The Effect of Aliphatic Alcohols and Organic Solvents on Reactions Catalyzed by 5-Hydroxy-N-methylpyroglutamate Synthetase*

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SUMMARY

5-Hydroxy-N-methylpyroglutamate synthetase (HMPC synthetase) catalyzes hydrolysis and acyl transfer reactions with \( \delta \)-substituted \( \alpha \)-ketoglutarates, presumably through the formation of an \( \alpha \)-ketoglutaryl enzyme intermediate. With \( \alpha \)-ketoglutarate as the substrate, maximal velocities of 18, 26, and 36 are observed for acyl transfer to water, methylamine, and ethanolamine, respectively. The corresponding maximal velocities with ethyl \( \alpha \)-ketoglutarate as the acyl donor are 19, 38, and 68. These results suggest that both acylation and deacylation are partially rate limiting for reactions involving \( \alpha \)-ketoglutaramate and ethyl \( \alpha \)-ketoglutarate. Aliphatic alcohols increase the rate of hydrolysis of \( \alpha \)-ketoglutarate and ethyl \( \alpha \)-ketoglutarate. These rate accelerations increase with increasing chain length of the alcohol until a maximal value is reached and are interpreted as a specific effect on the rate of deacylation of the acyl enzyme. Based on this assumption, the rate of transfer of the acyl group from the enzyme to water, to methylamine, and to ethanolamine has been calculated and found to be independent on the nature of the acyl donor. In contrast to the effects observed on hydrolysis, aliphatic alcohols act as competitive inhibitors with respect to amines in acyl transfer reactions and thus provide evidence for an amine-binding site on the enzyme. Organic solvents increase both hydrolysis and transfer reactions involving amines; however, these effects can be distinguished from those of alcohols by several kinetic criteria.

During the course of investigating the substrate specificity of the enzyme, we noted significant increases in the rate of hydrolysis of ethyl \( \alpha \)-ketoglutarate in the presence of alcohols and organic solvents. The present paper describes a detailed investigation into the nature of these rate accelerations.

MATERIALS AND METHODS

Materials—\( \delta \)-Ketoglutaramate, \( \delta \)-ethyl \( \alpha \)-ketoglutarate, and \( \delta \)-methyl \( \alpha \)-ketoglutarate were prepared by oxidation of the corresponding glutamic acid derivative with \( \beta \)-amino acid oxidase (2, 4). Amines, alcohols, and organic solvents were recrystallized or redistilled before use. Bovine liver glutamate dehydrogenase (ammonium free) was obtained from Sigma Chemical Co. \( ^{14} \)C-Ethanolamine was obtained from Amersham-Searle Corp. 5-Hydroxy-N-methylpyroglutamate synthetase was prepared as previously described (2).

The hydrolysis of the \( \delta \)-amide and esters of \( \alpha \)-ketoglutarate were routinely measured spectrophotometrically by coupling the reaction to the glutamate dehydrogenase reaction (2) and following the oxidation of DPNH at 340 nm. Except when noted, all kinetic runs were performed at 30°. The pH of reaction mixtures was measured at the end of each kinetic run and was found not to deviate more than \( \pm 0.05 \) pH units from the desired pH. The extinction coefficient of DPNH does not significantly change in the presence of alcohols or organic solvents. The \( K_m \) for ethyl \( \alpha \)-ketoglutarate (3.8 \( \times 10^{-5} \) M) (3) is too low to permit a detailed kinetic study by the spectrophotometric assay. Alternatively, we used the glutamate dehydrogenase coupled assay but followed DPNH oxidation fluorometrically on an Eppendorf fluorometer, as previously described (3). No difference was observed for DPNH standard curves prepared in buffered reaction mixtures containing alcohols or organic solvents as compared to standard curves prepared in buffer alone.

Transfer reactions between \( ^{14} \)C-amines and \( \alpha \)-ketoglutarate or ethyl \( \alpha \)-ketoglutarate were measured by the Dowex 50-H+ chromatographic assay previously described (2). The transfer reaction between higher molecular weight amines and ethyl \( \alpha \)-ketoglutarate was assayed by measuring the disappearance of the ester. Reaction mixtures containing 50 mM potassium Tris\( \cdot \)HCl buffer, pH 8.6, 2 mM ethyl \( \alpha \)-ketoglutarate, amine and enzyme, in a final volume of 1.5 ml were incubated

The enzyme, 5-hydroxy-N-methylpyroglutamate synthetase, catalyzes hydrolysis and acyl transfer reactions with \( \delta \)-substituted \( \alpha \)-ketoglutarates (1–3) as shown in Scheme I where \( X = \text{NH}_2, \text{NHCH}_3, \text{OEt}, \text{OPr}, \text{and OBz, and } E = \text{CH}_3, \text{HOCH}_2\text{CH}_3, \text{and CH}_3(\text{CH}_2)_n\text{CH}_3(n = 0 \text{ to } 3)\). The K_m for ethyl \( \alpha \)-ketoglutarate (3.8 \( \times 10^{-5} \) M) (3) is too low to permit a detailed kinetic study by the spectrophotometric assay. Alternatively, we used the glutamate dehydrogenase coupled assay but followed DPNH oxidation fluorometrically on an Eppendorf fluorometer, as previously described (3).

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* This work was supported in part by grants from NIAMD (Grant No. 13443) and the Robert A. Welch Foundation (Grant No. 1-301).
at 30°. At the desired time periods, an aliquot was withdrawn and placed in a boiling water bath for 1 min. A portion of this aliquot was used to measure α-ketoglutarate with glutamate dehydrogenase and unreacted ester with rat liver amidase (9) coupled to glutamate dehydrogenase, as previously described (3). Both the hydrolytic and transfer reactions are linear for at least 1 hour.

The maximal velocity for the hydrolytic reactions in the presence of organic solvents or alcohols and the concentration of alcohol or solvent required to give half-maximal rate acceleration (K_a) was obtained from double reciprocal plots of 1/(v - v_0) versus 1/(alcohol or solvent), where v_0 is the velocity observed at a given concentration of alcohol or solvent and v is the velocity observed in the absence of alcohol or solvent. V_max was obtained by addition of the velocity in the absence of solvent or alcohol to the maximal velocity obtained from these double reciprocal plots. The negative reciprocal of the 1/(alcohol or solvent) intercept yielded K_a. In all cases, double reciprocal plots were linear. (Since ethyl α-ketoglutarate was used at a concentration greater than 250 times its K_m, maximal velocities were assumed to prevail.)

Activation parameters were determined by measuring the effect of temperature on the rate of hydrolysis of ethyl α-ketoglutarate in the presence or absence of alcohols or organic solvents. Over the temperature range of 15-33°, plots of log v versus 1/T were linear.

Exchange between [14C]ethanol and ethyl α-ketoglutarate was measured in the following manner. A 0.25-mL solution containing 1.0 mM ethyl α-ketoglutarate, 0.25 to 1.0 α-C[14C]ethanol (specific activity, 1 x 10^4 cpm per μmole), 50 mM potassium Tricine buffer, pH 8, 1.5 mM dithiothreitol, and 1 μg of glutamate dehydrogenase was incubated for 30 min at 30°. The reaction was terminated by immersing the reaction mixture in a boiling water bath for 2 min. The reaction mixture was transferred to a 20-mL scintillation vial and taken to dryness by gently blowing air over the solution. Absolute ethanol (1 mL) was added, and the solution was again taken to dryness. The above procedure was repeated five times, after which 10 mL of scintillation fluid was added and the 14C content was determined. Control experiments showed that no nonenzymatic exchange reaction occurred, nor was the ethyl ester hydrolyzed by this procedure.

### Table I

| Compound         | Concentration | α-Ketoglutarate (20 μM) | α-Methyl α-ketoglutarate (0.2 μM) | α-Ethyl α-ketoglutarate (0.2 μM) | α-Ethyl α-ketoglutarate (0.2 μM) |
|------------------|---------------|-------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Methanol         | 1.0           | 1.1                     | 1.2                               | 1.1                               | 0.9                               |
| Ethanol          | 1.0           | 1.6                     | 1.6                               | 1.5                               | 0.9                               |
| n-Propyl alcohol | 1.0           | 2.4                     | 2.5                               | 2.9                               | 0.8                               |
| Isopropyl alcohol| 1.0           | 2.2                     | 2.2                               | 2.6                               | 0.8                               |
| Normal butyl alcohol | 1.0       | 2.6                     | 2.6                               | 2.9                               | 0.8                               |
| tert-Butyl alcohol| 1.0           | 1.9                     | 2.6                               | 2.9                               | 0.8                               |
| n-Pentyl alcohol | 1.0           | 1.9                     | 3.3                               | 0.9                               | 1.9                               |
| tert-Pentyl alcohol| 0.1          | 1.8                     | 2.4                               | 0.9                               | 1.9                               |
| Acetone          | 1.0           | 1.9                     | 2.1                               | 0.9                               | 1.9                               |
| Dioxane          | 1.0           | 2.0                     | 2.7                               | 0.8                               | 2.0                               |
| Dimethyl sulfone | 1.0           | 1.5                     | 1.5                               | 1.7                               | 1.5                               |
| Dimethyl formamide| 1.0           | 1.9                     | 2.1                               | 0.8                               | 1.9                               |
| Acetonitrile     | 1.0           | 1.3                     | 1.6                               | 1.5                               | 1.6                               |

1. v/(alcohol or solvent) in the velocity observed in presence of alcohol or solvent and v_0 is the velocity in absence of solvent or alcohol.
2. purified rat liver amidase (5), 0.56 μg, used in place of HMPG synthetase.
3. Purified rat liver amidase was used in a concentration greater than 250 times its K_m, maximal velocities were assumed to prevail.

In order to determine whether acyl transfer from ethyl α-ketoglutarate or α-ketoglutarate was occurring in the presence of alcohols, we measured the rate of exchange of [14C]ethanol with ethyl α-ketoglutarate by a different enzyme, rat liver α-amidase (9). In this control experiment, one notes a slight decrease in the hydrolytic reaction, thus illustrating that the rate accelerations observed in the HMPG synthetase reactions are specific for this enzyme and are not derived from artifacts in the assay system.

The maximum rates obtained for α-ketoglutarate and α-ethyl α-ketoglutarate hydrolysis, in the presence of aliphatic alcohols and solvents, is shown in Table II. All organic solvents gave the same maximal rate enhancement for a particular substrate, whereas the rate accelerations with primary alcohols increase with increasing chain length until a maximum value is reached.

In order to establish whether alcohols and organic solvents increase the hydrolytic reactions by the same mechanism, the effect of these compounds on the kinetics of ethyl α-ketoglu- tarate hydrolysis was examined. As shown in Fig. 1, alcohols increase both the K_m of ethyl α-ketoglutarate and the maximal
TABLE II
Maximal rate of acceleration for α-ketoglutarate and ethyl α-keto-
glutamate hydrolysis by organic solvents and alcohols

The values of $K_A$ and $V_{\text{max}}$ were obtained as described under "Materials and Methods," utilizing the reaction mixtures described in Table I.

| Solvent or alcohol | Ethyl α-ketoglutarate | α-ketoglutarate |
|--------------------|-----------------------|----------------|
|                    | $K_A$ | $V_{\text{max}}$ | $K_A$ | $V_{\text{max}}$ |
| None               |       |               |       |               |
| Tetrahydrofuran    | 0.5   | 19.0          | 0.5   | 19.0          |
| Dioxane            | 0.7   | 75.2          | 0.5   | 46.0          |
| Dimethyl formamide | 1.0   | 79.2          | 0.9   | 42.0          |
| Acetone            | 2.4   | 78.5          | 0.7   | 45.0          |
| Acetonitrile       | 3.0   | 72.0          |       |               |
| Dimethyl sulfoxide | 4.0   | 71.0          |       |               |
| Ethanol            | 0.27  | 73.0          |       |               |
| Propanol           | 0.34  | 73.0          |       |               |
| Butanol            | 0.08  | 106.0         | 0.05  | 47.0          |
| Pentanol           | 0.04  | 132.0         | 0.008 | 46.0          |
| Hexanol            | 0.01  | 133.0         | 0.006 | 48.0          |
| Heptanol           | 0.009 | 137.0         | 0.003 | 49.0          |
| Octanol            | 0.008 | 139.9         | 0.008 | 49.0          |

Fig. 1. The effect of alcohols and solvent on the kinetics of ethyl α-ketoglutarate hydrolysis. The hydrolysis of ethyl α-ketoglutarate was measured fluorometrically in 2-ml reaction mixtures containing 50 mM sodium Tricine buffer, pH 8.0, 1.5 mM dithiothreitol, 2 mM ethyl α-ketoglutarate, [14C]methylamine (specific activity 1.7 X $10^{6}$ cpm per pmole) as indicated, 0.67 μg of enzyme, KCl to maintain the ionic strength (O), and when added, 0.2 M butanol (X), 0.2 M pentanol (Δ), or 0.5 M dioxane ( ● ). The samples were incubated for 10 min at 30°, after which time the reaction was terminated by the addition of 0.05 ml of 20% trichloracetic acid. [14C]-Amide formation was measured by the Dowex 50 chromatographic assay (see "Methods"). Control experiments showed that under the above conditions the reaction was linear for at least 40 min. $E/t$ expressed as μmoles per min per mg of protein.

Fig. 2. Effect of alcohols and solvents on the reaction of methylamine with ethyl α-ketoglutarate. Reaction mixtures contained 50 mM sodium Tricine buffer, pH 8.0, 1.5 mM dithiothreitol, 2 mM ethyl α-ketoglutarate, [14C]methylamine (specific activity 1.7 X $10^{6}$ cpm per pmole) as indicated, 0.67 μg of enzyme, KCl to maintain the ionic strength (O), and when added, 0.2 M butanol (X), 0.2 M pentanol (Δ), or 0.5 M dioxane ( ● ). The samples were incubated for 10 min at 30°, after which time the reaction was terminated by the addition of 0.05 ml of 20% trichloracetic acid. [14C]-Amide formation was measured by the Dowex 50 chromatographic assay (see "Methods"). Control experiments showed that under the above conditions the reaction was linear for at least 40 min. $E/t$ expressed as μmoles per min per mg of protein.

Table III
Inhibition constants ($K_I$) for effect of amines on hydrolysis of ethyl α-ketoglutarate

Inhibition constants were calculated from plots of $1/v$ versus $(I)$ in reaction mixtures identical with those described in Table I, utilizing 0.2 mM ethyl α-ketoglutarate and amines in place of alcohols or organic solvents.

| Amine RNH₂ | $K_{I}$ (apparent) | $K_{I}$ (free base) |
|------------|-------------------|---------------------|
|            | mM                | mM                  |
| Methyl     | 78.0              | 190.0               |
| Ethyl      | 67.0              | 171.0               |
| n-Propyl   | 28.0              | 72.0                |
| n-Butyl    | 4.0               | 10.0                |
| n-Pentyl   | 1.8               | 4.6                 |
| n-Hexyl    | 1.4               | 3.6                 |
| n-Heptyl   | 0.8               | 2.0                 |
| n-Octyl    | 0.9               | 2.3                 |
| n-Nonyl    | 1.1               | 2.8                 |
| n-Decyl    | 1.3               | 3.3                 |
| n-Dodecylamine | 1.6 | 4.1 |

velocity, whereas solvents exert an effect only on the maximal velocity.

In addition to the hydrolytic reaction, HMG synthetase catalyzes acyl transfer reaction between δ-substituted α-ketoglutarates and amines (2, 3). The effect of alcohols on the transfer reaction, with δ-ethyl α-ketoglutarate and methyleneamine as substrates, is shown in Fig. 2. In contrast to the results obtained for the hydrolytic reaction, butanol and pentanol are competitive inhibitors with respect to methyleneamine, while ethanol and propanol have no effect. Also shown in Fig. 2 is the effect of dioxane on the transfer reaction. In this case, dioxane acts as a noncompetitive activator of the reaction. Using a different amine acceptor, ethanolamine, competitive inhibition between the amine and propanol, butanol, and pentanol was observed, while noncompetitive activation occurred in the presence of dioxane, acetone, and dimethyl formamide. These results serve to illustrate that kinetically the effect of alcohols can be distinguished from those of organic solvents.

The observed competitive inhibition between alcohols and amines suggests the presence of an amine-binding site on the enzyme. To establish more firmly the presence of such a bind-
The nature of the active form of the amine substrate (i.e., free base or protonated amine) was investigated by studying the hydrolysis for a series of amines with respect to ethyl α-ketoglutarate hydrolysis. The results of one such experiment, shown in Table III, indicate that there is a marked change in the pH over the range of 7.2 to 8.1.

In a similar type of experiment we found that the apparent Michaelis-Menten constant of heptylamine as the inhibitor, is shown in Fig. 3. It is apparent that both acylation and deacylation are partially rate limiting for the reaction studied. Table IV lists the maximal velocities for the hydrolysis and transfer reactions measured as described under "Materials and Methods." The activation parameters were determined by measuring the temperature dependence for the rate of hydrolysis of ethyl α-ketoglutarate (2 × 10⁻⁴ M) spectrophotometrically in the presence and absence of the solvents or alcohols, as described under "Materials and Methods."

**Table IV**

Maximal velocities for reaction of series of aliphatic amines with ethyl α-ketoglutarate

| Amine         | Concentration | V<sub>transfer</sub> | V<sub>hydrolysis</sub> |
|---------------|---------------|----------------------|------------------------|
| Methylamine   | 0.20          | 38                   | <1.0                   |
| Ethylamine    | 0.18          | 38                   | <1.0                   |
| Propylamine   | 0.07          | 16                   | ~1.0                   |
| Butylamine    | 0.01          | 8                    | ~1.4                   |
| Pentylamine   | 0.05          | 5                    | 3                      |
| Heptylamine   | 0.02          | 2                    | 4                      |
| Mixture       | 0.18          | 10                   | 10                     |

**Table V**

Maximal velocities for reaction of series of aliphatic amines with ethyl α-ketoglutarate

| Amine   | Concentration | V<sub>transfer</sub> | V<sub>hydrolysis</sub> |
|---------|---------------|----------------------|------------------------|
| Methylamine | 0.20          | 38                   | <1.0                   |
| Ethylamine | 0.18          | 38                   | <1.0                   |
| Propylamine | 0.07          | 16                   | ~1.0                   |
| Butylamine | 0.01          | 8                    | ~1.4                   |
| Pentylamine | 0.05          | 5                    | 3                      |
| Heptylamine | 0.02          | 2                    | 4                      |
| Mixture   | 0.18          | 10                   | 10                     |

**Table VI**

Activation parameters for hydrolysis of ethyl α-ketoglutarate in water and in presence of alcohols or organic solvents

The activation parameters were determined by measuring the temperature dependence for the rate of hydrolysis of ethyl α-ketoglutarate (2 × 10⁻⁴ M) spectrophotometrically in the presence and absence of the solvents or alcohols, as described under "Materials and Methods."

**Table VII**

Activation parameters for hydrolysis of ethyl α-ketoglutarate in water and in presence of alcohols or organic solvents

| Alcohol | E<sub>a</sub> (cal/mole) | ΔH<sup>a</sup> (cal/mole) | ΔS<sup>a</sup> (cal/mole K) |
|---------|--------------------------|---------------------------|-----------------------------|
| Control | 9,300                     | 8,700                     | 15,800                      |
| Acetone (0.5 M) | 9,600                   | 9,000                     | 15,400                      |
| Dioxane (0.5 M) | 9,400                   | 8,800                     | 15,300                      |
| Propanol (0.5 M) | 9,300                   | 8,900                     | 15,200                      |
| Pentanol (0.05 M) | 9,400                   | 8,800                     | 15,000                      |

* Parameters calculated at 300 K.

The HMPG synthetase-catalyzed hydrolysis of δ-substituted α-ketoglutarates is accelerated up to 10-fold by organic solvents forming an acyl enzyme (2, 3). In order to gain insight into the mechanism of the observed effects of alcohols and solvents, it was desirable to establish whether acylation, deacylation, or perhaps both, were rate limiting for the reaction studied. Table V lists the maximal velocities for the hydrolysis and transfer reactions (utilizing methylamine and ethanolamine as acceptors) with ethyl α-ketoglutarate and α-ketoglutarate as acyl donors. These results, along with the observation that at low amine concentrations the transfer reaction proceeds with a stoichiometric decrease in the hydrolytic reactions, suggest that both acylation and deacylation are partially rate limiting for the reactions involving both ethyl α-ketoglutarate and α-ketoglutarate. In the presence of a series of aliphatic amines the rate of acyl transfer decreases with increasing chain length of the amine, Table V.

Table VII lists the activation parameters for the hydrolysis of ethyl α-ketoglutarate in water, acetone, dioxane, propanol, and pentanol. The primary effect of these activators is to increase the entropy of activation, with little or no effect on the enthalpy of activation.

**DISCUSSION**

We have previously presented evidence that the hydrolysis and acyl transfer reactions proceed through the intermediate formation of an acyl enzyme (2, 3). In order to gain insight into the mechanism of the observed effects of alcohols and solvents, it was desirable to establish whether acylation, deacylation, or perhaps both, were rate limiting for the reaction studied. Table V lists the maximal velocities for the hydrolysis and transfer reactions (utilizing methylamine and ethanolamine as acceptors) with ethyl α-ketoglutarate and α-ketoglutarate as acyl donors. These results, along with the observation that at low amine concentrations the transfer reaction proceeds with a stoichiometric decrease in the hydrolytic reactions, suggest that both acylation and deacylation are partially rate limiting for the reactions involving both ethyl α-ketoglutarate and α-ketoglutarate. In the presence of a series of aliphatic amines the rate of acyl transfer decreases with increasing chain length of the amine, Table V.

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and aliphatic alcohols. Similar solvent- and alcohol-mediated rate accelerations have been observed for a number of proteolytic enzymes (6-15). The increase in hydrolytic activity cannot be a consequence of acyl transfer to the alcohol or solvent, since the assay system employed specifically measures the product of the hydrolytic reaction, α-ketoglutarate. Any such transfer reaction would result in no effect or apparent inhibition of the hydrolytic reaction (16).

The data presented in this paper provide quantitative evidence for the previously proposed two-step mechanism involving an acyl enzyme intermediate (2, 3) as shown in Scheme 2 where $K_X$ represents the equilibrium constant for binding of the substrate $X$ to the enzyme, and $k_1$, $k_2$, etc. represent the rate constants for the covalent bond-making and bond-breaking steps.

If a common intermediate (i.e. acyl enzyme) is formed in the reaction, then the rate of reaction of this intermediate with water or with a nucleophile (such as an amine) should be independent of the acyl donor (see Scheme 3). Although this criterion can easily be tested when formation of the acyl enzyme is fast relative to its decomposition (3, 17), it is more difficult to demonstrate when acylation is rate limiting or partially rate limiting.

That acylation and decylation are both partially rate limiting for the hydrolysis and acyl transfer reactions with α-ketoglutarate as the acyl donor and for acyl transfer reactions with ethyl α-ketoglutarate as the substrate is demonstrated by the observation that both substrates are hydrolyzed at nearly identical rates but show rate differences of 2-fold for acyl transfer to ethanolamine (Table IV). If decylation were entirely rate limiting, both α-ketoglutarate and ethyl α-ketoglutarate would exhibit identical maximal velocities for acyl transfer to ethanolamine. On the other hand, if acylation were entirely rate limiting, the same maximal velocity for hydrolysis and acyl transfer should occur for each substrate.

The rate of hydrolysis of ethyl α-ketoglutarate (and to a much smaller extent, α-ketoglutarate) increases with increasing chain length of the alcohol until a maximal value is reached. This observation is consistent with an increase by alcohols on the rate of acylation, such that decylation becomes rate limiting, or an effect on decylation such that acylation becomes rate limiting. If the effect were on acylation, α-ketoglutarate and ethyl α-ketoglutarate would show identical maximal velocities. The fact that these rates differ by a factor of 2 suggests that the effect of alcohols is to increase the decylation step, and that the maximum rate that can be obtained in the presence of an alcohol reflects the rate of the acylation step.

The rate of acyl transfer from the enzyme to water or to amines can be calculated by the equation

$$V_{\text{obs}} = \frac{V_{\text{acylation}} \times V_{\text{deacylation}}}{V_{\text{acylation}} + V_{\text{deacylation}}}$$

where $V_{\text{obs}}$ corresponds to the observed maximal velocity, $V_{\text{acylation}}$ corresponds to the rate of acylation of the enzyme by either ethyl α-ketoglutarate or α-ketoglutarate, and $V_{\text{deacylation}}$ corresponds to the rate of transfer of the acyl group from the enzyme to an acceptor molecule. Assuming that $V_{\text{acylation}}$ is equal to the highest maximal velocity obtained in the presence of alcohols, we have calculated $V_{\text{deacylation}}$ for transfer to water, transfer to methylamine, and transfer to ethanolamine. As seen in Table VII, identical values are obtained for both the ester, ethyl α-ketoglutarate, and the amide, α-ketoglutarate, and thus provide strong evidence for both the acyl enzyme mechanism and the postulate that alcohols specifically effect the rate of hydrolysis of the acyl enzyme.

The inclusion of an amine-binding site in the proposed mechanism is required to account for the observed competitive
inhibition of alcohols with respect to amines in acyl transfer reactions (Fig. 2). The failure to observe exchange between ethanol and ethyl α-ketoglutarate indicates that alcohols do not react with the acyl enzyme at an appreciable rate. Thus alcohols do not compete with amines for reaction with the acyl enzyme (a situation which could occur by direct nucleophilic attack), but instead interfere with the binding of amines to the enzyme.

The observation that propanol has no effect on the transfer reaction with methylamine as acceptor, yet is a competitive inhibitor toward ethanolamine when the latter compound is the acyl acceptor, suggests alcohols bind to a part of the amines-binding site which is not occupied by methylamine, i.e. not to the site which is directed toward the amino group. If alcohols were to bind to the amid group site, competitive inhibition would be independent of the nature of the amine. We interpret alcohol inhibition as primarily a steric effect in which the alcohol binds to a hydrophobic region of the amine site, and, depending on the size of the amine or the alcohol, overlap between the two molecules can occur resulting in competitive inhibition. The binding of alcohols to the hydrophobic site presumably results in the increased hydrolytic rates. The data in Table V shows that the rate of the reaction of a series of aliphatic amines with ethyl α-ketoglutarate decreases with increasing chain length. The linearity of the 1/v versus (I) plots for amine inhibition of ethyl α-ketoglutarate hydrolysis suggests that only 1 molecule of amine is bound per active site. We thus interpret the decrease in amine reactivity with increasing chain length as binding of the amine to both the amino group site and the hydrophobic site, which results in nonproductive binding.

The present data does not permit us to determine whether alcohols bind sequentially (as do amines) or randomly to the enzyme. However, the data is consistent with a specific effect of alcohols on the deacylation step.

The solvent-mediated rate accelerations of hydrolysis can be distinguished from those of aliphatic alcohols by several criteria. (a) Alcohols increase both $K_m$ and $V_{\text{max}}$ for the hydrolysis of ethyl α-ketoglutarate, whereas solvents effect only $V_{\text{max}}$. (b) Alcohols are competitive inhibitors with respect to amines in the transfer reactions while organic solvents are noncompetitive activators of these reactions. (c) The maximal rate acceleration for ethyl α-ketoglutarate hydrolysis shows a dependence on the size of the alcohol, yet is identical for all organic solvents.

Although it is quite clear that solvent-mediated rate accelerations differ from those produced by aliphatic alcohols, the nature of these solvent effects is unclear at the present time.

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The Effect of Aliphatic Alcohols and Organic Solvents on Reactions Catalyzed by 5-Hydroxy- N-methylpyroglutamate Synthetase
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J. Biol. Chem. 1971, 246:7804-7809.

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