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Transfer Reactions in Ice. Inhibition of Nonenzymatic Hydroxylaminolysis of Amino Acid Esters by Structural Analogs*

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ABSTRACT: Rates of hydroxylaminolysis of several amino acid esters were measured in water at 1° and in ice at −18°. The rate at −18° exceeded that at 1° for each ester. In the presence of structural analogs of phenylalanine, inhibition occurred only in the frozen state. The following systems showed the inhibition: L-phenylalanine ethyl ester plus β-2-thienylalanine or β-phenyllactic acid; L-tyrosine ethyl ester plus tyramine, β-phenylpropionic acid, 2-amino-3-phenylbutanoic acid, or phenylacetic acid; L-phenylalanine methyl ester plus 2-amino-3-phenylbutanoic acid, *m*-fluoro-DL-phenylalanine, β-phenylpropionic acid, or DL-β-phenylserine; L-glutamic acid γ-methyl ester plus β-phenyllactic acid. Phenylalanine analogs failed to inhibit when DL-serine methyl ester or L-lysine methyl ester was the substrate. Inhibition of the tyrosine ethyl ester reaction by tyramine or β-phenylpropionic acid increased with inhibitor concentration and could be shown to be partly reversed by raising the substrate concentration. With β-phenylpropionic acid or 2-amino-3-phenylbutanoic acid, plots of reciprocal of initial velocity *vs.* reciprocal of tyrosine ethyl ester concentration resembled those expected for competitive inhibition of an enzyme. The results are viewed as providing evidence for catalytic activity in the ice and possibly in liquid systems containing highly structured water.

Recent studies have shown that various reactions proceed more rapidly in frozen systems than in water (Grant *et al.*, 1961, 1962; Wang, 1961; Prusoff, 1963; Bruice and Butler, 1964; Alburn and Grant, 1965). Some of these enhanced rates do not appear to be ascribable to a concentration mechanism. Factors suggested as contributing to unexpectedly high rates in ice have been enhanced proton mobility and substrate–catalyst orientational constraint (Grant *et al.*, 1961), catalytic action by the ice (Bruice and Butler, 1964), and association of nucleophile molecules (Alburn and Grant, 1965). Irrespective of whether the reaction occurs within the frozen solid or in liquid inclusions, the present available evidence does indicate that the reactants are brought into a favorable juxtaposition in the vicinity of ice structure. This suggests the applicability, in some degree, of the concepts of catalyst–substrate complex formation, catalytic active sites, and catalytic specificity. It appeared likely that, if any of these play a role, then inhibition by structural analogs of the substrate would be demonstrable.

This paper describes the effects of phenylalanine analogs on the reaction between amino acid esters and hydroxylamine in frozen solutions. Hydroxylaminolysis is a transfer reaction which has been shown to be catalyzed by chymotrypsin (Jencks *et al.*, 1963) and by various tissue amidases (Meister *et al.*, 1955).

Experimental Section

The hydroxylamine hydrochloride was Baker Analyzed reagent, two preparations of which gave assay values of 99.4 and 100.3%. The β-phenylpropionic (hydrocinnamic) acid, β-phenyllactic acid, and phenylacetic acid were recrystallized from warm water. The L-tyrosine ethyl ester, L-phenylalanine ethyl ester, and

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TABLE I: Effects of Substrate Structural Analogs on Several Hydroxylaminolysis Reactions.[a]

Methyl Ester Substrate	Inhibitor	[I]	Temp., °C.	k ($10^5 k_{obsd}$, min^{-1})	ki	Change (%)
L-Phenylalanine	β-Phenylpropionic acid	0.04	1	59.6	60.3	+1
		0.04	−18	124	61.8	−50
L-Phenylalanine[b]	β-2-Thienylalanine	0.06	1	20.8	20.2	−3
		0.06	−18	22.2	12.6	−43
L-Phenylalanine	*m*-Fluoro-DL-phenylalanine	0.08	1	59.6	73.5	+23
		0.08	−18	124	74.8	−40
L-Phenylalanine	2-Amino-3-phenylbutanoic acid	0.08	1	59.6	60.8	+2
		0.08	−18	124	92.1	−26
L-Tyrosine[b]	Phenylacetic acid	0.05	1	11.4	11.9	+4
		0.05	−18	22.4	16.8	−25
L-Phenylalanine	DL-β-Phenylserine	0.04	1	59.6	58.7	−2
		0.04	−18	124	110	−11
L-Phenylalanine[b]	β-Phenyllactic acid	0.025	1	11.2	11.4	+2
		0.025	−18	13.1	10.3	−21
L-Glutamic acid (γ)	β-Phenyllactic acid	0.025	1	11.8	12.0	+2
		0.025	−18	64.6	55.3	−14
L-Leucine	β-Phenyllactic acid	0.025	1	30.7	30.7	0
		0.025	−18	95.3	89.5	−6
L-Glutamic acid (γ)	β-Phenylpropionic acid	0.04	1	25.2	31.1	+23
		0.04	−18	93.7	93.7	0
DL-Serine	β-2-Thienylalanine	0.04	1	34.2	34.6	+1
		0.04	−18	69.2	74.1	+7
L-Lysine	DL-β-Phenylserine	0.04	1	82.9	95.2	+15
		0.04	−18	139	151	+9

[a] Hydroxylamine concentration is 0.6 M. In the phenylacetic and phenyllactic acid experiments the substrate concentration is 0.005 M and the pH is 7.2. In the others, the substrate concentration is 0.01 M and the pH is 7.7. [b] Ethyl ester.

L-glutamic acid γ-methyl ester were chromatographically pure preparations from Mann Research Laboratories. The other analogs and substrates were purchased from Mann or Nutritional Biochemicals Corp. and were used directly.

Reaction mixtures contained 0.002–0.01 M substrate, 0.6–0.9 M hydroxylamine hydrochloride, and 0.02–0.10 M inhibitor. Sufficient sodium hydroxide was added finally to provide a self-buffering system of the desired pH. The solutions were distributed into at least six test tubes, stoppered, and set at 1° (refrigerator) and −18° (deep freeze). Except where noted, solutions for experiments at −18° were initially immersed in a dry ice–acetone mixture. All samples held at −18° appeared to be frozen solid. Frozen samples were thawed by immersion in a room temperature water bath and then mixed again by holding against a Vortex mixer. The pH after incubation was checked on a Beckman Expanded Scale pH meter and at both temperatures remained within 0.1 pH unit of the initial value. The total time between the beginning of thawing and termination of the reaction by acid addition was 4–5 minutes. (Corrections were not introduced for this period, during much of which the physical state was changing; rates of reactions run between pH 7 and 8 at −18, 1, and 22° indicated that less than 2% of either phenylalanine methyl ester or tyrosine ethyl ester would react.) For assay, 3-ml samples were mixed successively with 3 ml of 0.935 M HCl and 1 ml of 15% ferric ammonium sulfate in 1 N H_2SO_4, giving the iron complex whose absorbance was read on a Klett–Summerson spectrophotometer with a 540 mμ filter. Depending on the rate, samples were assayed at intervals ranging from two/hour to two/day.

Results

In the presence of a large excess of hydroxylamine, the reaction with various amino acid esters followed first-order kinetics both in the liquid solution at 1° and in the frozen solution at −18°. Table I compares the effects of several phenylalanine analogs on the hydroxylaminolysis rates. As expected, the additives caused no inhibition in the liquid systems. The rate enhancements found in several 1° experiments seem to be real, since they appeared repeatedly and cannot be explained by the negligible hydroxylaminolysis of the inhibitor. The major finding is that structural analogs significantly inhibit the reaction with phenylalanine or tyrosine esters in the frozen systems.

When tested with the methyl esters of serine or lysine, phenylalanine analogs failed to inhibit the formation of

hydroxamic acids. β-Phenylpropionic acid lowered the rate with L-phenylalanine methyl ester by one-half, but it had no inhibitory activity at all with L-glutamic acid γ-methyl ester as substrate. β-Phenyllactic acid showed decreasing inhibition with phenylalanine ethyl ester, glutamic acid γ-methyl ester, and leucine methyl ester. In other control experiments not included in Table I, the following systems showed no inhibition: 2,5-diketopiperazine with 0.04 M tyramine or 0.08 M *m*-fluoro-DL-phenylalanine; L-glutamic acid γ-methyl ester with 0.08 M L-glutamic acid; L-phenylalanine methyl ester with 0.04 M L-arginine; and L-leucine methyl ester with 0.04 M L-valine or DL-norleucine.

With L-tyrosine ethyl ester as substrate, the effects of varying concentrations of tyramine and β-phenylpropionate were determined. Table II shows that at

TABLE II: Effect of Various Concentrations of Tyramine on L-Tyrosine Ethyl Ester Hydroxylaminolysis at −18°.[a]

Inhibitor (M)	Substrate 0.006 M ($10^5 k_{obsd}$, min^{-1})	Substrate 0.01 M ($10^5 k_{obsd}$, min^{-1})
0.00	23.1	23.0
0.02	20.6	24.4
0.04	18.4	24.2
0.05	18.4	19.6
0.06	17.3	19.3
0.08	14.6	17.3

[a] Conditions are: pH 7.7, 0.6 M NH_2OH.

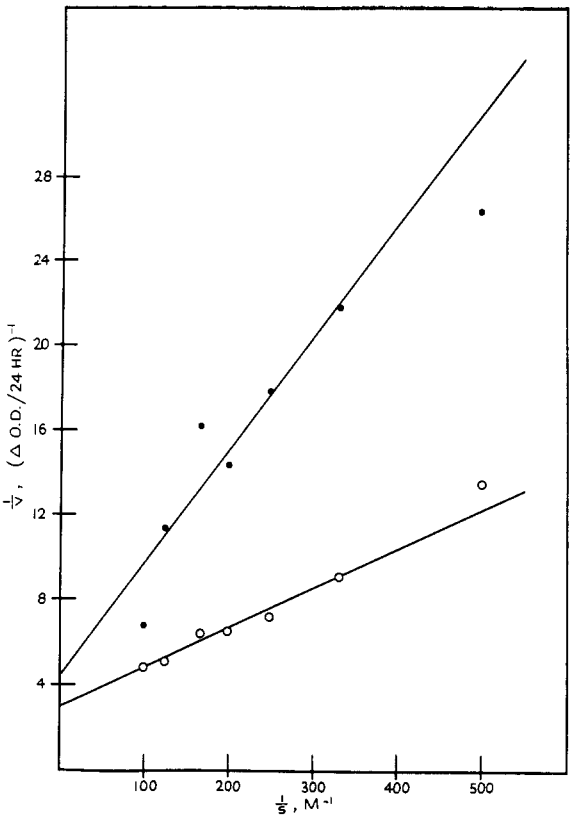

FIGURE 1: Effect of β-phenylpropionic acid on the plot of reciprocal of initial velocity against reciprocal of L-tyrosine ethyl ester concentration. Conditions: 0.6 M NH_2OH, pH 7.7, −18°, rapid freezing. ○, control; ●, 0.08 M inhibitor.

two levels of substrate increasing amounts of tyramine gave increasing inhibition. A dependence upon substrate concentration was shown by the consistently smaller effect of tyramine at the higher tyrosine ethyl ester concentration.

Table III gives the results obtained from varying β-phenylpropionate concentration. As in the case with tyramine, inhibition increases with concentration of inhibitor. In the range of 0.02–0.06 M β-phenylpropionate, evidence for reversal of the inhibition by increased substrate concentration appears on comparing the last two columns but not the first two. In the range of 0.07–0.10 M β-phenylpropionate, rates at all these substrate concentrations provide support for partial reversal of the inhibition by additional substrate.

All of the foregoing data suggested that the idea of competitive inhibition might be relevant to these systems. Therefore, initial reaction velocities were determined at various concentrations of L-tyrosine ethyl ester in the absence of any inhibitor and in the presence of β-phenylpropionic acid and 2-amino-3-phenylbutanoic acid. Figures 1 and 2 show the resulting double reciprocal plots. The experiments were performed under different conditions (concentration of hydroxylamine; rate of freezing), and in both cases the control data show good linearity. Although the inhibitor data in Figure 1 are only approximately linear, the inhibition is clearly dependent upon the substrate concentration. In Figure 2 both sets of data are linear, and the relationship between the two resembles Lineweaver–Burk plots for competitive inhibition.

Discussion

Inhibition by some additives and not by others at equal concentrations indicates that the mechanism is not a nonspecific freezing point depression (*i.e.*, ice melting and consequent dilution). Lack of inhibitory activity or possession of an activating capacity by an agent in liquid solutions indicates that factors other than concentration of the inhibitor by removal of water are responsible for inhibitory activity in frozen solutions.

Inhibition of a reaction by a structural analog of one of the reactants suggests the formation of an intermediate complex, the existence of a "catalytically active site," and a degree of specificity. The nature and number of "active sites" for nonenzymatic reactions occurring in the frozen state are not apparent. These

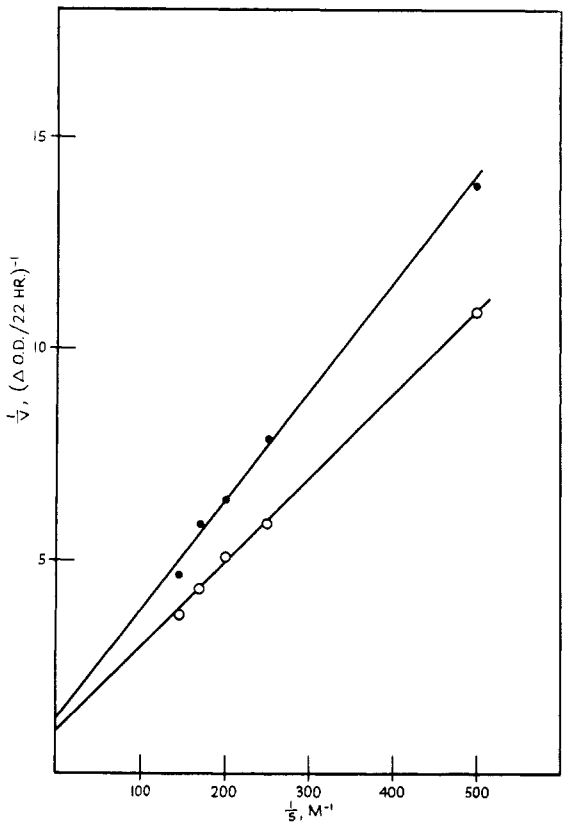

FIGURE 2: Effect of 2-amino-3-phenylbutanoic acid on the plot of reciprocal of initial velocity against reciprocal of L-tyrosine ethyl ester concentration. Conditions: 0.8 M NH_2OH, pH 7.7, −18°, slow freezing. ○, control; ●, 0.05 M inhibitor.

TABLE III: Effect of Various Concentrations of β-Phenylpropionate on L-Tyrosine Ethyl Ester Hydroxylaminolysis at −18°.[a]

Inhibitor (M)	Substrate 0.005 M	0.007 M	0.01 M
	($10^5 k_{obsd}$, min^{-1})		
0.00	36.0	34.4	33.0
0.02	31.7	26.8	
0.03	26.1	21.8	
0.04	25.6	24.6	27.0
0.05	25.6	18.8	
0.06	21.0	11.9	16.5
0.07	10.8	12.8	
0.08	10.4	10.6	14.2
0.09	3.8	9.6	
0.10		8.9	11.2

[a] Conditions are: pH 7.7, 0.9 M NH_2OH.

questions may possibly be clarified by further studies on the concentration of ice "cells," as proposed by Bernal and Fowler (1933), or of the flickering hydrogen-bonded water clusters, as proposed by Némethy and Scheraga (1962), and possibly by the application of inhibitor and substrate methods for estimating the number of catalytic centers (Bender *et al.*, 1962; Myers, 1952). Conversely, studies on frozen state reactions may throw some light on the questions of water structure. The plots in Figures 1 and 2 lend themselves to calculations of equilibrium constants for substrate and inhibitor, but until much more is known about the microstructure of the frozen reaction mixture mechanistic aspects of the reactions will remain a matter of conjecture. However, if the requirements for catalysis consist of promoting the formation and breakdown of an intermediate, then it appears reasonable to view the ice as acting catalytically.

A catalytic role for ice is consistent with many of the current ideas on water structure and its effect on the properties of biopolymers. Frank (1965) has suggested the idea of water as a mixture and solutes as dissolving partly in the structured species in the form of "quasi-clathrates." Klotz (1965) has stressed the likelihood that apolar side chains of proteins could form clathrate-like hydrates resembling those formed by small hydrocarbons. Richards (1963) has proposed that "icelike" water at the surface of a protein would be impossible but that structures similar to a hydrate cage could exist within holes in the surface. The enzymatically important factors of orientational constraint, high proton mobility, and catalytic activity might be envisioned as converging at such sites.

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