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Synthesis and Purification of Racemic 2-(1-Hydroxyethyl)thiamine Revisited[1]

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A procedure is reported for the rapid (≤1 day) and reproducible purification of 2-(1-hydroxyethyl)thiamine (HET) from contaminating thiamine (≤50%) using aqueous cation exchange chromatography. The unhydrated product contains ≤0.2% thiamine on the basis of its ^{1}H NMR spectrum, visualization on silica gel TLC by fluorescence quenching after development in 60% aqueous ethanol, and the failure to observe the hydrolysis of thiamine using an iodine trapping assay under conditions where hydrolysis of ≤0.2% contaminating thiamine would have been detected. This purification procedure is also applicable to the resolved *R* and *S* stereoisomers of HET. Several literature procedures for the synthesis and purification of racemic HET are discussed and critically evaluated.

INTRODUCTION

Thiamine diphosphate (TDP)[3] (**1a**), the physiologically active form of thiamine (**1b**), functions in carbohydrate metabolism as a coenzyme for the decarboxylation of α-keto acids, the formation of α-ketols, and transketolase reactions (*1*). Thiazolium carbinols such as 2-(1-hydroxyethyl)thiamine (HET) (**2b**) or HETDP (**2a**) have been implicated as reaction intermediates in several nonenzymatic and enzyme-catalyzed aldol-type addition reactions between thiamine, or TDP in the enzymatic reactions, and carbonyl compounds. For example, 2-(1-hydroxyethyl)thiamine diphosphate (HETDP, **2a**) is an intermediate in the conversion of pyruvate to acetaldehyde catalyzed by the TDP-dependent enzyme pyruvate decarboxylase (2-oxo-acid carboxy-lyase; EC 4.1.1.1) (*2*).

We are interested in the mechanism and catalysis of several nonenzymatic reactions involving thiamine and HET. The rate constants for several of the slow model reactions involving HET are conveniently determined from initial rate measurements. However, small amounts of reactive impurities, such as thiamine,

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[3] Abbreviations used: TDP, thiamine diphosphate; HET, 2-(1-hydroxyethyl)thiamine; HETDP, 2-(1-hydroxyethyl)thiamine diphosphate; T, thiamine.

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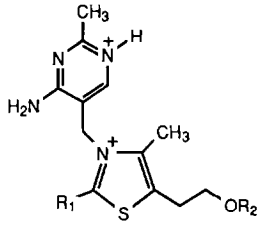
cause errors in kinetic measurements by the method of initial rates because these reactions are typically followed to less than 2% of completion (*3*). Because our



1, R_1 = H a, R_2 = $P_2O_6^{3-}$

2, R_1 =  b, R_2 = H

mechanistic investigations require relatively large amounts of highly purified HET, we have undertaken the development of a rapid and convenient procedure for the purification of racemic HET from contaminating thiamine and reexamined the procedures for the synthesis of racemic HET.

EXPERIMENTAL PROCEDURES

Materials. Thiamine chloride hydrochloride, acetaldehyde, and deuterium oxide (≥99 atom% D) were purchased from Aldrich. The acetaldehyde was always distilled immediately before use. For the synthesis of HET in basic absolute ethanol, thiamine was dried *in vacuo* at 80°C against P_2O_5 for 5 h (mp 242–243°C dec). All water was prepared on a four-bowl Milli-Q water system including an Organex-Q cartridge (Millipore). All other organic and inorganic chemicals were reagent grade and were used without further purification. SP-Sephadex C-25 cation exchange resin (Na^+ form) was purchased from Pharmacia. Baker-flex silica gel IB-F thin-layer chromatography plates (200 μm) were purchased from J.T. Baker and stored at ambient temperature and humidity.

Methods. Proton NMR spectra were recorded on a Bruker WM-300 300-MHz spectrometer in D_2O using sodium 4,4-dimethyl-4-silapentane-1-sulfonate as an internal reference. Melting points were obtained using an Electrothermal digital melting point apparatus and required no stem correction. Ultraviolet absorbance measurements were made using a Hitachi U-2000 spectrophotometer and matched quartz cuvettes with a 1-cm path length. Conductivity measurements were made using a Radiometer CDM 83 conductivity meter and a type CDC 314 cell. Hydrolysis of the thiazolium ring of thiamine was followed at 351 nm by trapping the enethiolate hydrolysis product with iodine as described elsewhere (*4*).

Synthesis of racemic 2-(1-hydroxyethyl)thiamine chloride hydrochloride. The aqueous synthetic method of Oka and Yurugi (*5*) was followed with several minor modifications which were adapted from Gruys *et al.* for the purification of HETDP from TDP (*6*). Thiamine chloride hydrochloride (19.9 g, 0.059 mol) was dissolved in 90 ml of water in a 500-ml round-bottom flask, the thiamine solution was

cooled to 4°C in a refrigerator, and then 113 ml (1.6 mol) of ice-cold 80% aqueous acetaldehyde was added to the cold thiamine solution; it is important that the temperature of these solutions be ≤20°C to prevent boiling of the acetaldehyde. The acetaldehyde/thiamine solution was warmed to room temperature and adjusted to pH 8.0 with 5 M NaOH, and 1 M NaOH was added dropwise to give a clear, pale yellow solution of pH 8.6. The flask was stoppered and the acetaldehyde/thiamine solution was stirred for 2 h in a 42–45°C water bath. It is important not to exceed 45°C during the incubation because higher temperatures result in boiling and excessive loss of acetaldehyde. After 2 h the reaction solution was cooled to room temperature and the pH of the solution was adjusted from pH 7.9 to 8.6 by the dropwise addition of 1 M NaOH. The reaction flask was resealed after the pH adjustment and incubated for an additional hour at 42–45°C. The reaction solution was then adjusted to pH 2.0 by the dropwise addition of 5 M HCl, and the solvent was removed by rotary evaporation at 50°C under reduced pressure to give an orange oil. The oil, which contained HET, thiamine, NaCl, and trace impurities, was dissolved in 225 ml of boiling 5% HCl/ethanol (prepared by diluting 12.5 ml of 12.0 M aqueous HCl in a 250-ml volumetric flask with absolute ethanol) and then cooled at −20°C in a freezer for 45–60 min to precipitate the NaCl. Care must be taken not to exceed this time limit because HET will also begin to precipitate under these conditions. The insoluble NaCl was removed by filtering the solution through a 250-ml sintered glass funnel (10–15 μm) and the NaCl-free filtrate was concentrated by rotary evaporation as described above. The orange oil was then dissolved in a minimum amount of boiling methanol and 12.5 g (56% yield) of crude product was obtained after the addition of acetone to the methanol solution and cooling to room temperature; the crude product was 90% racemic HET and 10% unreacted thiamine on the basis of ^{1}H NMR. The crude product (mp 214–216°C dec) was routinely stored desiccated over anhydrous $CaSO_4$ at room temperature after drying *in vacuo* at 60°C over P_2O_5 to a constant weight. Further precipitations of the crude product from methanol/acetone could only improve the purity of the product to 98% racemic HET and 2% thiamine on the basis of ^{1}H NMR. Consequently, the crude HET was purified by cation-exchange chromatography as described below.

Cation exchange chromatography of crude HET. Twenty-five grams of filtered, swollen SP-Sephadex C-25 cation exchange resin (Na^+ form) was allowed to swell at room temperature in a starting buffer containing 0.58 M NaCl in 1% aqueous formic acid for 48 h. A 2.5 × 50-cm glass chromatography column was packed at room temperature under gravity with continual addition of slurry to avoid layering defects in the column. The column was further packed at room temperature to a bed height of 35 cm by passing 500 ml of starting buffer through the column at a gravity-driven flow rate of 1 ml/min. For routine separation of HET from mixtures containing 2–10% contaminating thiamine, between 0.4 and 1.0 g of crude HET was dissolved in 1–2 ml of starting buffer, loaded onto the column, washed into the column with 2–4 ml of starting buffer, and eluted at room temperature with a linear gradient developed from 250-ml volumes each of 0.58 and 0.70 M NaCl in 1% aqueous formic acid at 1 ml/min into 2.5-ml (100-drop) fractions. Pure HET was typically found to elute between gradient fractions 82 and 100 corresponding

to 0.66 and 0.68 M NaCl, respectively. The NaCl gradient was extrapolated from conductivity measurements made from the starting and ending buffers and early gradient fractions that contained no HET or thiamine to avoid significant contributions of these divalent organic cations to the overall conductivity. Fractions containing pure HET were pooled on the basis of TLC analysis performed as described below. The pooled fractions were evaporated to dryness by rotary evaporation *in vacuo* at 50°C. The resulting white solid, which contained both HET and NaCl, was suspended in 200 ml of boiling 5% HCl/ethanol (prepared as described above) and cooled at −20°C in a freezer for 45–60 min to precipitate the NaCl. The insoluble NaCl was removed by filtering the suspension through a sintered glass filter (10–15 μm). The filtrate, which contained HET and trace NaCl, was evaporated to dryness by rotary evaporation *in vacuo* at 50°C, the white solid was redissolved in 60 ml of a boiling solution of 5% HCl/ethanol, and the remaining NaCl was precipitated and removed as described above. The filtrate was evaporated to dryness by rotary evaporation at 50°C and the white solid was precipitated[4] from methanol/acetone, collected on a sintered glass funnel (40–60 μm), and washed with a small amount of acetone. The product was dried *in vacuo* over P_2O_5 at 60°C for approximately 6 h (mp 231–232°C dec) and was routinely stored desiccated over anhydrous $CaSO_4$ at room temperature. The ^1H NMR spectrum agreed with that reported previously (*8*). The racemic HET chloride hydrochloride is unhydrated and free of NaCl on the basis of the calculated molecular weight obtained from a mercurimetric titration of chloride using diphenylcarbazone (*9*).

Analytical thin-layer chromatography. A 0.5-μl aliquot of each column fraction was spotted, using a Drummond microcapillary pipet, on a commercially available thin-layer chromatography (TLC) plate that consisted of a 200-μm-thick layer of silica gel in an inert binder containing a fluorescent indicator. The TLC plate was developed at ambient temperature in 60% aqueous ethanol. The developed plate was dried for about 30 s using warm air and viewed under short-wavelength UV light; the strong fluorescence quenching properties of HET chloride hydrochloride (R_f = 0.72) and thiamine chloride hydrochloride (R_f = 0.51) easily allow the detection of ≥0.2% contaminating thiamine (see below).

RESULTS AND DISCUSSION

Although racemic HET has been synthesized using reactions that are based on the Hantzsch pyrrole synthesis modified for cyclization to thiazole derivatives (*10*) and the decarboxylation of α-lactylthiamine (*11*), it is more conveniently synthesized by the reaction of thiamine with acetaldehyde in aqueous solution at pH 8.6 at 45°C (*5*) or, more recently, in basic absolute ethanol at −5°C (*8, 11*)

[4] Crystalline HET (thin white plates) has been formed upon dropwise addition of acetone to a solution of HET in methanol (*7*).

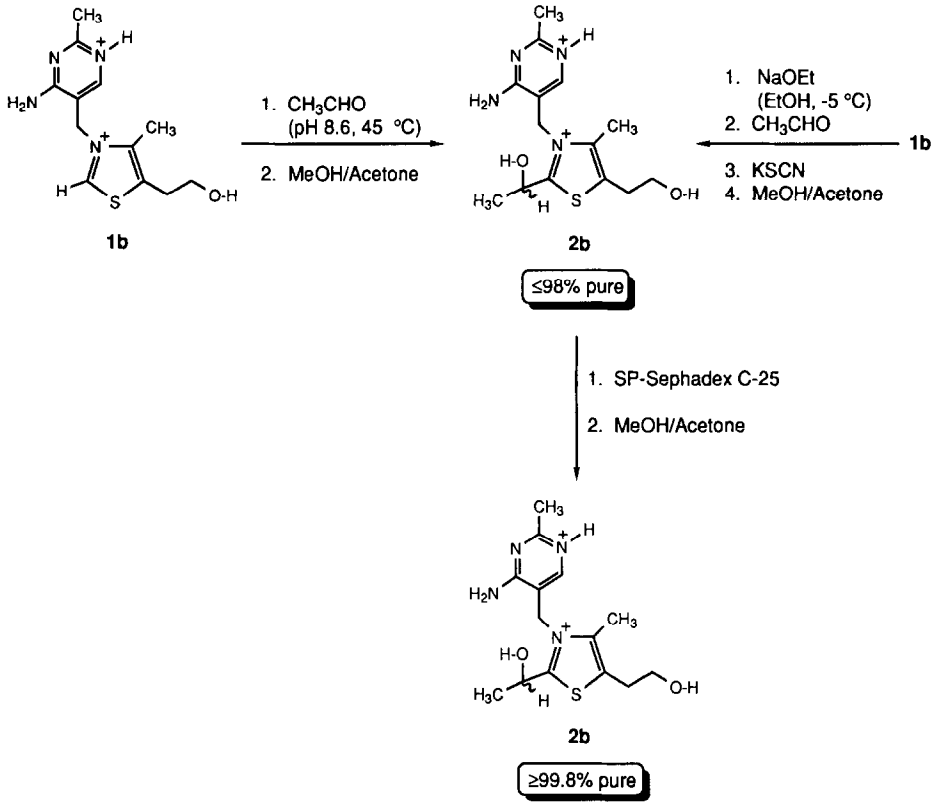


FIG. 1. Summary of the literature procedures for the preparation of racemic 2-(1-hydroxyethyl)thiamine chloride hydrochloride (HET) (**2b**) from acetaldehyde and thiamine. There are two commonly used synthetic routes for the synthesis of HET: (left) thiamine (**1b**) is reacted with acetaldehyde in aqueous solution at pH 8.6 and 45°C and (right) in basic absolute ethanol at −5°C (see text). Both procedures can provide HET that is 95–98% pure where the primary contaminant is unreacted thiamine; the 2–5% remaining thiamine can be removed using the SP-Sephadex C-25 cation exchange chromatography procedure reported here.

(Fig. 1). An advantage of the modified Hantzsch synthesis and the decarboxylation of α-lactylthiamine over the procedures that use an aldol-type addition reaction is that they avoid a difficult separation from contaminating reactant thiamine because HET is the only thiazolium cation formed and it is easily separated from the other reactants.

It is our experience that the addition reaction in basic ethanol (*8, 11*) does not give HET of sufficient purity for use in our mechanistic investigations. The amount of contaminating thiamine remaining could be easily and substantially reduced by reacting the crude HET with potassium thiocyanate, which takes advantage of the selective reactivity of thiamine to nucleophilic attack at the C(2) position of the thiazolium ring (*10*). However, even after treatment with thiocyanate and subsequent precipitation, HET of only 95–98% purity was obtained, which was unacceptable for our investigations.

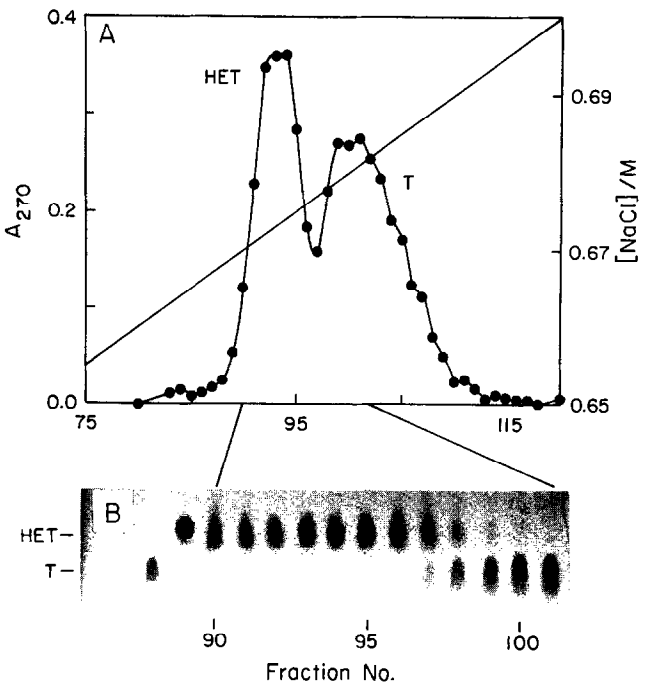
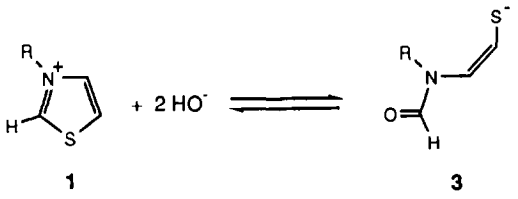


FIG. 2. SP-Sephadex C-25 cation exchange chromatography (2.5 × 35 cm) of a 1.0-ml sample containing 0.52 M HET and 0.59 M thiamine in 0.58 M NaCl and 1% aqueous formic acid. HET and thiamine (T) were eluted with a linear gradient developed from 250-ml volumes each of 0.58 and 0.70 M NaCl in 1% aqueous formic acid at a gravity-driven flow rate of 1 ml/min into 2.5-ml fractions. (A) Ultraviolet (UV) absorption at 270 nm of 1000-fold diluted aliquots of gradient fractions 80–120; the NaCl gradient is also shown. (B) TLC analysis of gradient fractions 90–101. A constant amount of UV-absorbing material (as determined by the absorbance at 270 nm) was spotted on the TLC plate in 0.5-μl aliquots. A thiamine (R_f = 0.51) or HET (R_f = 0.72) reference standard is shown on the left of each row.

The method that we have found most reliable and convenient for the synthesis of racemic HET is the aqueous procedure described by Oka and Yurugi (*5*). This simple method does not require the hazardous reagents (sodium metal or sodium ethoxide; HCl gas), specialized equipment (glassware to maintain an inert atmosphere; refrigerated bath), and expertise that are required for the synthesis of α-lactylthiamine (*12*) and HET (*8, 11*), and provides consistently good overall yields (30–50%) of 98% pure product after two precipitations from methanol/acetone. However, even substrate that contained only 2% thiamine proved unacceptable for our mechanistic work because of a rapid, complicating side reaction involving hydrolysis of the contaminating thiamine. Consequently, we developed a simple and rapid aqueous cation exchange column chromatography procedure for resolving HET from thiamine that provides HET of sufficient purity for our investigations of reactions involving reactive intermediates in thiamine catalysis.

The results of SP-Sephadex C-25 cation ion exchange chromatography of a 1 : 1 mixture (by weight) of HET and thiamine is shown in Fig. 2. Figure 2A shows the



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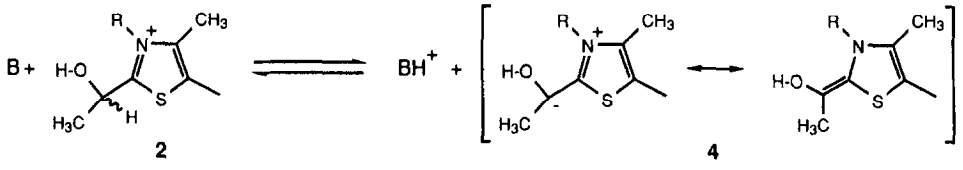
SCHEME 1

absorbance profile at 270 nm of gradient fractions 80–120. A TLC analysis of the peak fractions 90–101, including thiamine and HET standards, is shown in Fig. 2B. These results show that the cation exchange chromatography procedure can resolve thiamine and HET on a preparative scale even under conditions where HET and thiamine are present in equal mass amounts. In routine application of this chromatographic procedure, when 2–3% contaminating thiamine is present, HET which contains ≤0.2% thiamine can be obtained in 85–90% yield (based on the total amount of HET loaded on the column). The high level of purity can be confidently assigned on the basis of (1) the failure to detect the signal from C(2)-H of thiamine in the ^{1}H NMR spectrum of a 1.0 M solution of the purified HET in acidic D_2O under conditions where the C(2)-H signal from ≥1.5% contaminating thiamine would have been observed; (2) the observation of a single spot in the TLC analysis of 0.19 mg of the purified HET under conditions where ≥51 ng (≤0.03%) of contaminating thiamine could be visualized by fluorescence quenching; and (3) the failure to observe hydrolysis of thiamine using an iodine trapping assay under conditions where ≥0.2% contaminating thiamine would have been detected.

Initial rate kinetic assays require substrates of the highest purity. In our studies of the mechanisms of the hydrolysis of the thiazolium ring of thiamine (*4b*) and thiazolium C(α)-proton transfer from HET (*13*), a sensitive initial rate assay has been used in which either the enethiolate hydrolysis product of thiamine (Scheme 1) or the C(α)-carbanion/enamine derived by proton transfer from HET (Scheme 2) is irreversibly trapped with iodine (*4, 13*). These reactions are followed spectrophotometrically by measuring the zero-order disappearance of triiodide absorption at 351 nm, which is in equilibrium with iodine (*14*). Substituents at the thiazolium C(2) position slow the rate of nucleophilic attack at C(2) approximately 100-fold (*15*) and the C(2)-ylide derived from thiamine reacts rapidly and reversibly with iodine in aqueous solution (*4a*). The 50-fold greater rate of the thiazolium ring



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SCHEME 2

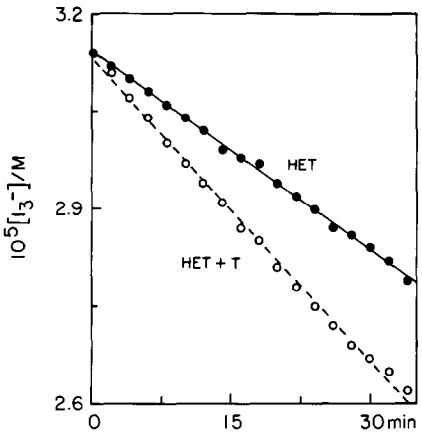

FIG. 3. Reaction of iodine with equal mass amounts of highly purified HET (●) or crude HET (○), which contains 1.5% contaminating thiamine on a molar basis, in H_2O at 25°C, I = 1.0 M (KNO_3). Final conditions were 4.0×10^{-5} M iodine-triiodide, 0.20 M KI, and 20 mM highly purified HET (●) in 0.30 M potassium acetate buffer containing 0.30 fraction free base. Iodine, which is in equilibrium with triiodide ion, reacts with both the enethiolate hydrolysis product of thiamine (**3**) and the C(α)-carbanion/enamine (**4**) derived from HET. The broken line through the data for crude HET was calculated (*14*) from the rate constants for C(α)-proton transfer from highly purified HET (*13*) (solid line) and hydrolysis of 0.3 mM thiamine (**4b**), which corresponds to 1.5% contaminating thiamine. The fit of the calculated line to the data for crude HET demonstrates that the difference in the observed rates is due to the rapid hydrolysis of the small concentration of contaminating thiamine present in the crude HET.

hydrolysis reaction of thiamine compared to thiazolium C(α)-proton transfer from HET (*13*) requires ≤0.2% contaminating thiamine in reactions containing highly purified HET if the hydrolysis reaction is to represent ≤5% of the observed rate of triiodide consumption; we estimate the error in our values of k_{obsd} to be ≤±5%.

Figure 3 shows how sensitive the iodine trapping assay for C(α)-proton transfer from HET is to trace amounts of contaminating thiamine. The slope of the solid line is the initial rate of triiodide consumption observed when 20 mM purified HET (treated using the chromatographic procedure described here) is used in the iodine trapping assay for C(α)-proton transfer. The broken line is the calculated initial rate of triiodide consumption if 1.5% contaminating thiamine were present (*4, 13*); the open circles are data points from a kinetic run using 20 mM crude HET that contained 1.5% contaminating thiamine based on the ^{1}H NMR spectrum of a 1.0 M solution of the crude HET. The fact that the calculated line fits the observed data demonstrates that all of the "extra" observed rate seen with the crude HET sample compared to the highly purified HET sample can be attributed to the presence of contaminating thiamine.

There is considerable inconsistency in the literature regarding the melting point of racemic HET. We measured a (corrected) melting point of 231–232°C (dec) after precipitation from methanol/acetone for unhydrated racemic HET that was synthesized using the modified aqueous synthetic procedure described here and purified by aqueous cation exchange chromatography. We have found that the melting point for HET can vary widely depending on the amount of contaminating

thiamine and/or NaCl which is present in the final product; it is very easy to carry over small amounts of these contaminants if care is not taken and the presence of these contaminants markedly depresses the melting point. Comparisons with uncorrected literature melting point values, which are in the range 217–236°C (*5, 8, 11, 16*), are not likely to be useful as a criterion of purity because these relatively high temperatures in uncorrected apparatuses can lead to discrepancies merely because of the lack of calibration.

An inherent weakness in this purification scheme is that the removal of NaCl from the purified HET relies on the difference in solubility of NaCl and HET in 5% HCl/ethanol at −20°C, which will not be reproducible if the procedure is not followed in detail. Particular attention must be paid to the preparation of the 5% HCl/ethanol solution to reproduce our product yield and purity. We routinely perform a chloride assay (*9*) on the final product and typically find ≤1% contaminating NaCl. For applications where contaminating NaCl is not a problem, only a single precipitation from 5% HCl/ethanol is necessary to remove the majority of the contaminating NaCl. The presence of contaminating NaCl would make an elemental analysis problematic.

To our knowledge this is the first report of a preparative column chromatography procedure for the resolution of HET and thiamine. Aqueous cation exchange chromatography was used previously to resolve the various phosphorylated esters of thiamine and HET on the preparative scale (*2b, 6, 17*). Krampitz reported a method for resolving HET from thiamine using preparative paper chromatography (*16*), but this method is relatively slow and provides lower product yields than the cation exchange chromatography procedure reported here. We examined several other methods for separating HET from thiamine on the preparative scale including silica gel column chromatography, preparative TLC on a 2000-μm layer of silica gel, C_{18}-reverse-phase HPLC, and rotary TLC using a Chromatotron[5] on a rotor with a 2000-μm thickness of silica gel and have not found a procedure that offers greater resolution on a preparative scale than the cation exchange chromatography method reported here. We have also used this purification procedure to remove contaminating thiamine from samples of the resolved (*11*) *R* and *S* stereoisomers of HET.

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