Activation Parameters for Thiolate-Disulfide Interchange Reactions in Aqueous Solution[1]

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We have measured activation parameters for thiolate–disulfide interchange reactions in water. Two disulfides were examined (Ellman's reagent and oxidized glutathione) and three thiols (2-mercaptoethanol, 1,3-dithiopropan-2-ol, and 1,4-dithiothreitol). These values are consistent with the interpretation of this reaction as an uncomplicated S_N2 reaction of thiolate anion on the disulfide bond.

Introduction

Thiol–disulfide interchange is central to a number of important biochemical processes, including formation and cleavage of structural cystine disulfide bonds and disulfide-mediated redox reactions.[3-9] Examination of the physical-organic chemistry of simple thiol–disulfide interchange reactions in aqueous solution has established the reaction to be a very simple one.[3-5,10-14] Interchange involves three steps (eq 1–3; in these equations $R_{nuc}S$ is the

$$R_{nuc}SH \rightleftharpoons R_{nuc}S^- + H^+ \quad (1)$$

$$R_{nuc}S^- + R_cS\text{-}SR_{lg} \rightleftharpoons [R_{nuc}S\cdots S(R_c)\cdots SR_{lg}]^{-(*)} \rightleftharpoons R_{nuc}S\text{-}SR_c + {}^-SR_{lg} \quad (2)$$

$$R_{lg}S^- + H^+ \rightleftharpoons R_{lg}SH \quad (3)$$

attacking (nucleophilic) thiol group, R_cS is the attacked (central) thiol group, and $R_{lg}S$ is the leaving thiol group): initial ionization of the thiol, nucleophilic attack of the resulting thiolate anion on a sulfur atom of the disulfide group, and protonation of the product thiolate anion. The nucleophilic attack appears to be a simple S_N2 reaction that occurs along the sulfur–sulfur bond axis of the disulfide group.[15,16] Charge in the transition state is distributed over all three sulfur atoms but is concentrated on the terminal two.[10-13] Both rate and equilibrium constants follow the Brønsted relation in the pK_a's of the participating thiol groups.[10-14,17]

This paper summarizes activation parameters for representative thiolate–disulfide interchange reactions and is intended to contribute detail to the present mechanistic model for the transition state for these reaction. Activation parameters are particularly helpful in inferring changes in the organization of the solvent water on going from ground to transition state. In this instance, the values of $\Delta S^\ddagger$ are similar to those observed in other S_N2 reactions of tholate anions.

Results

We examined the reduction of two disulfides (Ellman's reagent, ESSE, 5,5′-dithiobis(2-nitrobenzoic acid); oxidized glutathione, GSSG), each by three thiols (2-mercaptoethanol, ME; 1,3-dithiopropan-2-ol, DTP; dithiothreitol, DTT). Reactions were carried out in oxygen-free solutions containing 50 mM phosphate buffer, 1 mM EDTA, pH 7.0. The kinetic methods used have been described in detail elsewhere[13] and are summarized in the Experimental Section. Figure 1 shows representative kinetic plots. Figure 2 shows Arrhenius plots derived from the resulting kinetic constants. In these plots, k_1 for monothiols is the rate constant for the reaction of eq 4, and k_1 for dithiols

$$RS^- + R'SSR' \xrightarrow{k_1} RSSR' + R'S^- \quad (4)$$

$$HSRS^- + R'SSR' \xrightarrow{k_1} HSRSSR' + R'S^- \quad (5)$$

is that for eq 5: in these equations R′SSR′ = ESSE or GSSG. The detailed rate constants on which the plots are based are listed in supplementary material to this article. All calculations required a knowledge of the temperature dependence of the thiol pK_a values. These were measured independently and are summarized in Figure 3. Table I summarizes thermodynamic parameters.

The data for reduction of ESSE were obtained by using a straightforward UV assay; we believe these data to be reasonably accurate. The appreciable experimental uncertainties in the values of $\Delta S^\ddagger$ (log A) reflect primarily the relatively small temperature ranges (40–50 °C) over which data were collected. Kinetic measurements involving GSSG used both UV (for DTT) and enzymatic (for ME and DTP) assays. Both of these assays were less precise than that used for ESSE. This lower precision, combined with smaller temperature ranges (20–30 °C) for the kinetic measurements, underlie the larger experimental uncertainties for $\Delta S^\ddagger$ (log A) for these data.

Discussion

The activation enthalpies summarized in Table I are not surprising: values for $\Delta H^\ddagger$ are indistinguishable within the limits of error for attack of the three thiols used as reducing agents on a single disulfide; the difference of ~5 kcal/mol between reactions involving ESSE and GSSG as substrate

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Table I. Activation Parameters for Thiolate–Disulfide Interchange Reactions[a]

RS⁻	R'SSR'	$\Delta G^{\ddagger}$ [b]	$\Delta H^{\ddagger}$ [b]	$\Delta S^{\ddagger}$ [c]
$HOCH_2CH_2S^-$	ESSE	10.6 ± 1	9 ± 1	−4.4 ± 4
$HSCH_2CHOHCH_2S^-$		10.7 ± 1	10 ± 1	−1.3 ± 4
$HSCH_2CHOHCHOHCH_2S^-$		10.9 ± 1	9 ± 1	−4.7 ± 4
$HOCH_2CH_2S^-$	GSSG	15.2 ± 1	16 ± 1	3.2 ± 4
$HSCH_2CHOHCH_2S^-$		15.7 ± 1	16 ± 1	−0.1 ± 4
$HSCH_2CHOHCHOHCH_2S^-$		15.3 ± 1	14 ± 1	−1.9 ± 5

[a] Values are calculated for 30 °C. [b] kcal/mol. [c] cal/(mol·deg).

Table II. Activation Parameters for Representative S_N2 Reactions Involving Sulfur

electrophile	nucleophile	pH	$\Delta H^{\ddagger}$ [a]	$\Delta S^{\ddagger}$ [b]
Cys-S-S-Cys [c]	CN^-	12.5	13	−24
ovomucoid-S-S	OH^-	12.8	18	−7
$(HOCH_2CH_2S)_2$ [d]	OH^-	13.6	18	−18
$(HO_2CCH_2CH_2S)_2$ [d]	OH^-	13.8	19	−20
ESSE [d]	OH^-	11.6	14	−12
$2\text{-}NO_2PhSOCH_2CH_3$ [e]	OH^-		17	−12
H_2O_2 [f]	SCN^-		15	−25
$(PhS)_2$ [g]	PhS^-		15	−9
$(CH_3CH_2CH_2S)_2$ [g]	*n*-BuS^-		14	−14
$SCH_2CH_2CH_2S$ [g]	*n*-BuS^-		12	−4
CH_3CH_2Br [h]	PhS^-		18	−8

[a] Units are kcal/mol. [b] Units are cal/(mol·deg) = eu. [c] Wagner, E. S.; Davis, R. E. *J. Am. Chem. Soc.* **1966**, *88*, 7-12. [d] Donovan, J. W.; White, T. M. *Biochemistry* **1971**, *10*, 32-8. [e] Hogg, D. R.; Vipond, P. W. *Int. J. Sulfur Chem.*, **1971**, *6*, 17-22. Measured in 50% ethanol/H_2O. [f] Curci, R.; Edwards, J. O. In "Organic Peroxides"; Swern, D., Ed.; Wiley: New York, 1970; Vol. I, pp 199-264. 264. [g] Reference 24. Measured in methanol:water (88:12 v/v). [h] Measured in methanol: Hine, J.; Brader, W. H., Jr. *J. Am. Chem. Soc.* **1953**, *75*, 3694-70.

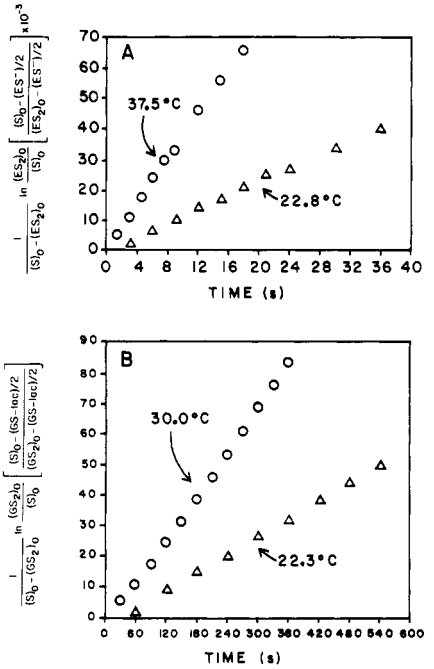

Figure 1. Kinetic plots for reduction of ESSE (A) and GSSG (B) by DTP in aqueous solution (phosphate buffer, 50 mM, pH 7.0) under argon. The vertical axes are based on kinetic equations summarized in the Experimental Section.

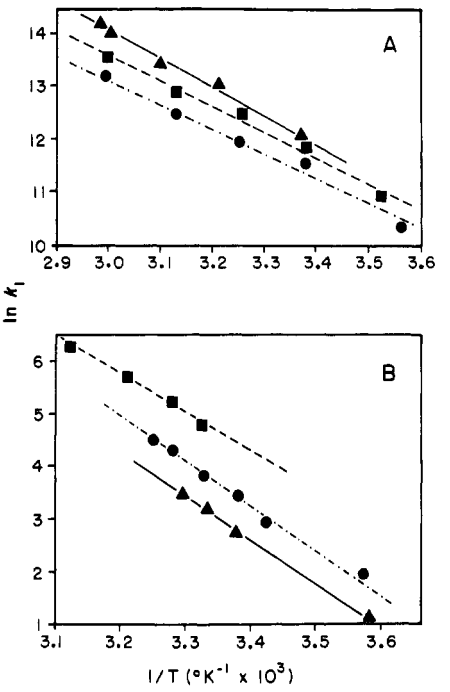

Figure 2. Arrhenius plots for the reduction of ESSE (A) and GSSG (B) by thiols in aqueous solution (phosphate buffer, 50 mM, pH 7.0) under argon: 2-mercaptoethanol (●); 1,3-dithiopropan-2-ol (▲); 1,4-dithithreitol (■).

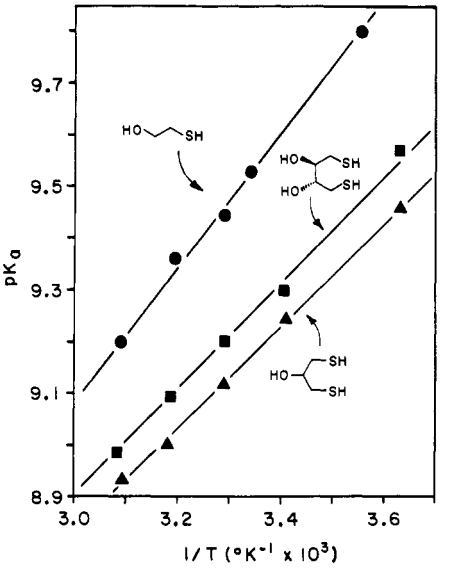

Figure 3. Temperature dependence of the thiol pK_a values. The values for DTT and for DTP are for pK_{a1} (i.e., for the first ionization).

is easily rationalized on the basis of the relative pK_a's of ESH and GSH.[10-14] The activation entropies are also similar to those observed previously for S_N2 reactions involving thiolate anions: Table II gives examples drawn from the literature.

In the absence of solvent effects, a bimolecular reaction is expected to have $\Delta S^{\ddagger} \sim -20$ to -30 eu, due to loss in translational and vibrational degrees of freedom in going

Table III. Values of Thiol pK_a and ΔH_{diss}

	pK_a (25 °C)		ΔH_{diss}, kcal/mol	
	this work	lit.	this work	lit.
$HOCH_2CH_2SH$	9.55	9.6 [a]	5.9	6.15 [b]
$HSCH_2CHOHCH_2SH$	9.17 (10.65)	9.0 (10.3) [a]	4.6 (6.7)	
$HSCH_2CHOHCHOHCH_2SH$	9.26 (10.34)	9.14 (9.94) [c]	4.9 (5.6)	

[a] Reference 14. [b] Danehy, J. P.; Noel, C. J. *J. Am Chem. Soc.* **1960**, *82*, 2511. [c] Loechler, E. L.; Hollocher, T. C. *Ibid.* **1980**, *102*, 7312-7321. See also: Fukada, H.; Takahashi, K. *J. Biochem.* **1980**, *87*, 1105-1110 (pK_a = 9.19; pK_{a_2} = 10.13 10.13).

from ground to transition state.[18-21] The usual rationalization for bimolecular reactions involving ionic species of positive values of $\Delta S^{\ddagger}$ is that the ground state is more tightly solvated than the transition state because charge is more localized in the ground state: the "release" of solvating solvent in going to the transition state (a concept whose details have, perhaps wisely, been allowed by mechanistic chemists to remain fuzzy) is argued to account for the positive contribution to $\Delta S^{\ddagger}$.[22,23] Thiolate–disulfide interchange would seem an excellent candidate for such solvent release: the charge in the transition state seems to be delocalized.

Experimental Section

General Methods. Biochemicals were obtained from Sigma Chemical. Thiols and 5,5′-dithiobis(2-nitrobenzoic acid) were from Aldrich. 1,3-Dithiopropan-2-ol was distilled under argon before use. The water used to prepare all solutions was deionized and doubly distilled, once from glass. Solution pH values were measured with a Radiometer PH M62 standard pH meter with REA 160 titrigraph module and REA 260 derivation unit and thermostated titration cell. Absorbance measurements were made on a Perkin-Elmer 552 spectrophotometer equipped with a constant-temperature bath and a strip chart recorder. The buffer system used throughout the study was dioxygen-free 50 mM phosphate plus 1 mM EDTA, pH 7.0, maintained under positive argon pressure. Assays for thiols,[26] ESSE,[26] GSSG,[27] methylglyoxal,[27] and GX-I[27] followed literature procedures.

Determination of pK_a Values. Degassed distilled water (10 mL) was allowed to equilibrate under argon in a thermostated titration vessel. Mercaptoethanol (70 μL), 1,3-Dithiopropan-2-ol (50 μL), or dithiothreitol (77.2 mg) was added to the vessel yielding a 0.1 N solution of the thiol. Thiols were titrated against 0.151 M carbonate-free potassium hydroxide.[24] For mercaptoethanol, the volume of KOH required to reach the equivalence point was determined by using the first derivative mode on the autotitrimeter. Additional mercaptoethanol solutions were titrated to the half equivalence point; the pH of the solution at this point was assumed to be the pK_a. For 1,3-Dithiopropan-2-ol and dithiothreitol complete titration curves at several temperatures were obtained by using the stepped curve mode on the autotitrimeter. The titrant was added to the thiol solution slowly enough to maintain the desired temperature throughout the titrations. Values of pK_a were obtained from the data giving pH vs. titrant added following a literature procedure[24] with use of a computer program. All titrations were done in triplicate. The pH meter was standardized against pH 7.00 and 10.00 MCB standard buffer solutions at each temperature. Values of thermodynamic parameters are summarized in Table III. Although a comparison value is available from the literature only for ME, values for similar compounds[25] are compatible with these data.

Kinetic Measurements. Methods. The reduction of ESSE by ME, DTP, and DTT was followed by measuring the increase in absorbance at 412 nm, the λ_{max} for the anion of 2-nitro-5-thiobenzoic acid (ES^-).[26] The reaction scheme used for analyzing the reduction is shown in eq 6–8 for monothiols.

$$RSH \rightleftharpoons^{K_a} RS^- + H^+ \quad (6)$$

$$RS^- + ESSE \xrightarrow{k_1} RS\text{–}SE + ES^- \quad (7)$$

$$RS^- + RSSE \xrightarrow{k_2} RS\text{–}SR + ES^- \quad (8)$$

In these reactions, $k_1 > 10k_2$,[14] so that k_1 can be measured if the concentration of thiol is less than half the concentration of ESSE (under conditions where ES^- production as a result of reaction 8 is insignificant). Under these conditions, eq 9 holds.

$$d[ES^-]\,dt \simeq k_1[RS^-][ESSE] = k_1^{obsd}([RS^-] + [RSH])[ESSE] \quad (9)$$

The observed rate constant, k_1^{obsd}, is related to the rate constant for reaction of thiolate, k_1, by eq 10.

$$k_1 = k_1^{obsd}\,[1 + 10^{pK_a - pH}] \quad (10)$$

The reaction scheme used for analyzing the reduction of dithiols is shown in eq 11–16.

$$HSRSH \rightleftharpoons^{K_a} HSRS^- + H^+ \quad (11)$$

$$HSRS^- \rightleftharpoons^{K_a'} {}^-SRS^- + H^+ \quad (12)$$

$$HSRS^- + ESSE \xrightarrow{k_1} HSRS\text{–}SE + ES^- \quad (13)$$

$${}^-SRS^- + ESSE \xrightarrow{k_1'} {}^-SRS\text{–}SE + ES^- \quad (14)$$

$$HSRS\text{–}SE \rightleftharpoons^{K_a''} {}^-SRS\text{–}SE + H^+ \quad (15)$$

$${}^-SRS\text{–}SE \xrightarrow{k_2} \overbrace{SRS} + ES^- \quad (16)$$

Previous work has shown that if the disulfide ring formed in reaction 16 is not strained, reaction 13 is rate limiting, and the reaction follows eq 17, where [S] = [HSRSH] + [$HSRS^-$] + [$^-SRS^-$]. A factor of 2 is introduced into eq 17 to reflect the fact that a second equivalent of ES^- is produced rapidly (eq 16) once the mixed disulfide HSRS–SE is formed. Again, k_1^{obsd} is related to k_1 by eq 10. Integration of eq 9 and 17 yields eq 18,[28] where

$$d[ES^-]/dt \simeq 2k_1([HSRS^-] + [{}^-SRS^-])[ESSE] = 2k_1^{obsd}[S][ESSE] \quad (17)$$

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(25) Values for AH_{diss} for other thiols are as follows (kcal/mol): C_6H_5SH, 4.02; $CH_3CHSHCH_3$, 5.3; $HSCH_2CO_2^-$, 6.2; $H_2NCH_2CH_2SH$, 6.08. (Christensen, J. J.; Hansen, L. D.; Izatt, R. M. "Handbook of Proton Ionization Heats"; Wiley: New York, 1976).
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for monothiols $[S]_0 = ([RSH] = [RS^-]_0)$, $n = 1$, and for dithiols $[S]_0 = ([HSRSH] + [HSRS^-] + [^-SRS^-])_0$, $n = 2$.

$$k_1^{obsd}t = 1/([S]_0 - [ESSE]_0) \times [\ln ([ESSE]_0/[S]_0)]([S]_0 - [ES^-]/n)/([ESSE]_0 - [ES^-]/n) \quad (18)$$

Two methods were used to measure the reduction of GSSG by thiols. The first, in which DTT was the reducing agent, relied on the UV absorbance of oxidized DTT (DTT^{ox}, $\epsilon = 110\ M^{-1}\ cm^{-1}$ at 310 nm),[29] a species formed during the course of the reduction. Equations 11–16 apply in this case, with GSSG (GS^-) substituted for ESSE (ES^-). The observed rate constants are calculated by using eq 19 and 20 and are converted to rate constants for attack of thiolate anion by eq 10 ($[S] = [HSRSH] + [HSRS^-] + [^-SRS^-]$).

$$d[DTT^{ox}]dt \simeq k_1([HSRS^-] + [^-SRS^-])[GSSG] = k_1^{obsd}[S][GSSG] \quad (19)$$

$$k_1^{obsd}t = 1/([S] - [GSSG]_0) \ln ([GSSG]_0/[S]_0 \times ([S]_0 - [DTT^{ox}])/[GSSG]_0 - [DTT^{ox}]) \quad (20)$$

The reduction of GSSG by ME and DTP was measured by using the procedure described previously.[13] Liberated GS^- is converted in a fast enzymatic step (catalyzed by glyoxalase I (GX-I)) into the UV-absorbing species (*S*)-lactoylglutathione (GS-lac; $\epsilon_{240\ nm} = 3370\ M^{-1}\ cm^{-1}$; eq 21 and 22).

$$GSH + CH_3COCHO \rightleftharpoons GSCHOHCOCH_3 \quad (21)$$

$$GSCHOHCOCH_3 + GX\text{-}I \xrightarrow{k_3} \underset{\text{(GS-lac)}}{GSCOCHOHCH_3} + GX\text{-}I \quad (22)$$

The concentrations of species present in the assay are adjusted such that k_1 (eq 7 and 13, with GSSG instead of ESSE) is rate limiting. Thus, for monothiols

$$d(GS\text{-}lac)/dt \simeq k_1[RS^-][GSSG] = k_1^{obsd}([RSH] + [RS^-])[GSSG] \quad (23)$$

and for dithiols

$$d(GS\text{-}lac)/dt \simeq 2\,k_1([HSRS^-] + [^-SRS^-])[GSSG] = 2k_1^{obsd}[S][GSSG] \quad (24)$$

where $[S] = [HSRSH] + [HSRS^-] + [^-SRS^-]$. An equation analogous to eq 18, with ESSE (ES^-) replaced by GSSG (GS-lac), was used to calculate k_1^{obsd}.

Kinetic Measurements. ESSE. Oxygen-free solutions of ESSE (1.49–37.9 μM) and ME (0.0307–1.58 mM) were prepared in phosphate buffer solution. Polystyrene cuvettes were sealed with serum stoppers and flushed with argon. The ESSE solution (2.0 mL) was added via a syringe. The cuvettes were equilibrated at the appropriate temperature for at least 15 min in the cell compartment of the spectrophotometer. After equilibration, 25–200 μL of an appropriate thiol solution was added to the cuvette with a spring-loaded calibrated Hamilton syringe; the resulting increase in absorbance at 412 nm was recorded. Solution temperatures were measured immediately after the reaction was complete with a thermometer capable of reading to a fraction of a degree. The concentration of ES^- was calculated by using the relation $[ES^-]\ (mM) = A_{412}/13.6$, with the resultant value used in eq 18 to calculate $k_1^{obsd}t$. The observed rate constant, k_1^{obsd}, was estimated from a graph of $k_1^{obsd}t$ vs. t.

Kinetic Measurements. GSSG. The method of Iyer and Klee was used to measure the rate of GSSG reduction by DTT.[29] Solutions of GSSG (~0.7 mM) and DTT (~13.5 mM) were prepared by using oxygen-free phosphate buffer. In an argon-flushed polystyrene cuvette sealed with a serum stopper was placed 2.0 mL of the GSSG solution. The cuvette was equilibrated at a specified temperature for at least 15 min. The spectrophotometer was set to measure absorbance at 310 nm with a full-scale absorbance of 0.10. A blank solution of oxygen-free GSSG was used to balance the instrument readings. After equilibration, 100–200 μL of the DTT solution was added with a spring-loaded syringe and the increase in absorbance ($\epsilon = 110\ M^{-1}\ cm^{-1}$) measured. The temperature of the solution was recorded immediately after completion of the reaction. Equation 10 was used to calculate k_1^{obsd}, and k_1 was calculated in the usual fashion.

The rate of reduction of GSSG by ME and DTP was measured by using the procedure described previously.[13] Dioxygen-free buffered solutions of DTP (~5 mM), ME (~5 mM), GSSG (~12 mM), methylglyoxal (~24 mM), and GX-I (1000 units/mL) were prepared by using deoxygenated phosphate buffer. In a quartz cuvette sealed with a serum stopper and flushed with argon were placed (via syringes) 2.0 mL of the thiol solution, 50 μL of methylglyoxal, and 10 μL of GX-I. A blank solution, lacking enzyme, was prepared similarly and was used to compensate for nonenzymatic reactions. The cuvettes were equilibrated at the proper temperature for at least 10 min, and then 50 μL of GSSG was added with a spring-loaded syringe to both cuvettes. The increase in absorbance at 240 nm, due to formation of (*S*)-lactoylglutathione ($\epsilon = 3370\ M^{-1}\ cm^{-1}$), was measured; the temperature was recorded following the reaction.

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Registry No. $HOCH_2CH_2S^-$, 57966-62-8; $HSCH_2CHOHCH_2S^-$, 83861-76-1; $HSCH_2CHOHCHOHCH_2S^-$, 59177-12-7; $HOCH_2CH_2SH$, 60-24-2; $HSCH_2CHOHCH_2SH$, 584-04-3; $HSCH_2CHOHCHOHCH_2SH$, 3483-12-3; ESSE, 69-78-3; GSSG, 27025-41-8.

Supplementary Material Available: Tables of the experimental rate constants as a function of temperature (2 pages). Ordering information is given on any current masthead page.

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