# Determination of the Thermodynamic Parameters of Individual Steps of Pyruvate–Oxime Formation by Rapid, Continuous Flow Microcalorimetry

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**Abstract:** A calorimetric method involving continuous flow at various flow rates has been used to follow the time course of heat evolution in the oxime formation reaction between pyruvate and hydroxylamine. Thermodynamic parameters for the two steps of the reaction have been evaluated. The first step of the reaction, which leads to the formation of a carbinolamine, is driven by a decrease in enthalpy ($\Delta H^\circ = -11.3$ kcal) which is largely entropy compensated. The second step, a dehydration, has a smaller enthalpy change ($\Delta H^\circ = -4.6$ kcal) with little change in entropy.

## Introduction

Addition reaction mechanisms for carbonyl compounds with nitrogen bases have been a subject of study for many years.[1-4] In 1959 Jencks demonstrated that such Schiff-base reactions involve a carbinolamine intermediate[5] (eq 1).

$$>C{=}O + H_2NR \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} >C\begin{matrix}\diagup OH \\ \diagdown NHR\end{matrix} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} >C{=}NR + HOH \qquad (1)$$

The overall rate of the reaction in the neutral pH range is limited by the acid-catalyzed dehydration step.

While some free-energy information is available for this class of reactions,[6,7] there is very little known of the corresponding enthalpies. Knowledge of this parameter is desirable since it may permit recognition of carbinolamine intermediates in enzyme catalyzed, as well as other reactions.

The first and second steps of the reaction in eq 1 can be separated in time, making it convenient to use continuous flow calorimetry to determine the enthalpy changes occurring in each of these two steps. While this method was used by Roughton[8] in 1941 to demonstrate the two-stage nature of the formation of $CO_2$ from $HCO_3^-$ and acid, and to measure the individual heats of formation of those two steps, its application has been limited by the lack of availability of apparatus with sufficient sensitivity. Recently Johnson and Biltonen[9] have demonstrated the ability of a modern, commercially available flow microcalorimeter to obtain both kinetic and thermodynamic data for reactions whose half-life exceeds a few seconds. In essence, variations in flow rate are used to vary the "residence time" of the reaction in the sensing chamber, so that the partial and total reaction heat, as a function of time, can be determined. We use the method here to determine the change in enthalpy of the two separate steps of the oxime formation reaction of pyruvate.

## Results

**1. Validation of the Method.** The measurement of the heat of protonation of Tris with excess HCl is shown to be independent of flow rate for residence times between 0.95 and 150 s in Figure 1 (□). Furthermore, when we compared the extent of the oxidation of NADPH by α-ketoglutarate and ammonia in the presence of a catalytic amount of glutamic dehydrogenase, using both spectrophotometric and flow calorimetric methods, we obtained good agreement as shown in Figure 1 (○).

**2. Oxime Formation.** The extent of oxime formation determined calorimetrically for several hydroxylamine concentrations at pH 6.0 is shown in Figure 2. In each case there is an initial exothermal burst followed by a much slower second exothermal, pseudo-first-order phase. A double reciprocal plot of these second-phase rate constants $k^q_{2,app}$ vs. hydroxylamine concentration is shown in Figure 3. The corresponding spectrophotometrically determined rate constants $k^s_{2,app}$ are plotted in the same figure. Good agreement between $k^q_{2,app}$ and $k^s_{2,app}$ is evident. For the mechanism portrayed by eq 1, the pseudo-first-order rate constant, $k_{2,app}$, is given by

$$k_{2,app} = k_2 a_H [NH_2R]/([NH_2R] + K_1) \qquad (2)$$

where $k_2$ is the pH-independent second-order rate constant for dehydration, $K_1 = k_1/k_{-1}$, $a_H$ is the activity of the hydronium ion measured at the glass electrode, and $[NH_2R]$ is the concentration of the free base form of the amine. Values of $k_2$ and of $K_1$ determined spectroscopically in this work, spectroscopically by Jencks, and calorimetrically are listed in Table I. From the agreement of these values it is evident that the second phase of the calorimetric time course of the reaction is in fact the dehydration step leading to the oxime.

To provide assurance that no extraneous phenomena are contributing to the calorimetric signal, amplitudes of the rapid initial phase of the thermal signal were measured and are also plotted in double-reciprocal form in Figure 3 (solid circles). It can be seen that they too follow a hyperbolic dependence on hydroxylamine concentration and yield the same value for $K_1$ as that given by the kinetic data. It would seem unlikely then that any process unrelated to oxime formation contributes to the calorimetric signal.

Having established the relationship between the calorimetric signal and the individual steps of the reaction, we can now calculate the thermodynamic parameters of those steps. The heat released in the first step, $q_1$, is expressed by

$$q_1 = \frac{\Delta H_1 [Pyr]_T [NH_2OH]}{[NH_2OH] + K_1} \qquad (3)$$

for the case where $[NH_2OH] > [Pyr]$, where $\Delta H_1$ is the enthalpy for carbinolamine formation, $[Pyr]_T$ is the initial concentration of pyruvate, and the remaining terms have been defined above. It follows from eq 3 that $\Delta H_1$ can be determined from the intercept of the dashed line in Figure 3 on the ordinate and that $K_1$ (and therefore $\Delta G_1^\circ$) can be determined from the intercept on the abscissa. $\Delta H^\circ_{overall}$ was determined from an average of six measurements of the heat evolved in the reaction between 60 and 159 s at a total hydroxylamine concentration of 0.29 M (upper curve, Figure 2). The second step of the reaction had proceeded for 12.8 half-lives. $\Delta H_2^\circ$ was calculated by subtracting $\Delta H_1^\circ$ from $\Delta H^\circ_{overall}$. $\Delta G^\circ_{overall}$, the value for which has not been previously published for this system, was

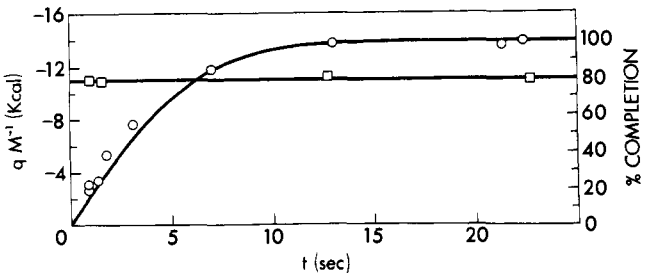


**Figure 1.** Validation of rapid-flow method. $t$ is the effective "residence time" of the reacting solution in the calorimeter cell. $q$ is the molar heat change calculated on the basis of the limiting reactant. □, neutralization of 140 $\mu$M Tris by 500 $\mu$M HCl, with the exception of the longest residence-time point, which was run with 500 $\mu$M Tris and 1.2 mM HCl; ○, oxidation of 300 $\mu$M NADPH by 6 mM $\alpha$-ketoglutarate, 60 mM ammonium chloride, and 0.1 mg mL$^{-1}$ glutamate dehydrogenase in 0.1 M phosphate buffer, pH 7.6. The curved solid line represents the average of five stopped-flow spectrophotometric runs of the same reaction, measuring the change in absorbance at 340 nm. The right-hand ordinate represents the degree of completion of the reaction, ($\Delta A_{340}/\Delta A_{340}$ total) 100.

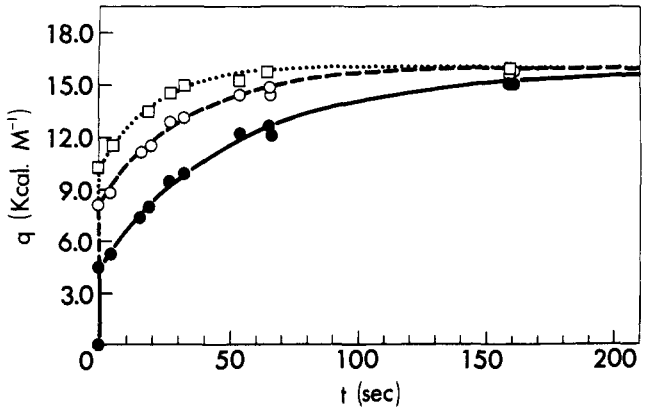


**Figure 2.** Time dependence of heat evolution on mixing 1 mM sodium pyruvate with 16 (-●-●-), 57 (-○-○-), and 290 nM (·□·□·) total hydroxylamine at pH 6.0, 25 °C (concentrations are final ones after mixing). Both solutions were made up in 0.6 M KCl. Experimental details are described in the text.

determined from spectrophotometric equilibrium measurements described in the Experimental Section. $\Delta G_2°$ was calculated by subtracting $\Delta G_1°$ from $\Delta G°_{overall}$. These equilibrium constants and the thermodynamic parameters are collected in Table II.

Because of the slowness of the reaction at pH 7.0, we have not obtained data over a sufficient time range to evaluate the rate constants at this pH. However, measurements of $q$ at a residence time of 1.93 s at a variety of hydroxylamine concentrations show that at pH 7.0, $K_1$ = 0.012 M and $\Delta H_1°$ = −11.1 kcal. These values do not differ significantly from those determined at pH 6.0; therefore, no significant pH effect on the enthalpy change in the neutral pH region is observed.

## Conclusion

We conclude that the first step of the reaction, the rapid formation of a carbinolamine, is driven by a very substantial decrease in enthalpy, compensated to a considerable extent by an unfavorable entropy change. The second step, a dehydration, is a much smaller enthalpy-driven reaction, accompanied by only a very small entropy effect.

The purpose of this investigation was to provide a set of thermodynamic parameters characterizing the carbinolamine intermediate, so that carbinolamine intermediates could be identified in more complex reactions. While this has been done, it must be recognized that the particular reaction investigated

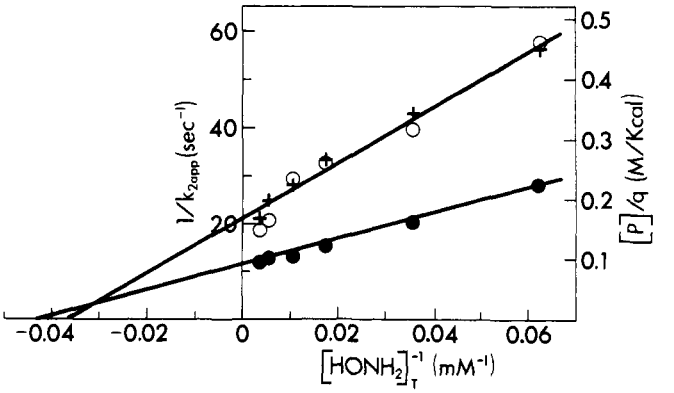


**Figure 3.** Dependence of kinetic and equilibrium parameters on hydroxylamine concentration. ○: $k^q_{2app}$ (calorimetrically measured constants). +: $k_2^s$ (spectrophotometrically measured constants). Solid line is a weighted least-squares fit to all spectrophotometric points omitting the leftmost point. Left ordinate applies to both +'s and ○'s. ●: amplitudes of initial phase of thermal signal. Right hand ordinate applies. Experimental conditions are as described for Figure 2.

**Table I.** Equilibrium and Rate Constants for Pyruvate–Hydroxylamine Carbinolamine Formation and Dehydration

| | Spectrophotometric value (this work) | Calorimetric value | Spectrophotometric value (Jencks)[5] |
|---|---|---|---|
| $k_2$, M$^{-1}$ min$^{-1}$ [a] | $2.7 \times 10^6$ | $2.5 \times 10^6$ | $4.0 \times 10^6$ |
| $K_1$, M | 0.014 | 0.011 | 0.017 |

[a] $k_2 = \lim_{[NH_2R]\to\alpha} k_{2,app}/a_H$.

**Table II.** Thermodynamic Parameters of the Reaction between Pyruvate and Hydroxylamine to Form a Carbinolamine and Its Subsequent Dehydration to an Oxime

| Carbinolamine | $\Delta G°$, kcal mol$^{-1}$ | $\Delta H°$, kcal mol$^{-1}$ | $\Delta S°$, eu |
|---|---|---|---|
| Formation step (1) | −2.5 ± 0.2 | −11.3 ± 0.3 | −29.5 ± 1.7 |
| Dehydration step (2) | −3.5 ± 0.2 | −4.6 ± 0.3 | −3.7 ± 1.7 |
| Overall reaction | −6.0 ± 0.2 | −15.9 ± 0.3 | −33.2 ± 1.7 |

involves an "$\alpha$ effect" and it is not known to what extent this effect contributes to $\Delta H°$. This can only be determined by comparing such enthalpy changes for individual steps in the reaction, as we have done here, with the corresponding parameters for non-$\alpha$-effector reactions of the same kind. It is clear that the approach used here is quite applicable to such a study.

We have not been able to find $\Delta H°$ measurements in the literature for reactions or individual steps of reactions similar to those described here with one exception. The early study by Roughton, which we have cited as the original demonstration of the ability of a continuous flow technique to dissect the overall $\Delta H°$ of a reaction into the component $\Delta H°$'s of its individual steps,[8] did show that the dehydration of $H_2CO_3$ to form $CO_2$ and water had a $\Delta H°$ (at 27.1 °C) of −0.890 kcal. This reaction has some formal resemblance to the second step of the reaction described here.

## Experimental Section

**Materials.** Hydroxylamine hydrochloride was purchased from Aldrich Chemical Co. Sodium pyruvate was obtained from Mann Research Labs and from Calbiochem. Tris(hydroxymethyl)aminomethane was also supplied by Calbiochem. $\alpha$-Ketoglutaric acid and

NADPH were purchased from Sigma Chemical Co. Glutamic acid dehydrogenase was supplied by Boehringer Mannheim Corp. Water was deionized and glass distilled.

**Methods.** Microcalorimetric equilibrium and kinetic measurements were determined at 25 °C on an LKB 10700-1 flow microcalorimeter, by the continuous flow method as described by Johnson and Biltonen.[9] The calorimeter is enclosed in a submarine, surrounded by water maintained within 0.001 °C by a Tronac Precision Temperature Controller. Thermopile voltage was amplified with a Keithley 150B microvolt-ammeter, with further amplification provided by a chart recorder.

Flow rates were determined by either one of two methods. At slower pumping rates, flow was determined by collecting and weighing the amount of water pumped through the calorimeter over a defined period of time. At high flow rates, the volume of water collected over a specified time was measured. At flow rates greater than about 1 mL/min, reactants were thermostated at 25 ± 0.2 °C prior to pumping through the flow system. Where flow rates exceeded 2.25 mL/min, the reactants were passed through a heat exchange coil consisting of 4 ft of stainless steel tubing (i.d. 0.047 in.) submerged in the water bath surrounding the calorimeter flow unit.

For the kinetic studies, flow rates ranged from about 0.141 mL $min^{-1}$ to 23.2 mL $min^{-1}$, corresponding to residence times of 160 and 0.97 s, respectively. At the very highest flow rates (residence times less than 5 s), an increase in temperature of the aluminum heat sink was observed. Block temperature slowly drifted upward with time. If such flow rates are maintained, block temperature can change by as much as 0.1 °C in several hours when the calorimeter is operated at 25 °C. At 15 °C, water friction heats are much larger than at 25 °C ($H_2O$ viscosity is greater at 15 °C); therefore, heat sink differential temperature is also much larger for a given flow rate. A temperature change of 0.3 °C was observed over a period of 2.5 h at a flow rate corresponding to a residence time of about 3 s. To circumvent any problems which might conceivably arise from a gradually warming heat sink, experiments were carried out as rapidly as possible.

Since the electrical calibration constant ($\epsilon$) varies with flow rate, calibrations were determined for each experiment. Measurements, as well as electrical calibrations, were conducted in triplicate, with the magnitude of electrical calibrations approximating the signal itself. Signal magnitude was determined by hand measurement of chart recordings. At high flow rates, chart speeds up to 10 in./min were used to artificially level baseline and signals, thus facilitating measurements.

**Determination of Effective Calorimeter Cell Volume.** Cell volume was determined using a modification of the method described by Johnson and Biltonen.[9] Instead of the hydrolysis of ethyl acetate reaction used by those authors, we employed an enzymatic reaction catalyzed by glutamate dehydrogenase from bovine liver, a reaction which does not attack the silicon rubber tubing in the peristaltic pumps, as was reported for ethyl acetate.

In this procedure, a solution of $\alpha$-ketoglutarate (6 mM), $NH_4Cl$ (60 mM), and NADPH (600 $\mu$M), prepared in 0.1 M potassium phosphate buffer pH 7.6, was pumped through the calorimeter with one peristaltic pump. The remaining pump was turned off after flushing the line with a solution consisting of $\alpha$-ketoglutarate (6 mM), $NH_4Cl$ (60 mM), and glutamate dehydrogenase (0.2 mg $mL^{-1}$), also in potassium phosphate buffer. Heat generated by the enzymatic reaction, represented by signal magnitudes from 0.22 to 0.55 $\mu$V, was followed through the cell until the volume element exited from the thermal sensing area, thereby causing loss of heat. The time between enzyme introduction and termination of active signal (measured at the point where signal starts to decay rapidly) was determined. With the flow rate and volume element transition time known, the effective cell volume can be calculated from the expression $V = ft_r$, where $f$ is the flow rate and $t_r$ is the volume element transition time.

Transition time was calculated at two separate flow rates. With a single pump flow rate equal to 0.152 mL $min^{-1}$, effective cell volume was 0.375 ± 0.007 mL (average deviation from mean); with a flow rate of 0.183 mL $min^{-1}$, cell volume was 0.376 ± 0.01 mL. Calculations in this paper are based on an effective cell volume of 0.375 mL.

Spectrophotometric rate measurements were made on a Gilford Model 2000 spectrophotometer at 25 °C using 1-cm path length quartz cells.

The equilibrium constant for the overall reaction was determined from spectrophotometric measurements at 230 and 240 nm on a Zeiss PMQII spectrophotometer. The disappearance of a stoichiometric amount of hydroxylamine was negligible at these wavelengths. Pyruvate absorbance, however, was found to be 60 and 22% of that of the oxime, at 230 and 240 nm, respectively. Differential molar absorptivity, $\Delta\epsilon$ (oxime–pyruvate), was, therefore, used to measure the extent of oxime formation in the individual experiments. $\Delta\epsilon$ was determined to be 1152 $M^{-1}$ $cm^{-1}$ at 230 nm and 1210 $M^{-1}$ $cm^{-1}$ at 240 nm.

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