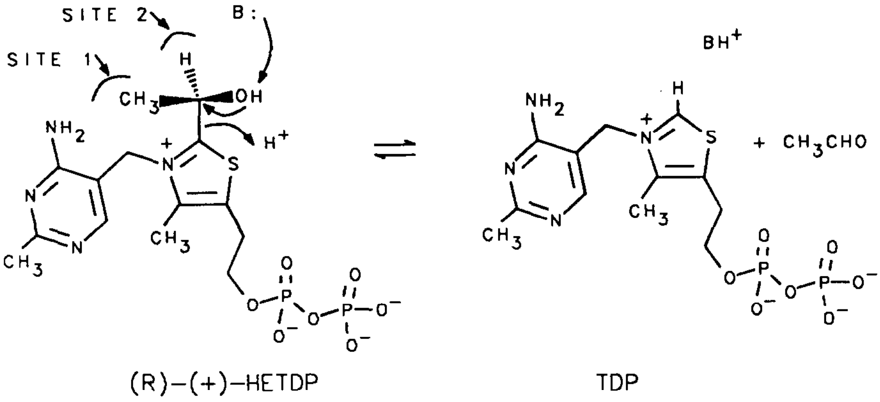
A mechanism in which the enzyme generates a single enantiomer of HETDP through decarboxylation of pyruvate and also reacts with either enantiomer of HETDP to form acetaldehyde does not violate the principle of microscopic reversibility. The intermediate (*E* or *Z* HETDP$^-$), generated by decarboxylation of LTDP, must be protonated stereospecifically in order to generate a single enantiomer of HETDP. (Scheme II is illustrated hypothetically with the *Z* isomer.)

In the reverse reaction, carboxylation of HETDP$^-$, resulting from deprotonation of HETDP, should be stereospecific. The deprotonation must generate the same geometric isomer of HETDP$^-$ (but which one is not yet known) and in a consistent reversible mechanism must result from the reaction of a single enantiomer of HETDP (Scheme II).

However, the elimination of acetaldehyde from HETDP does not involve HETDP$^-$. Instead, the proton is removed from the hydroxyl group of HETDP to lead to elimination of acetaldehyde. If a base on the enzyme associates with the proton of the hydroxyl group, the distinction between the *R* and *S* species is based



SCHEME II



SCHEME III

on the accessibility of the hydroxyl proton, the position of the base, and the relative affinity of the enzyme for the enantiomers (Scheme III).

The differing substituents on $C_{2\alpha}$ (H vs CH_3) can each fit into the arbitrary spaces indicated as "Site 1" and "Site 2." Since distinction between these does not affect the normal catalytic process, there would be no evolutionary pressure to make such a distinction in terms of catalysis. As a result, the distinction need not be complete. The generality of these conclusions and the development of methods for greater sensitivity in stereochemical analysis of thiamin derivatives are currently in progress.

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