Enzymatic Hydrolysis of Organic Cyclic Carbonates*

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Ethylene carbonate, a cyclic organic carbonate widely used industrially, is toxic when metabolically converted to ethylene glycol. A rat liver enzyme active in catalyzing the ring opening has been purified to electrophoretic homogeneity and found to be active in the hydrolysis of ethylene, vinylene, and propylene carbonates to \( \text{CO}_2 \) and the respective glycols. Neither thio carbonates nor open chain carbonates served as substrate nor did a variety of esters, lactams, lactones, and related heterocycles. The enzyme was active, however, with imides and appears to be identical to rat liver imidase. The identification was confirmed by copurification of enzyme activities, by similarities in the pattern of inhibition, and by the reactivity with a polyclonal antibody directed against the enzyme purified here.

The systemic toxicity observed with ethylene carbonate, a cyclic organic carbonate that serves as a major intermediate in the plastics industry, was recognized as being similar to the effects of ethylene glycol (1). When the cyclic carbonate was fed to rats, ethylene glycol accumulated as did more oxidized metabolic products (1).

Since simple hydrolysis of ethylene carbonate results in the formation of ethylene glycol (Reaction 1), we examined a number of commercially available esterases, expecting to find one capable of catalyzing the conversion. The survey included rabbit and porcine liver esterases and pancreatic lipase, the last enzyme having recently been shown to hydrolyze large, micellar organic carbonates (2). Ethylene carbonate was not a substrate of any of these enzymes. Because of an interest in the family of enzymes that are active in detoxication, enzymes that are generally characterized by extremely broad substrate specificity (3), we searched for activity toward the cyclic organic carbonate functional group.

![Chemical Structure](image)

**REACTION I**

\[
\begin{align*}
\text{O} & \quad \text{H}_2\text{O} \quad \text{HO} \quad \text{OH} \\
\text{O} & \quad \text{H}_2\text{C} \quad \text{CH}_2 \quad \text{CO}_2 \\
\text{C} & \quad \text{H}_2 \\
\end{align*}
\]

We report the isolation in homogeneous form of an enzyme from rat liver that catalyzes the hydrolysis of ethylene carbonate as well as certain other cyclic organic carbonates but that is inactive toward linear organic carbonates and carboxyesters. Of specific interest is the finding that the isolated enzyme appears to be identical to one variously described as dihydro pyrimidinase (EC 3.5.2.2), hydantoinase, dihydropyrimidine hydride, and dihydropyrimidine aminohydrolase, an enzyme previously identified as a nonspecific imidase active with a large number of cyclic and linear imides (4).

**MATERIALS AND METHODS**

Organic carbonates and their analogues were from Aldrich; sources of additional candidate substrates have been presented (4). Unless otherwise noted, other reagents and enzymes were from Sigma.

**Electrophoresis**

Polyacrylamide gel electrophoresis was performed with an X-cell Minigel Cell (Novex, San Diego) using the manufacturer's precast isoelectric focusing gels (pH 3–10 range) as well their 14% Tris-glycine gels; the latter were used with SDS by the method of Laemmli (5). Isoelectric focusing was conducted at 100 V for the first h, 200 V for the second h, and 500 V for the last 30 min. Protein standards for SDS, gradient-native, and isoelectric focusing gels were purchased from Novex, Amersham Pharmacia Biotech, and Bio-Rad, respectively.

**Standard Assay**

Ethylene carbonate was adopted as the standard assay substrate, and its hydrolysis was followed by estimating the formation of ethylene glycol. The product was oxidized by periodate to form 2 mol of formaldehyde, which was allowed to react with chromotropic acid to yield a colored product (7).

Enzyme in a total volume of 0.1 ml was incubated in 100 mM sodium pyrophosphate at pH 8.1 with 5 mM ethylamine carbonate for 1 h at 25 °C. The reaction was terminated by transfer to an ice bath and addition of 0.1 ml of \( 10 \times \text{H}_2\text{SO}_4 \) after adding 0.2 ml of 10 mM potassium periodate and incubation at 37 °C for 30 min, 0.25 ml of 0.1 M sodium arsenite was added, and the reaction mixture was maintained at room temperature for another 10 min. A 150-μl aliquot of each sample was mixed with 900 μl of chromotropic acid reagent. The reagent was prepared from 200 mg of 4,5-dihydroxy-2,7-naphthalene disulfonic acid in 110 ml of water that was cooled in ice and to which 90 ml of concentrated \( \text{H}_2\text{SO}_4 \) were slowly added. The sample was heated at 95 °C for 30 min, and its absorbance was measured at 570 nm in cuvettes of 1-cm light path.

A unit of activity is defined as the amount of enzyme catalyzing the hydrolysis of ethylene carbonate to form 1 μmol of ethylene glycol/min under standard assay conditions. Specific activity is in terms of units of activity/mg of enzyme. Protein was determined by the method of Bradford (8) using bovine serum albumin (Pierce) as standard and the Bio-Rad protein assay reagent (Bio-Rad).

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† The abbreviations used are: DTNB, 5,5'-dithiobis(nitrobenzoic acid); DTT, dithiothreitol; GSSG, glutathione disulfide.

Continuous Spectrophotometric Assay

The standard assay is effective only with substrates that yield a compound with vicinal nucleophilic groups as products (7). That is not the case for products derived from the linear carbonates of ethyl, propyl, and allyl alcohols that we followed by the formation of the respective alcohol. The alcohols were measured by coupling the hydrolysis of the carbonate with horse liver alcohol dehydrogenase and yeast aldehyde dehydrogenase and measuring the formation of NADH. Although ethylene glycol was not an effective substrate in this dehydrogenase-coupled assay, the formation of each of the other noted alcohols could be readily measured. Included in a total volume of 1.0 ml were 0.1 M Tris-HCl at pH 8.0, 2 mM NAD, 0.2 unit of horse liver alcohol dehydrogenase, and 0.2 unit of yeast aldehyde dehydrogenase. After 3 min, 0.04 units of the organic carbonate hydrolyzing enzyme was added followed by 5 mM substrate at 37 °C. Unless otherwise noted, all procedures were conducted in a cold room at 4 °C or in an ice bath. The pH of all buffers refers to measurements taken after equilibration at room temperature.

Purification

Livers from young male Sprague-Dawley rats (150–200 g) were shipped in DrIce by Fed-Express Biologicals (Rogers, AR) and stored for 3–5 days at −18 °C. Unless otherwise noted, all procedures were conducted in a cold room at 4 °C or in an ice bath. The pH of all buffers refers to measurements taken after equilibration at room temperature.

Step 1: Extract—About 300 g of liver were partially thawed and suspended in 3-fold their volume of Buffer A (20 mM Tris-Cl containing 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 2 µg/ml leupeptin adjusted to pH 7.8). About half of this suspension was homogenized for 30 s in a Waring blender and then filtered through a double layer of cheesecloth. The combined filtrate was centrifuged for 20 min at 16,000 × g, and the residue was discarded.

Step 2: Salting Out—Ammonium sulfate, 25.2 g/100 ml was added to the solution, which was stirred for 30 min. The suspension was centrifuged at 16,000 × g, and the supernatant fluid was treated with an additional 10.8 g of ammonium sulfate/100 ml. The resultant precipitate was collected by centrifugation (16,000 × g, 20 min), suspended in 100 ml of Buffer A, and dialyzed overnight against three changes of 1, 1, and 4 liters, respectively, of the same buffer.

Step 3: DEAE-Sephacel—The enzyme solution was applied to a column (4.5 × 50 cm) of DEAE-Sephasel (Amersham) that had been equilibrated with Buffer A and was washed with an additional liter of the buffer. Protein was eluted with a linear salt gradient of 3.0 liters of the same buffer containing from 0 to 0.4 M NaCl (Fig. 1, DEAE). Active fractions of approximately 7 ml each were pooled (fractions 290–320).

Step 4: Red Agarose—The enzyme from Step 3 was applied directly to a column (2.2 × 17 cm) of Red Agarose (Sigma) that had been equilibrated with Buffer B (Buffer A without EDTA). The column was subsequently washed with 150 ml of Buffer B and eluted with a linear salt gradient in Buffer B ranging from 0 to 1 M NaCl. Active enzyme was collected, pooled (Fig. 1, RA), concentrated to 20 ml with an Amicon Diaflo PM10 membrane, and dialyzed overnight against three changes of, respectively, 1, 1, and 2 liters of Buffer C (10 mM potassium phosphate containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, and 2 µg/liter antipain adjusted to pH 6.8).

Step 5: Hydroxyapatite—The dialysate was applied to a column (2.0 × 20 cm) of hydroxyapatite gel (Sigma) that had been equilibrated with Buffer C. After washing the column with 100 ml of Buffer C, enzyme was eluted with 400 ml of a linear salt gradient of Buffer C and Buffer B made 0.4 M in potassium phosphate at pH 6.8 (Fig. 1, HA). Pooled, active fractions were concentrated by ultrafiltration to 5 ml with a PM10 membrane and dialyzed against Buffer E (Buffer C at pH 6.5 made 1 mM in EDTA).

Step 6: CM-Sepharose—The dialyzed protein solution was loaded onto a column (1.0 × 15 cm) of CM-Sepharose previously equilibrated with Buffer E. The column was washed with 30 ml of Buffer E and eluted with 120 ml linear salt gradient from 0 to 400 mM NaCl in Buffer E (Fig. 1, CM).

Step 7: Sephacryl—After concentration to 1 ml, the enzyme solution was used to charge a column (2 × 80 cm) of Sephacryl S-400 (Amersham) that had been equilibrated with a solution of 20 mM Tris-HCl at pH 7.8, 0.1 M NaCl, 1 mM DTT, 0.1 mM EDTA, and the three proteinase inhibitors. The column was developed with the aforementioned Tris buffer, resulting in the elution of the enzyme close to the front.

RESULTS

Based on the standard assay with ethylene carbonate as substrate, the enzyme responsible for Reaction 1 was purified to electrophoretic homogeneity by essentially conventional means in yields of 4–5% from rat liver. The enzyme is a homotetramer with subunits of identical size that were estimated to be 55,000 Da by SDS gel electrophoresis; a single band of protein was seen (Fig. 2, lane 1) in the same position whether or not the enzyme was heated with 1% 2-mercaptoethanol (5). Using sedimentation equilibrium centrifugation, Mr, of approximately 220,000 was estimated; the Mr was the same when equilibrium was established in the presence or in the absence of 1 mM DTT.

Molecular Weight Determination

Centrifugation was carried out with a Beckman XL-A analytical centrifuge and a TI-60 rotor using a 12-mm double sector cell containing 3-mm columns of protein solution (300 µg/ml in 20 ml Tris-HCl at pH 7.8, 0.1 M NaCl, with or without 1 mM DTT) and diffusate (12) layered over FC-47 fluorocarbon. Exact values of loading absorbance at 280 nm were obtained from initial scans taken just after speed had been reached. Rotor speeds of 5000 and 7000 rpm were employed at temperatures of 5 and 25 °C. Radial scans were performed every 48 h. At equilibrium, differences between successive scans were interpreted as negligible, and the residue was discarded.

Antibody to the enzyme, purified as described below and homogeneously upon SDS-gel electrophoresis, was prepared by Lofstrand Labs Ltd. (Gaithersburg, MD) 10 weeks after the initial inoculation with five booster doses, yielding a titer of greater than 1:32,000. The antibody was purified by saltting out (between 0.3 and 0.5 saturation with ammonium sulfate), dialysis against 20 mM Tris-HCl at pH 8.5, and by elution with a 0–0.5 M NaCl gradient in Tris-HCl at pH 7.8 from a DEAE-Sephadex column. Antibody diluted with 100 mM potassium pyrophosphate at pH 7.8, was incubated with enzyme at 4 °C overnight, and the suspension was centrifuged in an Eppendorf centrifuge at the same temperature. The supernatant fluid was tested for enzyme activity.

Immunology

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Effects of pH—The enzyme was most stable at slightly alkaline pH but not below neutrality. At the pH of the purification buffer, 7.8, activity was unaffected by storage of the purified enzyme at 4 and 28 °C for 3 months. Storage at pH 6.3 and 4 °C led to a 14% loss of activity after 3 days.

As shown in Fig. 3, the pH optimum for the enzyme was at approximately 8.1 after correction for spontaneous hydrolysis. A correction was also required for the spontaneous hydrolysis of the other two active cyclic carbonates that were substrates.

Redox Effects—Although the purified enzyme presented as a single protein band on both SDS and gradient native gels, gel isoelectric focusing displayed multiple protein bands between a pH 7.2 and 7.4 (Fig. 2, lane 2). Treatment of the protein with bovine alkaline phosphatase, potato acid phosphatase, or Clostridium neuraminidase did not alter the pattern (not shown), and the determination of the presence of less than 1 mol of carbohydrate/mol of protein suggested that glycosylation was not responsible for the multiple bands. The multiple band pattern could be manipulated, however, with oxidizing and reducing reagents. In the presence of 1 mM DTT, major bands were apparent between pH 7.2 and 7.5. In contrast, 5 mM GSSG yielded at least nine protein bands with P_i values between 6.3 and 7.2; the shift to lower P_i was enhanced progressively over the course of a 3-h exposure to GSSG (lanes 3 and 4). A similar shift to lower P_i was observed with 1 mM DTNB (lane 5) or 2 mM hydrogen peroxide (lane 6) as oxidizing agents. The effect of GSSG could be reversed by subsequent incubation with DTT (not shown).

Changes in P_i were reflected in enzyme activity. Incubation with 2 mM DTT resulted in dramatic loss of activity (Fig. 4) that was irreversible if 20 mM DTT was used (not shown). Incubation of enzyme with 1 mM GSSG or 1 mM DTNB, however, resulted in a slight but distinct increase in activity. Observations of such changes in activity as the result of formation of mixed disulfides with glutathione have been made (13–16) and, in one instance, were found, due to the formation of a disulfide bond between two protein cysteine residues (16).

Carbonates as Substrates—The enzyme was capable of hydrolyzing each of three cyclic carbonates: ethylene carbonate, vinylene carbonate, and propylene carbonate. Stoichiometry was estimated for ethylene carbonate; at completion, 0.99 and 1.00 mmol of ethylene glycol, respectively, were formed from 1.0 mmol of ethylene carbonate in two trials.
ate, patulin, oxindole, 5-octotetrahdrofuran-2-carboxylate, luminal, and ethylene and vinylene trithiocarbonates. The enzyme did not display esterase activity with 4-nitrophenyl acetate or ethyl acetate nor phosphatase activity with 4-nitrophenyl phosphate. Hydrolysis of ethylene carbonate was not inhibited by the serine esterase inhibitor paraaxon at 3 mM.

As with many of the substrates for the enzymes of detoxication (3), $K_m$ values for the cyclic carbonates were in the millimolar range, although $V_{\text{max}}$ values were substantial. Thus, for ethylene, vinylene, and propylene carbonates, the respective $K_m$ values were 26, 37, and 4 mM, and the respective $V_{\text{max}}$ values were 65, 56, and 8 $\mu$mol min$^{-1}$ mg$^{-1}$.

Since the product of propylene carbonate has an asymmetric center, evidence was sought for stereoselective hydrolysis. Under conditions previously employed for measuring circular dichroism with imidase (4), no evidence could be obtained for the stereoselective appearance of an asymmetric product over the course of 18 h of incubation with enzyme.

Imides as Substrates—Although a variety of heterocyclic compounds were tested and found inactive as noted above, one group, the imides, was effective as substrates. Phthalimide, the standard assay substrate for imidase, as well as 3,4-pyridyl-dicarbboximide and 6-methyl dihydrouracil, were each substrates for the carbonate-hydrolyzing enzyme exactly as presented for rat liver imidase (4). The $K_m$ for phthalimide had previously been found to be 3 mM with imidase (4) and was 3.5 mM for the enzyme purified here.

When used as competitive inhibitors of propylene carbonate hydrolysis, phthalimide had a $K_i$ of 5 mM, and N-carbamoyl-$\beta$-alanine, another substrate for the imidase reaction, had a $K_i$ of 0.2 mM. Although ethylene carbonate appeared to be a competitive inhibitor of phthalimide hydrolysis, a $K_i$ could not be accurately estimated at these high concentrations, i.e. greater than 0.1 mM.

Comparison of Imidase and Carbonate Hydrolase—Examination of Table I will confirm that the two enzyme activities toward imides and carbonates co-purified throughout the various steps in the purification process (Table I). The isolated enzyme appears to be the only protein with either type of catalytic activity in rat liver; no second peak of active protein was apparent at any stage of purification (Fig. 1).

The mechanism previously outlined for the hydrolysis of imides (4) appears to apply equally to the activity toward cyclic organic carbonates. The reaction would be expected to take the form of protonation of the carbonyl group of the carbonate, thereby providing a strong electrophilic center for the addition of water. Upon such addition, ring opening would be followed by elimination of CO$_2$. The finding of this enzyme in rat liver provides a metabolic pathway for the conversion of cyclic organic carbonates to their respective glycols.

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### TABLE I

Summary of purification with ethylene carbonate and phthalimide as substrates

| Volume | Total protein | Specific activity | Imide/ECO$_2$ |
|--------|--------------|------------------|--------------|
|        | ml           | mg               | ECO$_2$      | Phthalimide |               |
| Extract| 600          | 13080            | 0.024        | 0.050      | 2.1           |
| Salt precipitation | 103 | 4720          | 0.081        | 0.110      | 1.4           |
| DEAE-Sephacel | 200 | 82             | 2.8          | 5.0        | 1.8           |
| Red Agarose | 112 | 26             | 4.0          | 7.7        | 1.9           |
| Hydroxyapatite | 32 | 6.4           | 5.9          | 15.6       | 2.6           |
| CM-Sepharose | 10 | 1.6           | 8.1          | 24.4       | 3.0           |
| Sephadex S400 | 15 | 1.2           | 8.7          | 21.7       | 3.2           |

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Based on the provided text, it seems that the document discusses the enzymatic hydrolysis of organic cyclic carbonates and includes the results and methods of purification. The enzyme purified here hydrolyzes various cyclic carbonates with different $K_m$ and $V_{\text{max}}$ values. The enzyme also shows selectivity towards imides, as indicated by the $K_i$ values. The mechanisms and implications of these findings are discussed in detail. The references cited provide a basis for further research and validation of the findings.
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