Kinetic Study of the Reaction of Acetoacetate with Glycine and Sodium Nitroprusside

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This paper describes an extensive kinetic study of the reactions involved in the determination of acetoacetate in body fluids. It is concluded that acetoacetate reacts with glycine to produce an imine intermediate that tautomerizes to an enamine. It is also concluded that nitroprusside reacts with the imine intermediate to produce an unstable product with an absorption maximum near 540 nm. This product decays slowly to produce a stable product with an absorption maximum near 393 nm. A proposed reaction pathway is used to develop kinetic equations, rate constants, equilibrium constants, and molar absorptivity of the unstable product that permit quantitative prediction of the kinetic behavior for a wide range of reactant concentrations.

The β-oxidation of fatty acids results in the formation of a small amount of acetoacetate, some of which is subsequently converted to acetone in the lungs or enzymatically reduced to β-hydroxybutyrate in the liver and other tissues.[1] These three species are collectively called the ketone bodies. Average values for their relative proportions in blood are 78% β-hydroxybutyrate, 20% acetoacetate, and 2% acetone.[2] Under certain metabolic conditions associated with a high rate of oxidation of fatty acids (starvation, diets low in carbohydrates, and diabetes mellitus), there is an increased production of ketone bodies that exceeds the capacity of the peripheral tissues to metabolize them.[3] Thus, they accumulate in the blood and urine, causing ketonemia and ketonuria, respectively. The overall condition is called ketosis and can be fatal if left untreated.

The most frequently used procedure for estimating acetoacetate in body fluids is the semiquantitative nitroprusside method.[4-6] This method involves the reaction of acetoacetate with glycine and sodium nitroprusside to generate a colored product with an absorption maximum near 550 nm. Problems with the reaction system result from the instability of the colored product and the very complex kinetics of the reaction system. However, despite the importance of this determination and the difficulties associated with it, there has been no detailed kinetic study of the reaction system.

An earlier paper from this laboratory described the development and evaluation of a predictive method for the quantitation of acetoacetate in body fluids.[7] This paper presents results of a detailed kinetic study of the reaction of acetoacetate with glycine and sodium nitroprusside. Results are used to propose a mechanism for the reaction system which in turn is used to develop a mathematical model that can be used to describe the kinetic behavior of the system for a wide range of conditions.

Amines can act as catalysts in the decarboxylation of β-keto acids.[8,9] This is a multistep process involving the formation first of a ketimine which, in the absence of other species with which it can react, either decays to form carbon dioxide and regenerate the amine or tautomerizes to the enamine, which has no absorption band in the ultraviolet (UV) region with a maximum near 285 nm. The rate at which the ketimine reacts to produce carbon dioxide is highly dependent on pH, being relatively fast for pH less than 7 and relatively slow for pH greater than 8.[9]

Our results indicate that, in the presence of nitroprusside, some of the ketimine reacts to form an unstable colored product with an absorption maximum near 540 nm. Thus, the acetoacetate/glycine/nitroprusside reaction is believed to proceed in at least three steps. The first step, identified herein as the *primary reaction*, involves the reaction of acetoacetate and glycine to produce the enamine. In the second step, identified herein as the *indicator reaction*, nitroprusside reacts with the product of the primary reaction to form an unstable colored product. In the third step, herein called the *decay reaction*, the colored product reacts to form species with very low molar absorptivities at 540 nm. The simultaneous reaction of acetoacetate, glycine, and nitroprusside is identified as the *combined reaction*.

This paper presents the results on which these conclusions are based, a detailed proposal for the reaction pathway, and kinetic equations including the rate constants that result from the proposed pathway and experimental data.[10,11]

EXPERIMENTAL SECTION

Instrumentation. Several photometers, including an ultraviolet/visible diode-array-based spectrophotometer (HP 8450A, Hewlett-Packard Co., Palo Alto, CA), a multichannel centrifugal mixing/measurement system (Rotochem IIa/36, American Instrument Co., Deerfield, IL), and a stopped-flow spectrophotometer (D-110, Dionex Corp., Sunnyvale, CA), were used in different phases of this study. Kinetic data (absorbance vs time) were collected on-line by dedicated computers and then transferred to a central UNIX-based supermicrocomputer (MC-5500 workstation, Massachusetts Computer Corp., Westford, MA) for

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processing with software written in C language.[12] Multipoint, curve-fitting methods[13,14] were used to process the absorbance vs time data. Additional processing was done using a statistical package (SAS/STAT Software for Personal Computers, Release 6.03, SAS Institute Inc., Cary, NC) on a personal computer (PC XT, IBM Corp., Armonk, NY). With the rapid-scanning spectrophotometer (HP 8450A), solutions were mixed manually; with the centrifugal mixer (Rotochem IIa), solutions were mixed centrifugally in temperature-controlled cells; and with the stopped-flow instrument (Dionex D-110), solutions were mixed in 1:1 ratios in the mixing chamber after being thermostated for 15 min in the drive syringes surrounded by water circulating from a temperature-controlled water bath.

Reagents. All solutions were prepared in distilled deionized water.

Buffers. Phosphate buffer solution was prepared by dissolving 44.7 g of dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$; Mallinckrodt, Inc., Paris, KY) in water, adjusting the pH to 8.60 with 1 mol L^{-1} hydrochloric acid, and diluting to 1.0 L to give a final concentration of 0.17 mol L^{-1}. The resulting solution was filtered through a 5.0-μm filter (Type SM, Millipore Corp., Bedford, MA) and the pH was measured and adjusted to 8.60, if not already at that value. Results for the studies of the primary reaction were obtained with phosphate buffer. Borate would be a better buffer at that pH, but boric acid reacts slowly with acetoacetate.[15]

Unless stated otherwise, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS, pK_a = 8.4 at 25 °C; Sigma Chemical Co., St. Louis, MO) was used as a buffer in the studies of the indicator and combined reaction. The TAPS buffer solution was prepared by dissolving 24.33 g of TAPS in water to partial volume, adjusting the pH to 8.60 with 18 mol L^{-1} sodium hydroxide, and then diluting to 1.0 L for a final concentration of 0.1 mol L^{-1}. When necessary, pH was readjusted to 8.60.

Acetoacetate Standards. Aqueous acetoacetate standards were prepared by distilling ethyl acetoacetate (Sigma Chemical Co.), taking 0.750 mL of the distillate boiling between 176 and 177 °C, and diluting to 25.0 mL with 0.2 mol L^{-1} sodium hydroxide. The ethyl acetoacetate was allowed to hydrolyze in a refrigerator at 4 °C for 48 h to form a solution nominally 0.236 mol L^{-1} in acetoacetate.[16] Spectrophotometric studies of the hydrolysis product showed the presence of residual ethyl acetoacetate in the hydrolysate at the 3–12 μmol L^{-1} level.[17] The hydrolysate decomposes at a rate of 2% per month at 5–7 °C[16] and was prepared weekly. Aqueous standards were prepared by diluting 0.100–0.400 mL (in 0.050-mL increments) of the hydrolysate to 50.0 mL with buffer. Standards were prepared just before an experiment was run and were kept in the refrigerator at 4 °C when not used.

Glycine. Glycine solutions in 0.1 mol L^{-1} TAPS (or phosphate) buffer were prepared by dissolving glycine (Fisher Scientific, Fair Lawn, NJ) and 24.33 g of TAPS (or 26.8 g of $Na_2HPO_4 \cdot 7H_2O$) in 0.9 L of water, adjusting to pH 8.60 with 18 mol L^{-1} sodium hydroxide, and then diluting to 1.0 L. Readjustment of pH was not usually necessary. All solutions were stored at 4 °C and renewed monthly.

Sodium Nitroprusside. Sodium nitroprusside (sodium nitroferricyanide, $Na_2[Fe(NO)(CN)_5] \cdot 2H_2O$) solutions were prepared fresh daily by dissolving 1.49 g of sodium nitroprusside (Sigma Chemical Co.) in water and diluting to 100 mL for a final concentration of 0.05 mol L^{-1}. Solutions were stored at room temperature in amber bottles to decrease photochemical reaction.

Procedure. Unless stated otherwise, conditions used for the various studies were pH 8.60 and temperature 25.0 ± 0.2 °C. Conditions were varied for other variables by using 11 different combinations of the concentrations of glycine, acetoacetate, and nitroprusside given in Table I.

Primary Reaction. The primary reaction was initiated by mixing 0.50 mL of acetoacetate solution with 0.50 mL of glycine solution in the thermostated reaction cuvet of the rapid-scanning spectrophotometer (HP 8450A). Absorbance values at 285 nm were recorded at appropriate time intervals from 10 s after the reaction was initiated until the reaction had reached equilibrium. The 10-s initial delay allowed time for mixing perturbations to subside.

Indicator Reaction. Aliquots (0.50 mL each) of acetoacetate sample and glycine solution were mixed in the cuvet of the rapid-scanning spectrophotometer and incubated to equilibrium (eight to nine half-lives). The indicator reaction was then initiated by adding 0.20 mL of sodium nitroprusside solution to the cuvet and monitored by following the absorbance at 540 nm. The indicator reaction exhibited pseudo-first-order behavior only during the first two half-lives. In the studies of effects of variables, data between 10 s and the time required to reach about 80% of the maximum absorbance value were used for first-order fits.

In the studies with the centrifugal mixing system (Rotochem IIa), 0.60 mL of the equilibrium 1:1 acetoacetate/glycine mixture was pipeted in the middle well and mixed with 0.12 mL of nitroprusside solution loaded in the inner well. Buffer (TAPS) was used as the reference solution. Absorbance (measured with an interference filter with the transmission band centered at 550 nm with a 10-nm bandpass) was monitored long enough to include the decay of the absorbance of the unstable product. Also, a dynamic reagent blank reaction was followed by substituting buffer solution for the acetoacetate standard because nitroprusside was found to react slowly with glycine, forming a product with a broad absorbance band (centered at 393 nm) that affected the absorbance at 540 nm.

Combined Reaction. The combined reaction was initiated by mixing 0.50 mL of acetoacetate standard with 0.20 mL of nitroprusside solution in the cuvet of the rapid-scanning spectrophotometer and then adding 0.50 mL of glycine solution. With the centrifugal mixing system (Rotochem IIa), the transfer disk was loaded with 0.30 mL of acetoacetate standard and 0.12 mL of nitroprusside solution in the middle well and 0.30 mL of glycine solution in the inner well. Absorbance measurements were recorded as described above for the indicator reaction.

Stopped-Flow Experiments. The monochromator of the stopped-flow instrument was set at 540 nm with an exit slit width of 0.50 mm corresponding to a spectral bandwidth of 1.5 nm. The bias voltage of the photomultiplier tube was adjusted to 450 V for a maximum output voltage reading of 9.80 V, using TAPS buffer solution as blank.

In the studies of the indicator reaction, 0.50 mL of 0.236 mol L^{-1} acetoacetate (original hydrolysate) was diluted with 1.0 mol L^{-1} glycine to a final volume of 25.0 mL and incubated to equilibrium (1 h). The indicator reaction was initiated by mixing equal volumes of the equilibrium mixture and 16.6 mmol L^{-1} nitroprusside solution. The procedure was repeated with 0.30-mL aliquots of 0.236 mol L^{-1} acetoacetate solution and 8.30 and 4.15 mmol L^{-1} nitroprusside solutions.

In the combined reaction experiments, 0.10-, 0.30-, and 0.50-mL aliquots of 0.236 mol L^{-1} acetoacetate hydrolysate were diluted to 25.0 mL with 16.6 mmol L^{-1} nitroprusside solution, and 0.50 mL of buffer was diluted to 25.0 mL with 1.0 mol L^{-1} glycine. The combined reaction was initiated by mixing equal volumes of the diluted solutions. The procedure was repeated with 0.30-mL aliquots of 0.236 mol L^{-1} acetoacetate solution and 8.30 and 4.15 mmol L^{-1} nitroprusside solutions.

RESULTS AND DISCUSSION

All uncertainties are reported at the level of one standard deviation (±1 SD). All concentrations are reported as those in the final reaction mixture.

Primary Reactions. The reaction between glycine and acetoacetate produces a stable product with a broad absorption band peaking near 285 nm. With an excess of glycine

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relative to acetoacetate, the response curve at 285 nm follows pseudo-first-order kinetics very closely (see Figure 1A in ref 7).

Based on earlier studies,[8,9,18,19] the most probable reaction sequence involves formation of carbinolamine to form an imine, which subsequently tautomerizes to form an enamine. Earlier studies with simple systems suggest that the carbinol-amine should be present in very low concentrations. Thus, the overall reaction is represented as follows

$$\text{acetoacetate} + \text{glycine} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{imine} \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} \text{enamine} \quad (1)$$

It is most likely that the enamine is the species which is monitored by the absorption at 285 nm.

Rate equations for the reaction sequence in eq 1 are

$$dC_{AA}/dt = k_{-1}C_{Im} - k_1C_{AA}C_{Gly} \quad (2)$$

$$dC_{Im}/dt = k_1C_{AA}C_{Gly} + k'_{-1}C_{Enam} - k_{-1}C_{Im} - k'_1C_{Im} \quad (3)$$

and

$$dC_{Enam}/dt = k'_1C_{Im} - k'_{-1}C_{Enam} \quad (4)$$

Because glycine is in large excess over acetoacetate, glycine concentration remains nearly constant during the course of the reaction. Also, if, as suggested earlier,[8,9,18,19] the formation of the imine is the rate-limiting step, then the imine concentration will likely reach a steady state during most of the reaction. Thus, setting eq 3 equal to zero, the resulting equation can be rearranged into the form

$$C_{Im}^{ss} = (k_1C_{AA}C_{Gly} + k'_{-1}C_{Enam})/(k_{-1} + k'_1) \quad (5)$$

Substituting this expression for the imine concentration into eqs 2 and 4, these expressions become

$$\frac{dC_{AA}}{dt} = k_{-1}\left(\frac{k_1C_{AA}C_{Gly} + k'_{-1}C_{Enam}}{k_{-1} + k'_1}\right) - k_1C_{AA}C_{Gly} \quad (6)$$

and

$$\frac{dC_{Enam}}{dt} = k'_1\left(\frac{k_1C_{AA}C_{Gly} + k'_{-1}C_{Enam}}{k_{-1} + k'_1}\right) - k'_{-1}C_{Enam} \quad (7)$$

The solution to these coupled differential equations using the Laplace transform method[21,22] is

$$C_{AA} = C_{AA}^0\left(\frac{1}{1 + K_{eq}C_{Gly}}\right) - \left(C_{AA}^0\left(\frac{1}{1 + K_{eq}C_{Gly}}\right) - C_{AA}^0\right)e^{-k_{obs}t} \quad (8)$$

and

$$C_{Enam} = C_{AA}^0\left(\frac{K_{eq}C_{Gly}}{1 + K_{eq}C_{Gly}}\right)(1 - e^{-k_{obs}t}) \quad (9)$$

in which C_{AA}^0 is the initial acetoacetate concentration, C_{Gly} is the glycine concentration, K_{eq} is the overall equilibrium constant for reaction 1, and k_{obs} is the apparent pseudo-first-order rate constant. The overall equilibrium constant is the product of the individual rate constants for the two steps as shown in eq 10.

$$K_{eq} = K_{eq,1}K_{eq,1'} = k_1k'_1/k_{-1}k'_{-1} \quad (10)$$

The overall rate constant can be written in the form

$$k_{obs} = \frac{k_{-1}k'_{-1}}{k_{-1} + k'_1} + \left(\frac{k_1k'_1}{k_{-1} + k'_1}\right)C_{Gly} \quad (11)$$

suggesting that the observed pseudo-first-order rate constant should vary linearly with glycine concentration, with intercept and slope related to the rate constants for the two steps in reaction 1.

This behavior was confirmed earlier (see Figure 4 in ref 7). Linear least-squares fits of k_{obs} vs C_{Gly} yielded a slope of 1.3×10^{-3} L mol^{-1} s^{-1} and an intercept of 4.4×10^{-4} s^{-1}. Assuming that $k'_1 \gg k_{-1}$, the slope term in eq 11 simplifies to $k_1 \simeq 1.3 \times 10^{-3}$ L mol^{-1} s^{-1}. When this approximate value of k_1 was used as an initial estimate in fits of more complete data sets to be discussed later in this paper, values of $k_1 = 1.1 \times 10^{-3}$ L mol^{-1} s^{-1} and $k_{-1} = 3.7 \times 10^{-1}$ s^{-1} were obtained as best-fit values of these constants. These results correspond to an equilibrium constant for the first step in the reaction of $K_{eq,1} = k_1/k_{-1} = 1.1 \times 10^{-3}$ L mol^{-1} s^{-1}/3.7×10^{-1} s^{-1} = 3.0×10^{-3} L mol^{-1}.

When the assumption that $k'_1 \gg k_{-1}$ is applied to the intercept term, the result is

$$\text{intercept} \simeq k_{-1}k'_{-1}/k'_1 = k_{-1}(1/K_{eq,1'}) \quad (12)$$

using the value of the intercept determined earlier (4.4×10^{-4} s^{-1})[7] and the value of k_{-1} (3.7×10^{-1} s^{-1}) determined in this study, it follows from eq 12 that the equilibrium constant for the second step in reaction 1 is $K_{eq,1'} = 3.7 \times 10^{-1}$ s^{-1}/4.4×10^{-4} s^{-1} = 8.4×10^2.

By combining the values of the equilibrium constants for the two steps in reaction 1, it is possible to estimate the overall value of the equilibrium constant as $K_{eq} = K_{eq,1}K_{eq,1'} = (3.0 \times 10^{-3}$ L mol$^{-1})(8.4 \times 10^2) = 2.5$ L mol^{-1}. To test for consistency, the value of the overall equilibrium constant was also computed by dividing the numerical value of the slope in eq 11 by the numerical value of the intercept. The result, $K_{eq} = 1.3 \times 10^{-3}$ L mol^{-1} s^{-1}/4.4×10^{-4} s^{-1} = 2.95 L mol^{-1}, is in reasonably good agreement with the value of 2.5 L mol^{-1} obtained by using results from fitted data.

Finally, the estimate of the equilibrium constant was used with computed absorbance changes for various glycine concentrations (see Figure 4 in ref 7)) to estimate the molar absorptivity at 285 nm of the product of reaction 1, assumed to be the enamine. The value obtained is $\epsilon_{285} = (3.0 \pm 0.2) \times 10^3$ L mol^{-1} cm^{-1}.

In summary, we find $k_1 = 1.1 \times 10^{-3}$ L mol^{-1} s^{-1}, $k_{-1} = 3.7 \times 10^{-1}$ s^{-1}, $K_{eq,1} = 3.0 \times 10^{-3}$ L mol^{-1}, $K_{eq,1'} = 8.4 \times 10^2$, and $K_{eq} = 2.5$ L mol^{-1}. Unfortunately we could gain no information about values of k'_1 and k'_{-1} from this treatment because the rate-determining step is the formation of the imine.

Indicator/Combined Reactions. The colored product (λ_{max} = 540 nm) could be formed either by adding nitroprusside to the reaction mixture after glycine and acetoacetate had reacted to equilibrium (indicator reaction) or by mixing all three reactants simultaneously (combined reaction). We had assumed initially that significantly different behavior would be observed for these different situations. However, as will be shown below, behavior was virtually identical. Accordingly, results for these situations are described together.

Response Curves. Figure 1 includes typical response curves for a blank reaction between glycine and nitroprusside (bottom plot), the indicator reaction (middle plot), and the combined reaction (top plot). To reduce effects of the blank reaction, response curves for the indicator and combined reactions were "corrected" by point-by-point subtraction of the blank absorbance from each data set before subsequent processing. Data for the kinetic blanks were obtained by including the blank reaction in the same rotor as used for the

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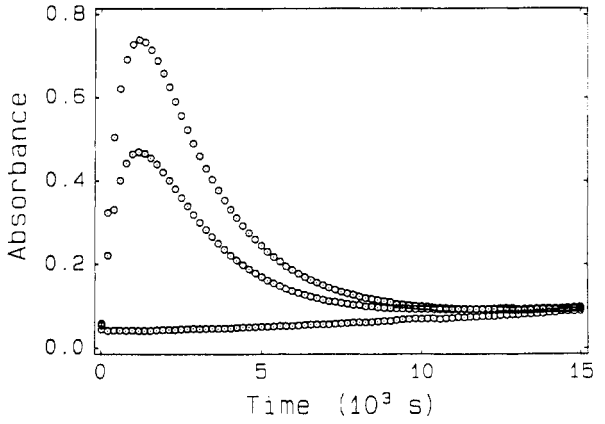


Figure 1. Response curves for combined (top), indicator (middle), and blank (bottom) reactions: pH 8.60, 25 ° C, 0.67 mol L^{-1} glycine, 1.18 mmol L^{-1} acetoacetate, and 8.3 mmol L^{-1} nitroprusside.

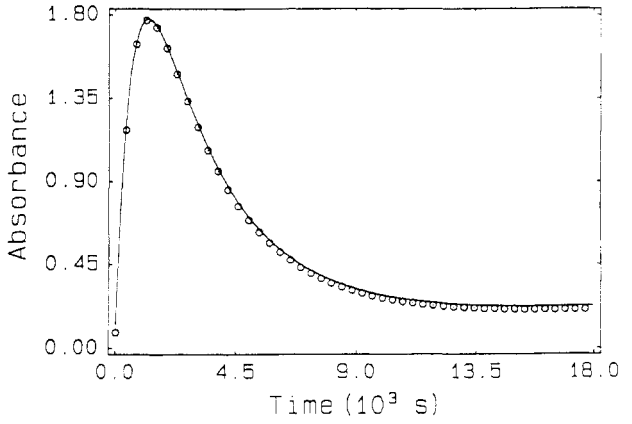


Figure 2. Response curves for combined (⊙) and indicator (—) reactions. First point at 10 s: initial acetoacetate concentration combined 3.02 mmol L^{-1}, indicator 4.72 mmol L^{-1}; pH 8.60, 25 °C, 0.49 mol L^{-1} glycine, and 8.3 mmol L^{-1} nitroprusside.

samples to which the blank corrections were applied. In this way, kinetic blanks were obtained at the same times and under virtually the same conditions as sample data. We had expected that blank-corrected response curves would decay to zero at long reaction times but they did not. Reasons for this are discussed in the next subsection.

It is observed that the responses for the indicator and combined reactions have different amplitudes for the same initial concentrations of the three reactants. We had expected that other aspects of the kinetic behavior of the indicator and combined reactions would be significantly different. However, preliminary processing of multiple data sets yielded very similar kinetic parameters for the two situations. For example, for a wide variety of conditions, average apparent first-order rate constants (10^{-3} s^{-1}) for the leading edge of response curves were 2.26 for the indicator reaction and 2.27 for the combined reaction. Analogous constants (10^{-3} s^{-1}) for the trailing edge were 0.36 for both situations. These results suggest that, except for some differences in signal amplitude, the kinetic behavior of the indicator and combined reactions was very similar.

Because this unexpected result was so critical to the mechanistic interpretation, we tested it further by adjusting the acetoacetate concentration to give the same maximum response for both situations. Results for the indicator and combined reactions are shown in Figure 2. The two plots are virtually superimposed, confirming that, except for some amplitude differences, the kinetic behaviors of the indicator and combined reactions are the same.

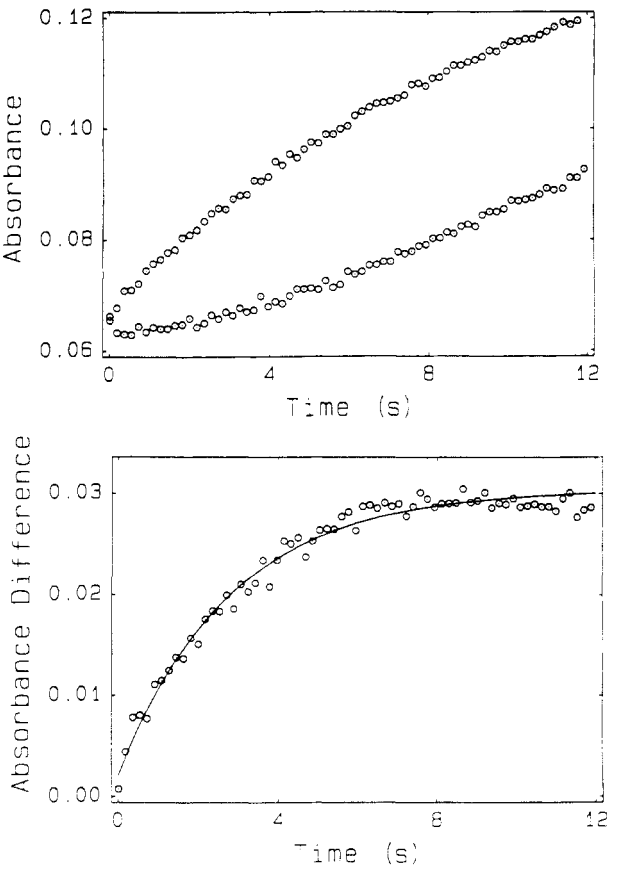


Figure 3. Response curves for combined and indicator reactions: initial acetoacetate concentration combined 2.84 mmol L^{-1}, indicator 4.72 mmol L^{-1}; pH 8.60, 25 °C, 0.49 mol L^{-1} glycine, and 8.3 mmol L^{-1} nitroprusside. (A, top panel) indicator (top) and combined (bottom) reactions; (B, bottom panel) point-by-point difference (top minus bottom) experimental (⊙) and fitted (—).

To obtain further insight into reasons for the differences and similarities between the indicator and combined reactions, we did fast-mixing (stopped-flow) studies on both reactions. Results are given in Figure 3A,B. The plots in Figure 3A show that, for approximately the same initial concentrations, the initial rate for the combined reaction (bottom plot) is much less than that for the indicator reaction (top plot) and that the reactions proceed at similar rates after the first few seconds. We conclude that the nitroprusside reacts rapidly with a product of the primary reaction when first added to the equilibrium mixture and that the subsequent slower reaction is essentially the same regardless of whether glycine and acetoacetate are permitted to react prior to addition of nitroprusside or are mixed simultaneously with nitroprusside.

To better characterize the fast component of the indicator reaction, we subtracted the response of the combined reaction from that of the indicator reaction. The difference response is shown in Figure 3B along with a plot obtained by fitting a first-order model to data between 0.003 and 11 s. The first-order model fits the data very well.

Similar experiments were run at two additional nitroprusside concentrations with similar results. Apparent first-order rate constants determined as best-fit values (solid curve in Figure 3B) varied linearly with nitroprusside concentration (Figure 4) between 2 and 9 mmol L^{-1}. The least-squares fit of the data yielded

$$k\ (s^{-1}) = (39.2 \pm 0.5)\ (L\ mol^{-1}\ s^{-1})C_{NP} + (2.7 \pm 0.3) \times 10^{-2}\ s^{-1}$$

with the standard error of the estimate, s_{yx}, of 2.1×10^{-3} s^{-1}

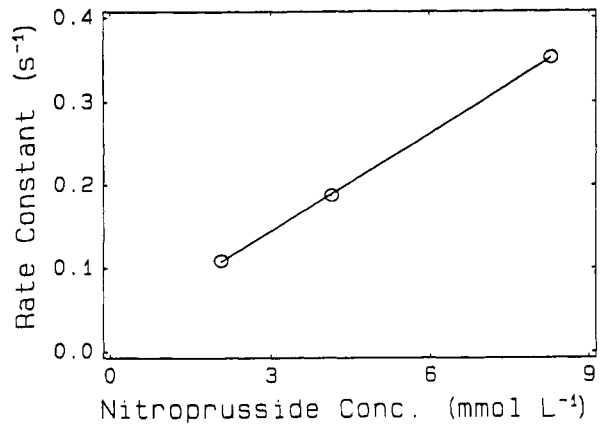


Figure 4. Effect of nitroprusside concentration on the rate constant of the reaction obtained by subtracting the combined reaction from the indicator reaction.

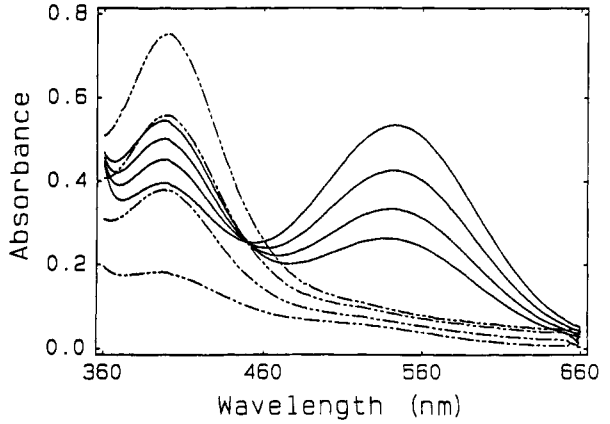


Figure 5. Absorption spectra for combined and blank reactions. Combined reaction (—): pH 8.6, 25 °C, 0.492 mmol L^{-1} acetoacetate, 0.667 mol L^{-1} glycine, and 8.3 mmol L^{-1} nitroprusside. Top to bottom at 540 nm: 1762, 2419, 3076, 3733 s. Blank reaction (···): pH 8.6, 30 °C, 0.67 mol L^{-1} glycine, and 8.3 mmol L^{-1} nitroprusside. Bottom to top at 390 nm: 10, 1560, 3060, 4960 s.

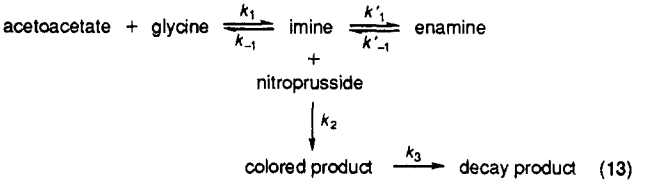
and $r = 0.999$. Although only three points are included in the plot, the linear fit is very good, with the standard derivation of the slope representing only 1.3% of the numerical value and the standard error of the estimate being only 2.7% of the smallest value and 0.6% of the largest value. For nitroprusside concentrations between 2 and 9 mmol L^{-1}, pseudo-first-order rate constants for this process vary between 0.10 and 0.36 s^{-1}.

Spectral Characteristics. The solid curves in Figure 5 show the time-dependent spectral responses in the visible region for the combined reaction. Similar spectra were obtained for the indicator reaction. The spectra include a well-defined isosbestic point near 450 nm, suggesting the presence of two interconverting absorbing species. The species with the peak near 540 nm is the unstable product of the indicator and combined reactions; the species with the peak near 393 nm is assumed to be the final decay product of the overall reaction.

The dotted plots in Figure 5 represent time-dependent spectra for the product of the blank reaction between glycine and nitroprusside (bottom plot in Figure 1). The shapes and positions of the spectra between 360 and 460 nm for the products of the combined and blank reactions are very similar. These spectra suggest but do not confirm the strong possibility that the products of the blank and decay reactions may be the same. If this were the case, it would explain why dynamic blank-corrected responses for the combined and indicator reactions do not reach zero after very long reaction times because the decay product would have a finite absorbance at 540 nm where those reactions were monitored.

Proposed Reaction Pathway. We assumed initially that production of the colored product (λ_{max} = 540 nm) involved reaction of nitroprusside with enamine produced in reaction 1. However, several facts were inconsistent with that assumption. First, if that were true, the indicator and combined reactions would be expected to exhibit significantly different behavior. Second, if it were true, the amount of colored product generated when high glycine concentrations were used should be independent of glycine because reaction 1 is fored to equilibrium with increasing glycine concentration (see Figure 4 in ref 7). The maximum absorbance, though, varied linearly with glycine concentration throughout the range examined, which included a 2000-fold excess relative to acetoacetate. Finally, attempts to fit the response curves with kinetic equations resulting from this pathway were unsuccessful in the sense that they did not predict consistent values of rate constants and molar absorptivity of the colored product.

These facts led us to consider an alternative pathway in which the nitroprusside reacts with the imine rather than the enamine. The proposed pathway is



(13)

We assumed that a steady-state condition would be established between the imine and the enamine, and accordingly, that step was not included in the rate equations for the overall reaction. With that assumption, the rate equations considered were

$$dC_{AA}/dt = k_{-1}C_{Im} - k_1C_{AA}C_{Gly} \quad (14)$$

$$dC_{Im}/dt = k_1C_{AA}C_{Gly} - k_{-1}C_{Im} - k_2C_{Im}C_{NP} \quad (15)$$

$$dC_{P_{540}}/dt = k_2C_{Im}C_{NP} - k_3C_{P_{540}} \quad (16)$$

$$dC_{DP}/dt = k_3C_{P_{540}} \quad (17)$$

Assuming a steady-state condition for the imine concentration, eq 15 is set equal to zero and is rearranged to give

$$C_{Im}^{ss} = (k_1C_{AA}C_{Gly})/(k_2C_{NP} + k_{-1}) \quad (18)$$

where C_{Im}^{ss} is the steady-state concentration of the imine. By using this steady-state assumption, it can be shown (see appendix A of ref 23) that the time-dependent product concentration is given by

$$C_{P_{540}} = [C_{AA}^0k/(k_3 - k)](e^{-kt} - e^{-k_3t}) \quad (19)$$

where

$$k = (k_1k_2C_{Gly}C_{NP})/(k_2C_{NP} + k_{-1}) \quad (20)$$

The time-dependent absorbance of the colored product is obtained by multiplying eq 19 by the product of the molar absorptivity, $\epsilon_{P_{540}}$, and the path length, b. This equation takes account only of the product with an absorption peak at 540 nm.

To include the decay product we use a simplified form of the reaction. Because eq 19 is identical to that for consecutive

(23) Laios, I. D. Predictive kinetic method for quantitation of acetoacetate in body fluids and kinetic study of the reaction of acetoacetate with glycine and nitroprussides. Ph.D. Thesis, Purdue University, 1991.

Table I. Final Concentration of Reagents Used in Different Combinations

level	acetoacetate (mmol L^{-1})	glycine (mol L^{-1})	nitroprusside (mmol L^{-1})
1	0.295	0.333	4.15
2	0.492	0.667	8.30
3	0.688	1.00	16.6

irreversible reactions,[22,24,25] we used the simplified reaction scheme

$$\text{acetoacetate} \xrightarrow{k} \text{colored product} \xrightarrow{k_3} \text{decay product} \quad (21)$$

By using this simplified equation, it can be shown that the time-dependent concentration of the decay product, C_{DP}, is given by

$$C_{DP} = C^0_{AA}\left\{1 + \left(\frac{ke^{-k_3t} - k_3e^{-kt}}{k_3 - k}\right)\right\} \quad (22)$$

for which all symbols have been defined.

Assuming additive absorbances and combining eqs 19 and 22, it can be shown that the time-dependent absorbance at 540 nm is given by

$$A_{540} = C^0_{AA}(\alpha e^{-kt} + \beta e^{-k_3t} + \epsilon_{DP}) + e_i \quad (23)$$

where α and β are

$$\alpha = (\epsilon_P k - \epsilon_{DP}k_3)/(k_3 - k) \quad (24)$$

$$\beta = (\epsilon_{DP} - \epsilon_P)\, k/(k_3 - k) \quad (25)$$

where ϵ_P and ϵ_{DP} are the molar absorptivities at 540 nm of the intermediate and decay products, k is given by eq 20, and e_i is a random error term. We used equations for single-component (eq 19) and two-component (eq 23) models in curve-fitting procedures described below.

Parameter Dependencies. Effects of reactant concentrations were evaluated by using 11 different combinations of the concentrations given in Table I. Curve-fitting methods were used to evaluate the validity of eqs 19 and 23. To obtain initial estimates of rate constants to be used in the fitting process, we fit portions of leading and trailing edges of response curves such as those in Figures 1 and 2 with a first-order model.

Assuming that the leading edges of the response curves were controlled by the formation of the unstable product and that the trailing edges were controlled by the decay of the product, we expected that apparent rate constants from the leading edges would be very dependent on reactant concentrations and that apparent rate constants from the trailing edges would be virtually independent of reactant concentrations. We were surprised to find the opposite effects. For example, for 11 different sets of reagent concentrations, average apparent first-order rate constants (10^{-3} s^{-1}) from the leading edge of response curves were 2.27 ± 0.30 and 2.26 ± 0.26 for the combined and indicator reactions, respectively. Average apparent rate constants (10^{-3} s^{-1}) from the trailing edges were 0.36 ± 0.16 for the combined and indicator reactions. The range of values (10^{-3} s^{-1}) from the leading edges was 1.90–2.96 with most being grouped much closer together and with a slight trend of increasing values with increasing glycine and nitroprusside concentrations. In contrast, the range of values (10^{-3} s^{-1}) from the trailing edges was 0.11–0.63 with a clear trend of increasing values with increasing glycine and nitroprusside concentrations.

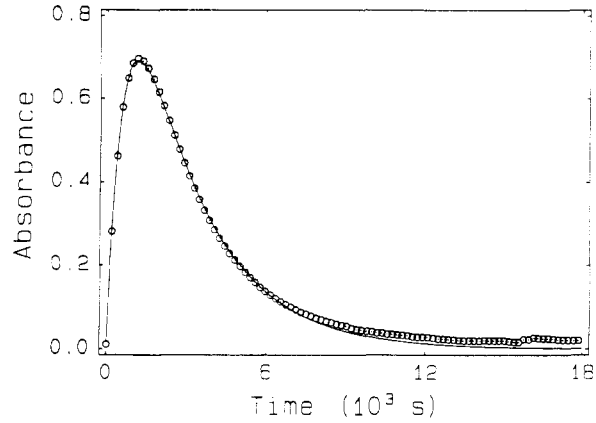


Figure 6. Experimental (⊙) and calculated (—) response curves of the combined reaction. One absorber, pH 8.6, 25 °C, 0.492 mmol L^{-1} acetoacetate, 0.667 mol L^{-1} glycine, and 8.3 mmol L^{-1} nitroprusside.

It has been noted that the rise and fall in signals for such situations do not necessarily reflect the rate constants for the formation and decay, respectively, of the intermediate.[22,24-31] Quoting Alcock et al.:[27] "... an optical density which rapidly increases and slowly declines does not necessarily imply a fast first and a slow second reaction". Following our intuition and using apparent rate constants from leading and trailing edges as initial estimates for the formation and decay reactions, respectively, we obtained very good fits of data but best-fit values of the molar absorptivities were inconsistent. For example, for separate fits for response curves of the combined reaction for the 11 sets of conditions mentioned above, the average value of the molar absorptivity (L mol^{-1} cm^{-1}) was 2.3×10^3 with a relative standard deviation (RSD) of 57%, a range from 420 to 4631, and a systematic dependence primarily on glycine concentration. Equally good fits were obtained when apparent rate constants from the trailing and leading edges were used as initial estimates for the formation and decay reactions, respectively. Moreover, best-fit values of molar absorptivities were more consistent. For separate fits to response curves for the 11 sets of conditions mentioned above, the average value of the molar absorptivity (L mol^{-1} cm^{-1}) was 9.1×10^3 with a relative standard deviation of 18% and a range from 6.7×10^3 to 13.1×10^3, with the trend depending primarily on nitroprusside concentration. We used this latter approach to obtain the remainder of results reported below.

Fitted Data. Excellent fits of experimental data for the combined reaction were obtained with both the single-absorber model (eq 19) and the two-absorber model (eq 22) applied to individual data sets without substitution of eq 20 for k. A typical set of experimental and fitted data is given in Figure 6 for the single-absorber model. Results for the two-absorber model are very similar except that the difference between experimental and fitted data at longer times is completely eliminated. These fits yielded consistent values of k_3 ($(1.67 \pm 0.14) \times 10^{-3}$ s^{-1}) and molar absorptivity ($(9.1 \pm 1.7) \times 10^3$ L mol^{-1} cm^{-1}) but inconsistent values of k ($(0.39 \pm 0.18) \times 10^{-3}$ s^{-1}). The inconsistent value of k is not surprising because it contains several variable dependencies (see eq 20). When k in eqs 19 and 22 was replaced by the expression in eq 20, inconsistent results were obtained for individual data

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Table II. Results of Fitting Full Models to Eleven Data Sets Simultaneously

points in data set	full model	$k_1 \pm$ SD (10^{-3} L mol^{-1} s^{-1})	$k_{-1} \pm$ SD (s^{-1})	$k_2^{a,b} \pm$ SD (L mol^{-1} s^{-1})	$k_3 \pm$ SD (10^{-3} s^{-1})	$\epsilon_{P_{340}} \pm$ SD (10^3 L mol^{-1} cm^{-1})	$\epsilon_{DP} \pm$ SD (L mol^{-1} cm^{-1})
1005	one absorber	1.12 ± 0.03	0.37 ± 0.02	39.2 ± 0.5	1.95 ± 0.04	12.0 ± 0.28	
1979	two absorbers	1.07 ± 0.02	0.34 ± 0.01	39.2 ± 0.5	1.98 ± 0.03	12.2 ● 0.24	8.6 ● 3.3

[a] Estimated independently from stopped-flow studies; kept constant during fitting process. [b] Constants in eq 13.

sets because they contained insufficient information to permit resolution of all the parameters.[32] This problem was resolved when multiple data sets (n = 11) were used simultaneously.

Figure 7A–C shows the effects of acetoacetate, glycine, and nitroprusside, respectively, on simultaneous fits of the single-absorber full model (eq 19 with substitution of eq 20 for k) to multiple data sets of the combined reaction. Similar fits (using more data points from the trailing edges) were obtained with the two-absorber full model (eq 22); deviations at longer times were not improved significantly. The models account quite well for different acetoacetate concentrations, less well for different nitroprusside concentrations, and significantly less well for different glycine concentrations.

Values of rate constants and molar absorptivities obtained from these fits are summarized in Table II. All the constants are determined with good precision, and inclusion of the second absorber makes little difference in either the rate constants or the molar absorptivity of the unstable product. These results suggest that the reaction of glycine and acetoacetate to produce the imine is the rate-limiting step and that the equilibrium constant for this reaction is very small ($\sim 3 \times 10^{-3}$ L mol^{-1}).

DISCUSSION

Some caveats should be kept in mind when interpreting and using the results of this study. First, although all the fitted curves (Figure 7) have the correct shapes, there are significant differences among experimental and fitted data. Second, solution conditions used to vary concentrations of the different reactants involved different ionic strengths, ranging from a low of 92 mmol/L to a high of 191 mmol/L. This variation in ionic strength may be the source of some of the variations among experimental and fitted data. In retrospect, it would have been better to have used an inert electrolyte to have controlled the ionic strength at the same level for all reaction conditions. Finally, even if the fits of the kinetic model to the data had been perfect, this would not prove the validity of the model but only that it may be the correct one. No attempt was made to identify either the unstable product or the decay product or to confirm the presence of the imine or enamine intermediates. However, despite these limitations, these results should be useful to anyone wishing to optimize conditions to quantify acetoacetate in body fluids or other samples.

During the course of this study, we learned that faulty data were incorporated into our earlier paper (Figure 5 in ref 7). Because absorbance values measured for some samples ($C^0_{AA} > 1.5 \times 10^{-3}$ mol L^{-1}) were outside the linenar range of the spectrophotometer used, response curves bent toward the time axis more rapidly than they should have. This resulted in artificially high values of rate constants and artificially low values of computed absorbance changes at high acetoacetate concentrations.

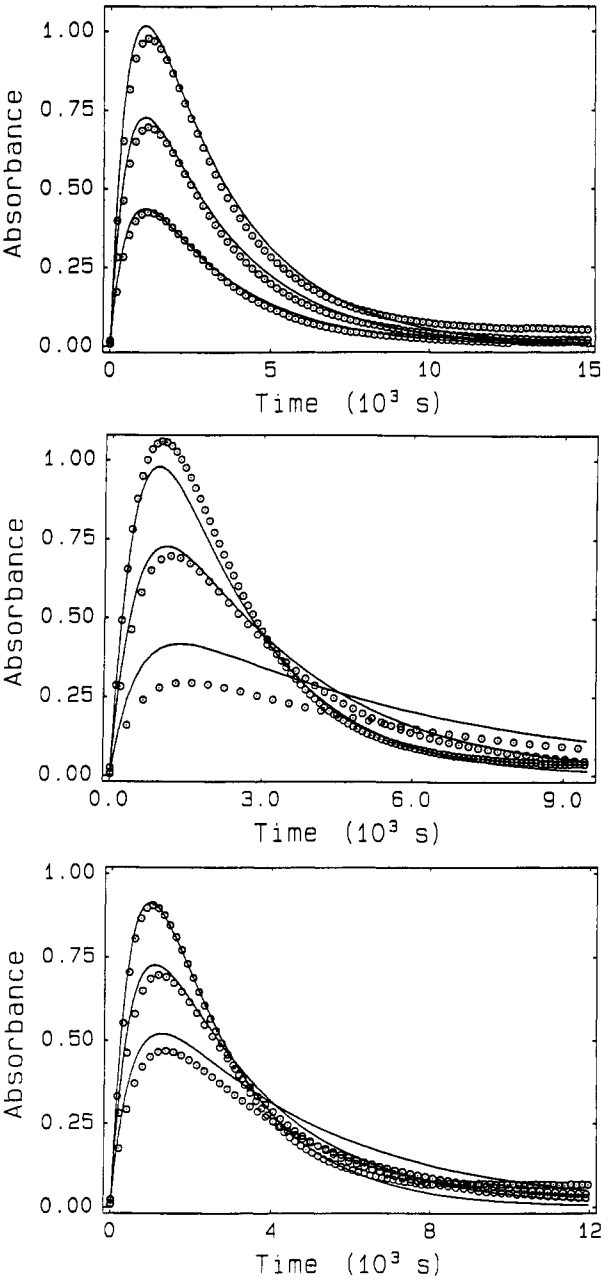

Figure 7. Experimental (⊙) and calculated (—) response curves of the combined reaction. All frames: pH 8.6, 25 °C, multiple data sets (11 runs, 1005 points); fixed concentrations are at level 2 in Table I. (A, top panel) Acetoacetate (bottom to top): 0.295, 0.492 0.688 mmol L^{-1}. (B, middle panel) Glycine (bottom to top): 0.333, 0.667, 1.00 mol L^{-1}. (C, bottom panel) Nitroprusside (bottom to top): 4.15, 8.30, 16.6 mmol L^{-1}.

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