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Stereochemical Control of Yeast Reductions. 2. Quantitative Treatment of the Kinetics of Competing Enzyme Systems for a Single Substrate

CHING-SHIH CHEN, BING-NAN ZHOU, GARY GIRDAUKAS, WOAN-RU SHIEH, FRANK VANMIDDLESWORTH, ARAVAMUDAN S. GOPALAN, AND CHARLES J. SIH[1]

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706

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Quantitative expressions have been developed for systems such as yeast reductions where competing enzymes act on one substrate to yield two enantiomeric products. These expressions relate the observed stereochemical variables, the extent of conversion (C), the optical purity expressed as enantiomeric excess (ee), and the initial substrate concentration (A_0) to the kinetic parameters K_R and K_S (apparent Michaelis constants) and y (V_R/V_S, the ratio of maximal velocities) of such competing enzymes. The expressions have been experimentally verified using a purified competing enzyme system of L- and D-lactic dehydrogenases. Furthermore, the enantioselective reduction of β-keto esters by intact yeast cells has been examined by means of this kinetic analysis.

INTRODUCTION

Microorganisms contain a wide variety of enzymes with relaxed substrate specificities (*1, 2*). Hence, microbial enzymes can often catalyze reactions on unnatural compounds yielding products of high optical purities. In particular, the asymmetric reduction of carbonyl compounds by bakers' yeast (*Saccharomyces cerevisiae*) has been widely used by synthetic chemists for preparing optically active alcohols (*3–5*) largely because the reaction is easy to carry out and bakers' yeast is commercially available in quantity. However, one common complication encountered with the use of resting yeast cells as a chiral catalyst is the presence of two or more competing enzymes with opposing stereochemical preferences, giving rise to products of opposite configurations at different rates (*6–8*). Consequently, yeast reductions of many ketonic substrates are only partially stereoselective (*9*).

Because the oxidoreductases of bakers' yeast can accommodate a variety of artificial substrates, one approach to controlling the stereochemical course of yeast reductions of carbonyl compounds would be to design substrates with large differences in the respective kinetic constants [apparent V (maximal velocity) and K (Michaelis constant)] for the competing enzymes. In theory, by making judi-

[1] To whom correspondence should be addressed.

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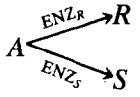
cious structural changes of substrate molecules, it may be possible to prepare carbinols of either configuration with high optical purity.

In the stereoselective reduction of ketones by two or more competing enzymes, the three variables of interest to the synthetic chemists are A_0, the initial substrate concentration; C, the extent of conversion; and *ee*, the enantiomeric excess of the product fraction. In this paper, we describe the derivation of a useful expression to relate these key parameters to the apparent kinetic constants (V and K) of competing enzymes for a given substrate. For intact cell systems, we will show that the values of apparent K_R, K_S, and $y = V_R/V_S$ may be calculated from the experimentally derived values of *ee* and C at fixed values of A_0. In turn, definition of the apparent kinetic constants, K_R, K_S, and y allows one to quantitatively follow the progress of the stereoselective reduction of ketonic substrates by yeast cells and to predict *ee* for any value of C and A_0. Moreover, structural changes of substrates may be correlated to changes in the relative ratios of V/K and V.

THEORY

A system consisting of two enzymes (ENZ_R, ENZ_S), each possessing absolute stereoselectivity, acting on one substrate, A, yielding two enantiomers, R and S, may be represented as



The rates of formation of R ($\bar{v}_R$) and S ($\bar{v}_S$) may be shown by steady-state kinetics to behave according to

$$\bar{v}_R = \frac{dR}{dt} = \frac{V_R A_t}{K_R + A_t} = \frac{V_R(A_0 - R - S)}{K_R + (A_0 - R - S)} \quad [1]$$

$$\bar{v}_S = \frac{dS}{dt} = \frac{V_S A_t}{K_S + A_t} = \frac{V_S(A_0 - R - S)}{K_S + (A_0 - R - S)} \quad [2]$$

assuming that the reaction is virtually irreversible and exhibits no product inhibition, and where V_R and V_S, and K_R and K_S denote maximal velocities and Michaelis constants for enzyme R and enzyme S, respectively; A_t is the remaining substrate concentration; A_0 is the initial substrate concentration; and R and S are the concentrations of the enantiomers formed by enzymes R and S, respectively.

Equation [3] may be obtained by solving Eqs. [1] and [2] (see Appendix),

$$R + S + \frac{y(K_R - K_S)}{(1 + y)} \ln \frac{(1 + y)A_0 + (yK_S + K_R)}{(1 + y)(A_0 - R - S) + (yK_S + K_R)} = (1 + y)S, \quad [3]$$

where $y = V_R/V_S$.

If the extent of conversion, C, is defined as

$$C = \frac{R + S}{A_0}, \quad [4]$$

then

$$A_t = A_0 - (R + S) = A_0(1 - C), \quad [5]$$

and Eq. [3] may be written as

$$A_0C + \frac{y(K_R - K_S)}{(1 + y)} \ln \frac{(1 + y)A_0 + (yK_S + K_R)}{(1 + y)A_0(1 - C) + (yK_S + K_R)} = (1 + y)S. \quad [6]$$

Equations [3] and [6] describe the general case where R and S are not necessarily enantiomeric. If R and S are enantiomers, their concentrations are related to the enantiomeric excess (*ee*), a measure of the optical purity of the product fraction

$$ee = \frac{R - S}{R + S}. \quad [7]$$

Combining Eqs. [4] and [7] affords

$$R = \frac{1 + ee}{2} A_0C \quad [8]$$

and

$$S = \frac{1 - ee}{2} A_0C. \quad [9]$$

Substituting Eq. [9] into [6] gives

$$ee = 1 - 2 \frac{\frac{A_0C}{1 + y} + \frac{y(K_R - K_S)}{(1 + y)^2} \ln \frac{(1 + y)A_0 + (yK_S + K_R)}{(1 + y)A_0(1 - C) + (yK_S + K_R)}}{A_0C}, \quad [10]$$

which may be rearranged to yield

$$ee = 1 - 2 \frac{A_0C + (K_R - x) \ln \frac{A_0 + x}{A_0(1 - C) + x}}{(1 + y)A_0C}, \quad [11]$$

where

$$x = \frac{yK_S + K_R}{1 + y}$$

and

$$\frac{y(K_R - K_S)}{1 + y} = (K_R - x).$$

Since in some situations, the action of each competing enzyme on an artificial substrate may be only partially stereoselective, Eq. [11] then may be modified to

accommodate this occurrence. For a single enzyme that is not absolutely stereoselective, the ratio of two enantiomers produced is constant (10). Hence, in this case, one can define

$$\left(\frac{R}{S}\right)_{ENZ_R} = \alpha \qquad [12]$$

and

$$\left(\frac{S}{R}\right)_{ENZ_S} = \beta. \qquad [13]$$

An equation similar to Eq. [11] can be derived for partially stereoselective enzyme systems (see Appendix),

$$ee = 1 - 2\frac{A_0C + (u - v)\ln\dfrac{A_0 + v}{A_0(1 - C) + v}}{wA_0C} \qquad [14]$$

where

$$u = \frac{(\beta + 1)V_RK_S + (\alpha + 1)\beta V_SK_R}{(\beta + 1)V_R + (\alpha + 1)\beta V_S}$$

$$v = \frac{V_R + V_S}{V_RK_S + V_SK_R}$$

$$w = \frac{(\alpha + 1)(\beta + 1)(V_R + V_S)}{(\beta + 1)V_R + (\alpha + 1)\beta V_S}.$$

RESULTS OF SIMULATIONS

Using Eq. [11] the theoretical effect of different enzymatic parameters on the optical purity of the product was examined. When the two competing enzymes have the same Michaelis constant, i.e., $K_R = K_S$, Eq. [11] reduces to

$$ee = 1 - \frac{2}{1 + y} = \frac{V_R - V_S}{V_R + V_S}. \qquad [15]$$

Hence, the rate of formation of the enantiomers, R and S, becomes independent of C and A_0, as in the case for a single enzyme. When $V_R < V_S$, ee has a negative value, indicating that the opposite enantiomer is preferentially formed. Figures 1A–C display the notional relationship of ee of the product fraction at complete conversion ($C = 1$) versus A_0 at constant K_R while K_S and $y = V_R/V_S$ are varied (1A, $y = 10$; 1B, $y = 1$; 1C, $y = 0.1$). As can be seen from Fig. 1, A_0 plays an important role in determining the value of ee. As $A_0 \rightarrow \infty$, the values of ee for all the curves also tend to converge to a value of $(V_R - V_S)/V_R + V_S$, and the rates of convergence depend on the K's of the two enzymes (Figs. 1A–C). At low A_0 concentrations (within the first-order region), the relative rates of the two reac-

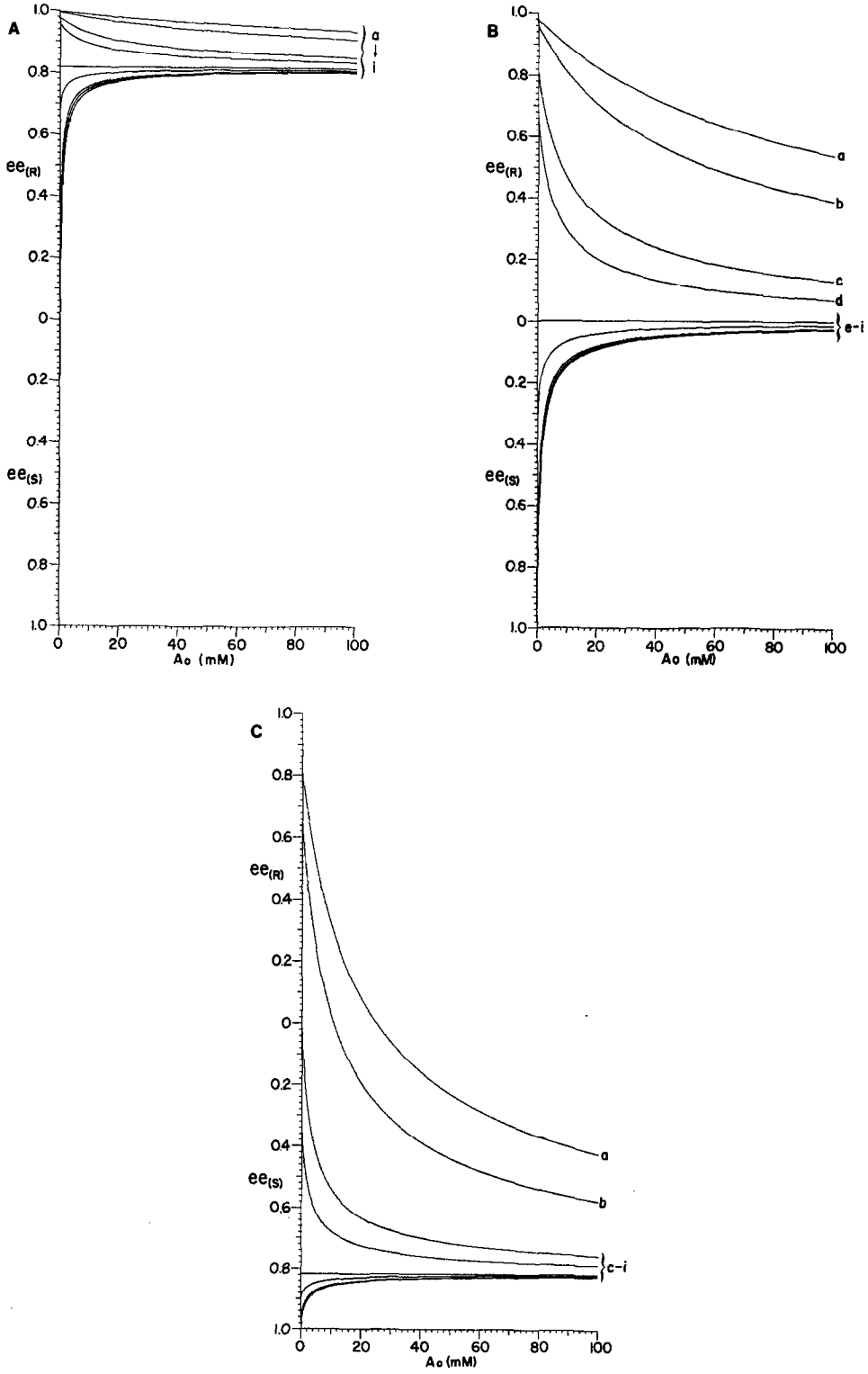


FIG. 1. Dependence of enantiomeric excess (*ee*) on the initial substrate concentration (A_0) at a fixed value of K_R (1×10^{-3} *M*) while *y* and K_S are varied. The curves were computer generated from Eq. [11]. (A) $y = 10$; (B) $y = 1$; (C) $y = 0.1$. The values of K_S were (a) 1×10^{-1} *M*; (b) 5×10^{-2} *M*; (c) 1×10^{-2} *M*; (d) 5×10^{-3} *M*; (e) 1×10^{-3} *M*; (f) 5×10^{-4} *M*; (g) 1×10^{-4} *M*; (h) 5×10^{-5} *M*; (i) 1×10^{-5} *M*.

tions depend on the V/K (first-order rate constant) ratio of the two enzymes, or as $A_0 \rightarrow 0$,

$$ee = \frac{\frac{V_R}{K_R} - \frac{V_S}{K_S}}{\frac{V_R}{K_R} + \frac{V_S}{K_S}}. \qquad [16]$$

Accordingly, the high V/K enzyme would be the major contributor in the competing reaction. As A_0 is raised, the relative values of V_R and V_S play a more dominant role in determining the enantioselectivity of the reaction. Thus, to obtain a product of high optical purity over a range of A_0, both a high V_R/V_S ratio as well as a large difference in the K values are essential.

Figures 2A–C show plots of *ee* vs. C at $K_R = A_0$ (0.001 M) while varying K_S and $y = V_R/V_S$ (2A, $y = 10$; 2B, $y = 1$; 2C, $y = 0.1$). As evidenced from the figures, changes in the extent of conversion do not affect the *ee* as dramatically as do changes in initial substrate concentrations (A_0). There is a tendency for the curves to migrate toward the side of the enantiomer produced by the lower K enzyme. This tendency is most pronounced in the two cases when (a) $K_R < K_S$ and $V_R < V_S$ (Figs. 2A and 2B, a–d); and (b) $K_R > K_S$ and $V_R > V_S$ (Fig. 2C, f–i).

KINETIC ANALYSIS OF TWO PURIFIED COMPETING ENZYMES

In order to test the experimental validity of our theoretical expressions, it was necessary to choose a suitable enzyme system. After careful analyses of the unique requirements of the competing enzyme systems, we selected the L- and D-lactic dehydrogenases (LDH) for this study. Both enzymes are known to confer absolute enantioselectivity (*11*) and the K and apparent V values of each enzyme may be accurately determined by continuous assay (*11*). Furthermore, no apparent substrate inhibition was noticeable (*12*), and the products, L- and D-lactic acids, can be conveniently assayed (*13, 14*).

As a first step, it was necessary to determine the K and apparent V of each enzyme under noncompetitive conditions. This was achieved by monitoring the initial velocity of the disappearance of NADH at 365 nm during the reduction of pyruvate catalyzed by each enzyme. The K and apparent V for the L-LDH were found to be 1.6×10^{-4} M and 5.18×10^2 μmol min^{-1} mg protein^{-1}, respectively, and those for the D-LDH were 5.94×10^{-4} M and 2.88×10^2 μmol min^{-1} mg protein^{-1}. Substitution of these kinetic parameters into Eq. [3] then allows one to generate two theoretical curves relating the amount of D- and L-lactic acids formed and the extent of conversion, C (solid curves, Fig. 3A), if these two enzyme reactions were conducted under a competitive situation.

To test the validity of Eq. [3], a competitive experiment was carried out using these same experimental conditions. Figure 3A clearly shows that the experimentally obtained values coincided fairly well with the predicted curves, although some deviation was observed at higher C values. This deviation may be attribut-

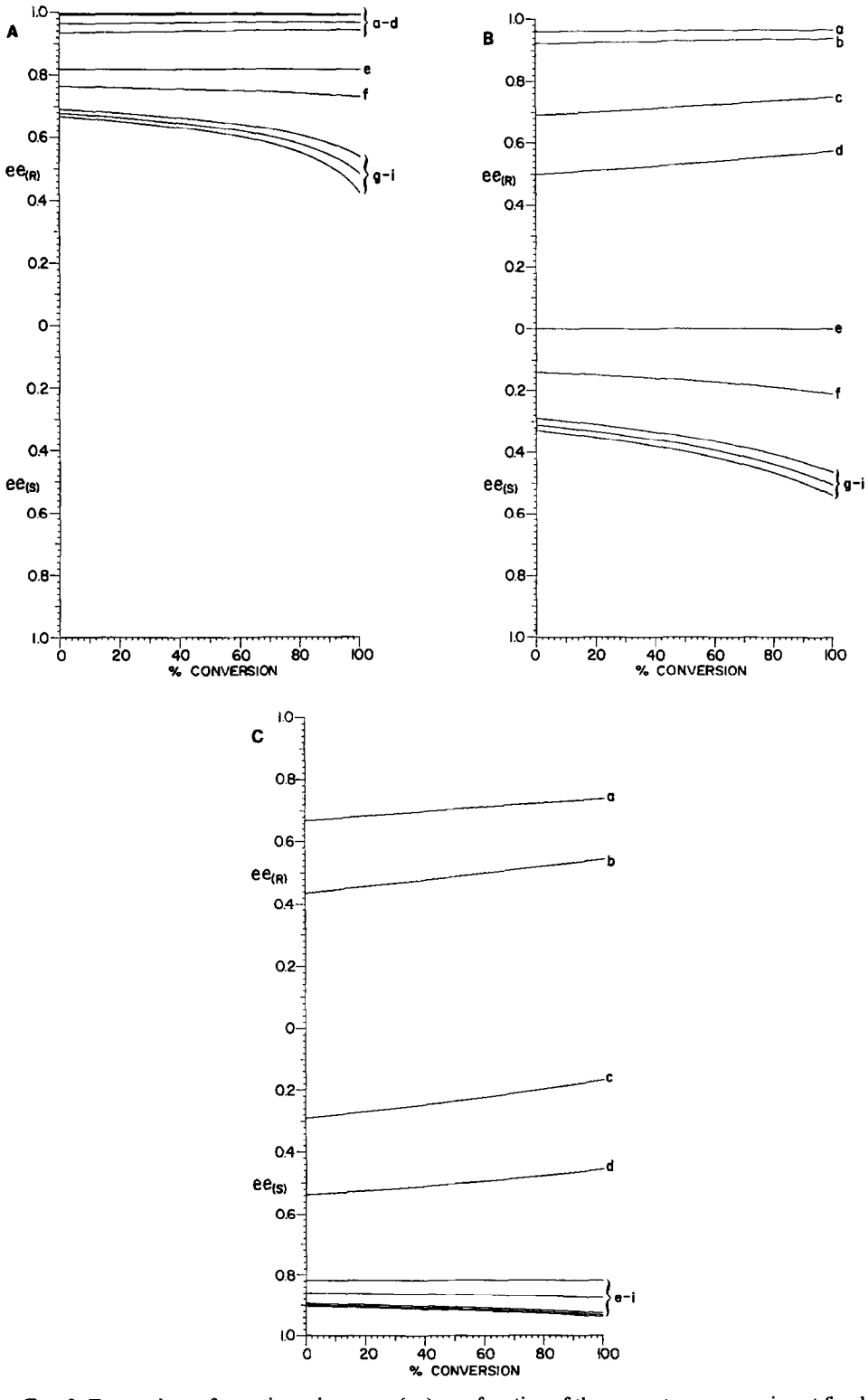


FIG. 2. Expressions of enantiomeric excess (*ee*) as a function of the percentage conversion at fixed values of A_0, K_R, and y while varying K_S. The curves were computer generated from Eqs. [11]. $K_R = A_0 = 1 \times 10^{-3}\,M$. (A) $y = 10$; (B) $y = 1$; (C) $y = 0.1$. The values of K_S were (a) $1 \times 10^{-1}\,M$; (b) $5 \times 10^{-2}\,M$; (c) $1 \times 10^{-2}\,M$; (d) $5 \times 10^{-3}\,M$; (e) $1 \times 10^{-3}\,M$; (f) $5 \times 10^{-4}\,M$; (g) $1 \times 10^{-4}\,M$; (h) $5 \times 10^{-5}\,M$; (i) $1 \times 10^{-5}\,M$.

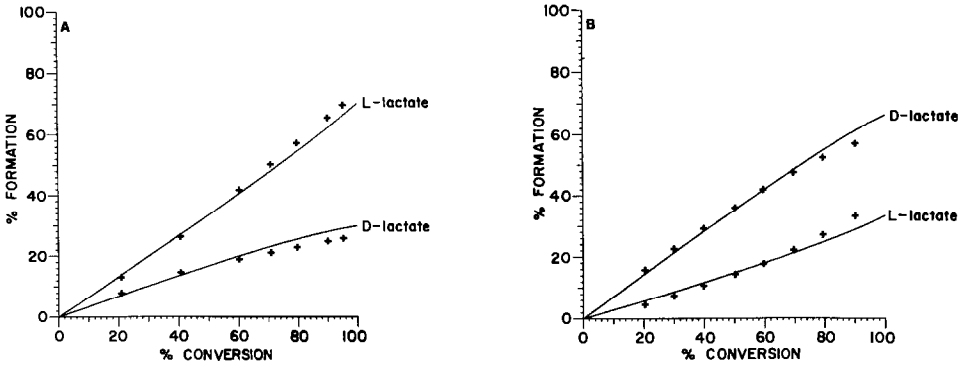


FIG. 3. Plots of percentage formation of L- and D-lactate as a function of the percentage conversion under competitive conditions. #, Experimentally determined values. The curves were computer generated from Eqs. [4] and [6] using the predetermined apparent kinetic constants of L-(*S*)- and D-(*R*)-lactic dehydrogenase. $K_R = 5.94 \times 10^{-4}\ M$, $K_S = 1.6 \times 10^{-4}\ M$. (A) $y = V_R/V_S = 0.635$; (B) $y = 3.18$.

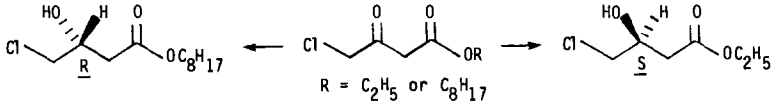
able to some error (≤5%) in the *V* determinations. Also, a small amount of product inhibition, as well as slight changes in kinetic constants with continuously decreasing NADH concentration, may have occurred. Further verification of Eq. [3] was derived from the results of another, similar competitive experiment using more of the D-LDH. Again, the experimental values were found to follow the same trend as the predicted curves (Fig. 3B).

KINETIC ANALYSIS OF COMPETING OXIDO-REDUCTASES IN INTACT YEAST CELLS

Having verified the experimental validity of our quantitative expressions on a purified enzyme system, it was of interest to apply this analysis to competing oxidoreductases in intact yeast cells. In many instances the reduction of carbonyl compounds by yeast is only partially stereoselective (*9*), which may be interpreted in two ways. The ketonic substrate may be reduced by a single oxidoreductase, which interacts with both faces of the carbonyl group to form two competing *R* and *S* transition states, one of which is more favored than the other. Alternatively, yeast may contain more than one oxidoreductase generating carbinols of opposite configurations at different rates (depending on *V* and *K*). From our kinetic analysis, these two conditions can be readily distinguished from each other if one conducts the reaction at several different substrate concentrations. In the former case, stereoselectivity is independent of changes in substrate concentrations whereas the latter is not.

In a recent publication (*15*), we disclosed that bakers' yeast contains more than one oxidoreductase catalyzing the reduction of γ-chloroacetoacetic esters. Interestingly, it was possible to alter the stereochemical course of the reduction by modifying the size of the ester grouping. For example, ethyl-γ-chloroacetoacetate

was reduced predominantly to the *S* isomer, whereas octyl-γ-chloroacetoacetate was transformed virtually all to the *R* isomer.



In order to follow quantitatively the stereochemical course of reduction in such systems, it is necessary to predict the value of *ee* for any values of *C* and A_0. Furthermore, it would be of interest to determine whether structural changes of substrates may be correlated to relative changes in binding or catalysis of competing enzymes. Consequently, quantitative expressions are needed to relate the variables *ee*, *C*, and A_0 to the apparent kinetic constants (*K* and *V*) of competing enzymes.

A) Special Considerations Affecting Analysis in Intact Yeast Cells

(i) For the above system to be a suitable model for our studies, the formation of the carbinolic products by bakers' yeast must be an irreversible reaction. If the reduction of γ-chloroacetoacetic esters was reversible or if there was the presence of interfering enzymes such as racemases in the yeast, the optical purities of the γ-chloro-β-hydroxybutyrates would be expected to change with time. However, it was found that, within the period of our investigations (24 hr), no significant changes in the optical purities of the γ-chloro-β-hydroxybutyrates (Fig. 4) nor the formation of γ-chloroacetoacetate was observed on their exposure to yeast.

(ii) The kinetic behavior of an enzymatic reaction for a particular substrate concentration depends on the individual values of *K* and *V*. Although several methods are available for the determination of *V* and *K* of mixed enzyme systems in crude cell extracts (*16–18*), these kinetic constants do not take into account additional parameters such as the number, relative concentration, and compartmentalization of competing enzymes; effective coenzyme concentration; or permeability factors. Hence, the *K* and *V* values thus obtained are not applicable to intact cell systems.

Because of the difficulty of obtaining accurate initial velocity measurements using intact cells at sufficiently low substrate concentrations, especially for water-immiscible substrates, an alternate approach is needed to estimate the apparent kinetic constants (*V* and *K*) of such systems.

Since the relationship of the parameters, *ee*, A_0, and *C* to the apparent kinetic constants *V* and *K* is given by Eq. [11], the three unknowns, *y*, K_R, and *x* may be solved by numerical analyses (see Appendix). Thus, by experimentally determining the values of *ee* and *C* at several A_0 concentrations, the apparent K_R, K_S, and $y = V_R/V_S$ may be calculated from Eq. [11].

(iii) Changes in *K* and *V* values have been traditionally used to evaluate structure–activity relationships of alternate substrates for enzymes. Such evaluations are based on the assumption that, under rapid equilibrium conditions, *K* is a measure of binding of substrate to the enzyme, and *V* is a measure of the rate of

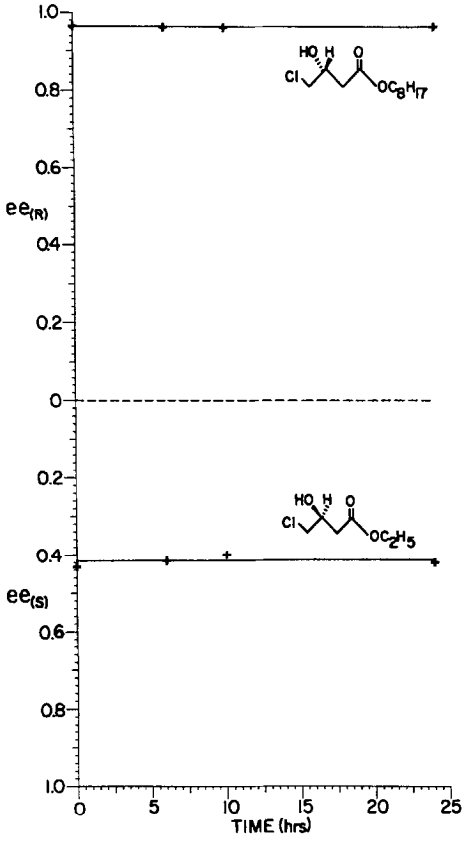


FIG. 4. Enantiomeric excess (*ee*) of γ-chloro-β-hydroxybutyrates versus time. Optically active ethyl-γ-chloro-β-hydroxybutyrate (ee_S = 0.43) and octyl-γ-chloro-β-hydroxybutyrate (ee_R = 0.97) were incubated separately with bakers' yeast, and the optical purities of the γ-chloro-β-hydroxybutyrates were analyzed at the indicated time intervals.

catalysis. However, because K is a complex function that has little diagnostic value, V and V/K data are used to distinguish between binding and catalytic changes (*19*).

In intact cells containing competing enzymes, Eq. [11] does not allow one to calculate the values of apparent V_R and V_S. Hence, structural changes of substrates cannot be related to V and V/K data of each competing enzyme. On the other hand, Eq. [11] does allow one to obtain two equally useful dimensionless constants $z = (V_R/K_R)/(V_S/K_S)$ and $y = V_R/V_S$, which dictate the stereoselectivity of the reaction. The value of z is the relative ratio of the apparent first-order rate constants for the combination of substrate with enzyme during catalysis when $A_0 \ll K_R$ and K_S, and y is a measure of the relative ratio of catalysis by the competing enzymes. Consequently, structural changes of substrates can be likewise correlated to changes in z and y, as a measure of the relative changes in binding and catalysis of competing systems.

B) Structure–Enantioselectivity Relationships in Reductions of β-Keto Esters

The enantioselective reductions of various γ-chloroacetoacetic esters at several different concentrations by bakers' yeast were examined, and the results are summarized in Fig. 5. A common trend was observed for the butyl, pentyl, hexyl, and octyl esters, in that as the substrate concentrations were raised, a decrease in the amount of *R* enantiomer was observed. On the other hand, the ethyl ester behaved differently, in that when the ethyl-γ-chloroacetoacetate concentration was raised, an increase in the formation of the *R* enantiomer was noted instead.

To gain an insight into the effect of the different ester substituents on the enantioselectivity of yeast reductions, these data were introduced into Eq. [11] for the calculation of the constants *z* and *y* (Table 1). It is apparent that as the size of the ester grouping was enlarged from C_4 to C_8, the value of *y* increased from 0.67 (C_4) to 8.2 (C_8). The results tend to suggest that the *R* enzyme system favors the

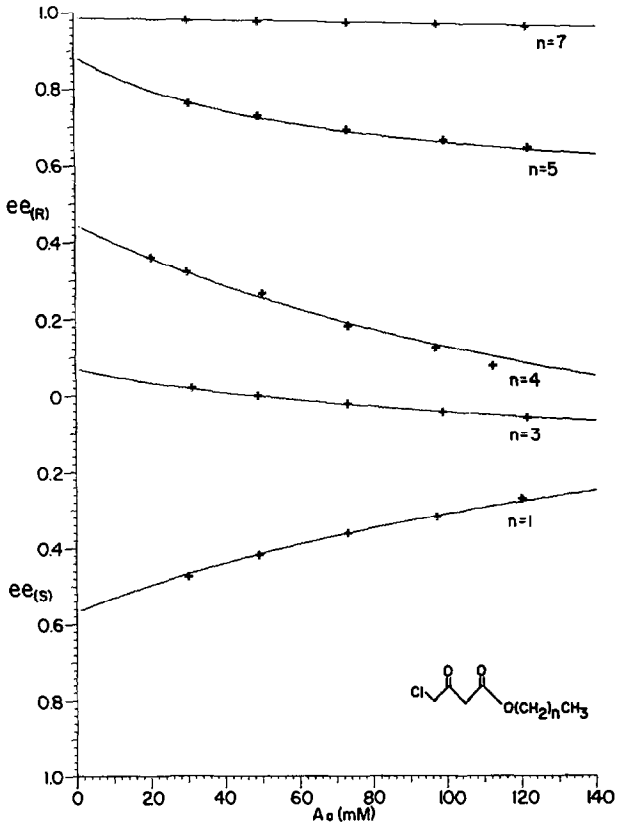
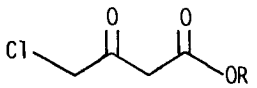


FIG. 5. Enantioselective reduction of various γ-chloroacetoacetic esters at different initial concentrations (A_0) by bakers' yeast. #, Experimental value. Experimental data for each ester were used to calculate the apparent kinetic constants of yeast cells by numerical analysis (Table 1). The curves depict the relationship between *ee* and A_0, calculated from the apparent kinetic constants K_R, K_S and *y* using a value of $C = 0.5$.

TABLE 1

APPARENT KINETIC CONSTANTS OF RESTING YEAST CELLS TOWARD VARIOUS ESTERS OF γ-CHLOROACETOACETATE

	K_R (M)	K_S (M)	$y = \frac{V_R}{V_S}$	$z = \frac{V_R/K_R}{V_S/K_S}$
R =				
$-C_2H_5$	0.341 ± 0.021	0.056 ± 0.001	1.671 ± 0.036	0.276 ± 0.001
$-C_4H_9$	0.076 ± 0.001	0.131 ± 0.002	0.669 ± 0.003	1.151 ± 0.001
$-C_5H_{11}$	0.054 ± 0.003	0.606 ± 0.099	0.315 ± 0.035	2.654 ± 0.033
$-C_6H_{13}$	0.012 ± 0.001	0.063 ± 0.003	3.027 ± 0.029	16.95 ± 0.67
$-C_8H_{17}$	0.048 ± 0.001	0.925 ± 0.095	8.186 ± 0.847	142.5 ± 1.0

rate of catalysis (i.e., enhancing the rate of release of products from the enzyme) of substrates bearing the large ester grouping, as compared to the *S* enzyme system. More striking, however, is the marked enhancement of z from a value of 1.2 (C_4) to 143 (C_8). If there are no differences in the access of various substrates to the *R* and *S* enzyme systems, these results would suggest that the *R* enzyme system has a higher binding affinity for substrates possessing larger ester groupings relative to the *S* enzyme system, which has a preference for smaller ester groupings. However, it is also possible to envisage that one of the enzyme systems (*R* enzyme) may be compartmentalized in the mitochondrial matrix, whereas the other one (*S* enzyme) resides in the mitochondrial membrane or the cytosolic fraction. As a consequence, it may be more facile for substrates with the bulky ester grouping (C_8) to penetrate the mitochondrial membrane than those possessing small ester groupings (C_2), leading to the preferential formation of the *R* enantiomer. Conversely, the small ester (C_2) may not reach the *R* enzyme system as effectively, thus yielding predominantly the *S* enantiomer. As one might expect, such differences in permeability are reflected in the values of z, because V/K in intact cell systems is also a measure of the effectiveness of substrate to reach the active site of the enzyme.

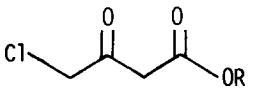
Having obtained the values of V_R/V_S, V_R/K_R, and V_S/K_S, it is now possible to calculate the *ee* as $A_0 \rightarrow 0$ and as $A_0 \rightarrow \infty$ (saturation) using Eqs. [16] and [15]. These values (Table 2) provide a useful overview of the upper and lower limits of optical purity that are theoretically achievable for each substrate.

CONCLUSION

Although the kinetics of two enzymes acting simultaneously on a single substrate yielding the same product have been adequately treated (*16–18*), the problem of two enzymes competing for the same substrate affording two different products has not been addressed until now. Equation [11], which we have derived and verified experimentally, should have general applicability in defining other

TABLE 2

THEORETICAL ENANTIOMERIC EXCESS (*ee*) OF γ-CHLORO-β-HYDROXYBUTYRATES AT ZERO AND INFINITE INITIAL SUBSTRATE CONCENTRATIONS (A_0)

	*ee* (%)	
	$A_0 \rightarrow 0$	$A_0 \rightarrow \infty$
R =		
$-C_2H_5$	56.7 (*S*)	25.1 (*R*)
$-C_4H_9$	7.0 (*R*)	19.8 (*S*)
$-C_5H_{11}$	45.3 (*R*)	52.1 (*S*)
$-C_6H_{13}$	88.9 (*R*)	50.3 (*R*)
$-C_8H_{17}$	98.6 (*R*)	78.2 (*R*)

competing enzyme systems, such as those engaged in branched pathways of amino acid biosynthesis, steroid hydroxylations, and inactivation of aminoglycoside antibiotics in bacteria. Moreover, it allows one to follow the kinetics of enantioselective reduction of ketones by two or more competing systems in intact cells. When the kinetic parameters K_R, K_S, and y are determined, the optical purity of the product may be predicted for any value of C and A_0. Furthermore, the kinetic parameters of competing enzyme systems may be correlated to subtle modifications of substrate structures. This information provides useful clues for the rational design of substrates toward enhancing the enantioselectivity of yeast reduction.

EXPERIMENTAL PROCEDURES

Materials. Fresh Red Star bakers' yeast was used throughout this work. (+)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid [(+)MTPA] was purchased from Aldrich. MN-Kieselgel 60 (0.05–0.2 mm; 70–270 mesh, Brinkmann) was used for column chromatography. All solvents were glass distilled prior to use. γ-Chloroacetoacetic esters were synthesized according to the method of Hamel (*20*). Silica gel plates (60F-254, 0.25 mm, E. Merck No. 5761) were used for thin-layer chromatography (TLC). L-Lactic dehydrogenase (EC 1.1.1.27, rabbit muscle), D-lactic dehydrogenase (EC 1.1.1.28, *Lactobacillus leichmannii*), β-nicotinamide adenine dinucleotide (β-NAD, Grade III), β-NADH (reduced form Grade III), and lithium L-lactate were products of Sigma. Lithium D-lactate, sodium pyruvate, and hydrazine sulfate were purchased from Aldrich.

^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer in deuterochloroform solution with tetramethylsilane as the internal standard. Gas chromatography (GC) was performed with a Varian Aerograph Model 2400 instrument. A Model M-6000 pump equipped with an U6K injector and a Model 77 double-beam uv (254 nm) detector (Waters Associates) were used for high-pressure liquid chro-

matography (HPLC). The diastereomeric MTPA esters were analyzed using an Alltech μPorasil (10 μm) column (4.6 mm i.d. × 50 cm).

Kinetic studies. The kinetic constants K and apparent V of L- and D-lactic dehydrogenase for pyruvate were calculated by computer-fitting of the initial velocity data to the Michaelis–Menten equation using a nonlinear regression program. (*21*). Initial velocity measurements were made using a reaction mixture consisting of 20 μl of 82.5 mM NADH, 5 μl of varying concentrations of sodium pyruvate, and 470 μl of 50 mM phosphate buffer, pH 7.0, in a 2-mm-light-path cell. After the system has been equilibrated to 21°C, the reaction was initiated by the addition of 5 μl of 0.05 μg of L-lactic dehydrogenase or 0.057 μg of D-lactic dehydrogenase. The disappearance of NADH was monitored by following the change in absorbance at 365 nm on a Gilford 240 recording spectrophotometer. The extinction coefficient of NADH at 365 nm was calculated to be 3388 M^{-1} cm^{-1}.

Competition studies. The system contained 3.3 mM NADH, 2 mM sodium pyruvate, 3 μg of L-lactic dehydrogenase, and either 3.4 or 17 μg of D-lactic dehydrogenase in a total volume of 30 ml of 50 mM phosphate buffer, pH 7.0. The reaction mixture was incubated at 21°C with stirring, and the extent of conversion was monitored by measuring the decrease in absorbance at 365 nm using a 2-mm-light-path cell. At the indicated intervals, a 2-ml aliquot of the reaction mixture was withdrawn and mixed with 0.2 ml of 0.6% perchloric acid to terminate the reaction. Aliquots (1 ml) of this mixture were used for the assay of L- and D-lactic acids separately.

For the quantitative enzymatic determination of L-lactic acid, the reaction system contained 1 ml of 1 M glycine–0.4 M hydrazine buffer, pH 9.5 (*13*), and the absorbance at 340 nm was measured using a 1-cm-light-path cuvette. To this was added 1 ml of the reaction sample, 20 μl of 0.5 M NAD^+, and 20 μl of L-lactic dehydrogenase (1 μg/μl). The reaction was allowed to proceed to completion and the final absorbance at 340 nm was determined. Because NAD^+ and hydrazine formed a complex which absorbed in the near-uv region, a control containing the same contents, except 1 ml of reaction sample was replaced by 1 ml of phosphate buffer, was used. For the estimation of D-lactic acid, the same procedure was used except 0.5 M glycine–0.4 M hydrazine buffer, pH 9.0 (*14*), and 57 μg of D-lactic dehydrogenase were used instead.

Yeast incubations. To each 125-ml Erlenmeyer flask was added 15 g of Red Star bakers' yeast and 35 ml of tap water. After incubating the flasks for 15 min on a gyrotary water bath shaker (215 rpm) at 21.5 ± 0.5°C, each of the γ-chloroacetoacetic esters was added to the flasks at five different concentrations (0.03, 0.05, 0.074, 0.097, and 0.122 M). Incubation was continued under the same conditions on the gyrotary shaker and the progress of the reaction was carefully monitored by GC analyses. When the conversion reached into the range 40–70%, the reaction was quenched by exhaustive extraction of the mixture with ethyl acetate. The combined ethyl acetate layers were dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of a solvent mixture of Skelly B–ethyl acetate (10 : 1 for hexyl and octyl esters; 8 : 1 for ethyl, butyl, and pentyl esters) and chromatographed over a silica gel column (14 g, 1.3 × 37 cm for extracts

derived from 0.03, 0.05, and 0.074 *M*; and 35 g, 1.8 × 32 cm for extracts from 0.097 and 0.122 *M* incubation mixtures). The columns were eluted with a solvent mixture consisting of Skelly B–ethyl acetate (10 : 1 for hexyl and octyl esters; 8 : 1 for ethyl, butyl, and pentyl esters) and 16-ml fractions were collected. The fractions were analyzed by TLC (Skelly B–ethyl acetate, 5 : 1) and the γ-chloro-β-hydroxybutanoate spots were revealed by spraying the TLC plates with $Ce(SO_4)_2$ in 2 *N* H_2SO_4; ethyl-γ-chloro-β-hydroxybutanoate was detected using $K_2Cr_2O_7$ in H_2SO_4 as the spray reagent.

GC analyses. A 3-ft OV-1 column was used throughout this work. The flow rate of nitrogen gas was 30 ml/min; the temperatures of injector and detector were 165 and 195°C, respectively. The temperature of the column, and retention times for the substrates and products were, respectively, ethyl ester, 80°C, 4 min 20 sec, 5 min 30 sec; butyl ester, 90°C, 2 min 29 sec, 3 min 9 sec; pentyl ester, 95°C, 3 min 45 sec, 4 min 42 sec; hexyl ester, 110°C, 3 min 17 sec, 4 min 2 sec; and octyl ester, 125°C, 5 min 18 sec, 6 min 22 sec.

Preparation and analyses of MTPA esters. To 20 mg of purified γ-chloro-β-hydroxybutanoate was added 20 mg of (+)MTPA chloride (*22*). After stirring for 1 min, 1 drop of anhydrous pyridine was added and the mixture was stirred for 16 hr. The contents were diluted with water and extracted with 10 ml of ether. The ethereal layer was washed with 1% HCl and then with water until neutral. After drying over Na_2SO_4, the ether layer was evaporated to dryness. The resulting (+)MTPA esters (30 mg) were analyzed by HPLC using an Alltech μPorasil (10 μm) column (4.6 mm i.d. × 50 cm). The column was eluted with a solvent mixture consisting of hexane–ether in the following ratios: ethyl ester, 20 : 1; butyl, pentyl, and hexyl esters, 22.5 : 1; and octyl ester, 25 : 1. The peak areas of diastereomers were carefully measured by the cut-and-weigh method.

APPENDIX

A. Two Enzymes Possessing Absolute Stereoselectivity

The rates of formation of two products, *R* ($\bar{v}_R$) and *S* ($\bar{v}_S$), catalyzed by two different enzymes, may be followed by Michaelis–Menten kinetics

$$\bar{v}_R = \frac{dR}{dt} = \frac{V_R A}{K_R + A} = \frac{V_R(A_0 - R - S)}{K_R + (A_0 - R - S)} \quad [1]$$

$$\bar{v}_S = \frac{dS}{dt} = \frac{V_S A}{K_S + A} = \frac{V_S(A_0 - R - S)}{K_S + (A_0 - R - S)}. \quad [2]$$

Assuming that the formation of *R* is a function of *S*.

$$\frac{dR}{dS} = \frac{V_R}{V_S} \times \frac{K_S + (A_0 - R - S)}{K_R + (A_0 - R - S)}, \quad [3]$$

let

$$\frac{V_R}{V_S} = y \qquad [4]$$

and

$$\theta = A_0 - R - S. \qquad [5]$$

Substituting Eqs. [4] and [5] into Eq. [3] affords

$$\frac{dR}{dS} = y \times \frac{K_S + \theta}{K_R + \theta}. \qquad [6]$$

From Eqs. [5] and [6], one can obtain

$$\frac{d\theta}{dS} = -\frac{dR}{dS} - 1 = -\frac{(K_R + yK_S) + (1 + y)\theta}{K_R + \theta}. \qquad [7]$$

Thus,

$$\frac{\theta + K_R}{(1 + y)\theta + (yK_S + K_R)}\, d\theta = -dS. \qquad [8]$$

The boundary conditions are

$$t = 0, \qquad \theta_0 = A_0, \qquad S_0 = 0$$

$$t = t, \qquad \theta = A_0 - R - S, \qquad S = S.$$

Integration of Eq. [8] affords

$$R + S + \frac{y(K_R - K_S)}{(1 + y)} \ln \frac{(1 + y)A_0 + (yK_S + K_R)}{(1 + y)(A_0 - R - S) + (yK_S + K_R)} = (1 + y)S. \qquad [9]$$

B. Two Enzymes Lacking Absolute Stereoselectivity

The rate of formation of the enantiomeric products may be written as

$$\frac{dR}{dt} = \frac{\alpha}{\alpha + 1}\, \bar{v}_R + \frac{1}{\beta + 1}\, \bar{v}_S \qquad [1]$$

and

$$\frac{dS}{dt} = \frac{1}{\alpha + 1}\, \bar{v}_R + \frac{\beta}{\beta + 1}\, \bar{v}_S. \qquad [2]$$

The reaction rate of each enzyme is described by

$$\bar{v}_R = \frac{V_R(A_0 - R - S)}{K_R + (A_0 - R - S)} \qquad [3]$$

and

$$\bar{v}_S = \frac{V_S(A_0 - R - S)}{K_S + (A_0 - R - S)} \qquad [4]$$

Substituting Eqs. [1] and [2] into [3] and [4] yields

$$\frac{dR}{dt} = \frac{\alpha(\beta + 1)\dfrac{V_R(A_0 - R - S)}{K_R + (A_0 - R - S)} + (\alpha + 1)\dfrac{V_S(A_0 - R - S)}{K_S + (A_0 - R - S)}}{(\alpha + 1)(\beta + 1)} \quad [5]$$

and

$$\frac{dS}{dt} = \frac{(\beta + 1)\dfrac{V_R(A_0 - R - S)}{K_R + (A_0 - R - S)} + (\alpha + 1)\beta\dfrac{V_S(A_0 - R - S)}{K_S + (A_0 - R - S)}}{(\alpha + 1)(\beta + 1)} \quad [6]$$

Similarly,

$$\frac{dR}{dS} = \frac{\begin{array}{c}\alpha(\beta + 1)[V_R(A_0 - R - S)](K_S + A_0 - R - S) \\ + (\alpha + 1)[V_S(A_0 - R - S)](K_R + A_0 - R - S)\end{array}}{\begin{array}{c}(\beta + 1)[V_R(A_0 - R - S)](K_S + A_0 - R - S) \\ + (\alpha + 1)\beta[V_S(A_0 - R - S)](K_R + A_0 - R - S)\end{array}}. \quad [7]$$

Let

$$\theta = A_0 - R - S. \quad [8]$$

Substituting Eq. [8] into Eq. [7] gives

$$\begin{aligned}\frac{dR}{dS} &= \frac{\alpha(\beta + 1)(V_R\theta)(K_S + \theta) + (\alpha + 1)(V_S\theta)(K_R + \theta)}{(\beta + 1)(V_R\theta)(K_S + \theta) + (\alpha + 1)\beta(V_S\theta)(K_R + \theta)} \\ &= \frac{[\alpha(\beta + 1)V_R + (\alpha + 1)V_S]\theta^2 + [\alpha(\beta + 1)V_RK_S + (\alpha + 1)V_SK_R]\theta}{[(\beta + 1)V_R + (\alpha + 1)\beta V_S]\theta^2 + [(\beta + 1)V_RK_S + (\alpha + 1)\beta V_SK_R]\theta} \\ &= \frac{e\theta^2 + f\theta}{g\theta^2 + h\theta} \quad [9]\end{aligned}$$

where

$$\begin{aligned}e &= \alpha(\beta + 1)V_R + (\alpha + 1)V_S \\ f &= \alpha(\beta + 1)V_RK_S + (\alpha + 1)V_SK_R \\ g &= (\beta + 1)V_R + (\alpha + 1)\beta V_S \\ h &= (\beta + 1)V_RK_S + (\alpha + 1)\beta V_SK_R.\end{aligned}$$

From Eq. [8], one can obtain

$$\frac{d\theta}{dS} = -\frac{dR}{dS} - 1 = -\frac{(e + g)\theta^2 + (f + h)\theta}{g\theta^2 + h\theta} = -\frac{i\theta^2 + j\theta}{g\theta^2 + h\theta} \quad [10]$$

where

$$\begin{aligned}i &= e + g = (\alpha + 1)(\beta + 1)(V_R + V_S) \\ j &= f + h = (\alpha + 1)(\beta + 1)(V_RK_S + V_SK_R).\end{aligned}$$

Thus,

$$-\frac{g\theta^2 + h\theta}{i\theta^2 + j\theta}\,d\theta = -dS. \quad [11]$$

The boundary conditions are

$$t = 0, \qquad \theta_0 = A_0, \qquad S_0 = 0$$

$$t = t, \qquad \theta = A_0 - R - S, \qquad S = S.$$

Integration of Eq. [11] affords

$$\frac{g}{i}(R + S) + \left(\frac{hi - gj}{i^2}\right) \ln \frac{iA_0 + j}{i(A_0 - R - S) + j} = S. \qquad [12]$$

Equation [12] can be rearranged to give

$$ee = 1 - 2\frac{A_0C + (u - v) \ln \dfrac{A_0 + v}{A_0(1 - C) + v}}{wA_0C} \qquad [13]$$

where

$$u = \frac{h}{g} \qquad v = \frac{j}{i} \qquad w = \frac{i}{g}.$$

C. Numerical Analysis

To solve the three unknowns, x, K_R and y, in Eq. [11], three sets of experimental data (A_{0i}, C_i, ee_i; $i = 1, 2, 3$) are inserted into the equation to yield three simultaneous equations

$$\frac{1 - ee_i}{2}(1 + y)A_{0i}C_i - A_{0i}C_i = (K_R - x) \ln \frac{A_{0i} + x}{A_{0i}(1 - C_i) + x}, \quad i = 1, 2, 3.$$

Solving the three simultaneous equations results in a differentiable form

$$f(x) = a \ln \left(\frac{A_{01} + x}{A_{01}(1 - C_1) + x}\right) - (b + c) \ln \left(\frac{A_{02} + x}{A_{02}(1 - C_2) + x}\right) + d \ln \left(\frac{A_{03} + x}{A_{03}(1 - C_3) + x}\right) = 0$$

where

$$a = \frac{1 - ee_2}{2} A_{02}C_2$$

$$b = \frac{1 - ee_1}{2} A_{01}C_1$$

$$c = \frac{(1 - ee_3)(ee_2 - ee_1)A_{01}C_1}{2(ee_3 - ee_2)}$$

$$d = \frac{(1 - ee_2)(ee_2 - ee_1)A_{01}A_{02}C_1C_2}{2(ee_3 - ee_2)A_{03}C_3}.$$

The value of x can be approximated using Newton's iterative method, i.e.,

$$x_{n+1} = x_n - \frac{f(x_n)}{f'(x_n)}$$

where

$$f'(x_n) = \frac{(b + c)A_{02}C_2}{(A_{02} + x_n)[A_{02}(1 - C_2) + x_n]} - \frac{aA_{01}C_1}{(A_{01} + x_n)[A_{01}(1 - C_1) + x_n]} - \frac{dA_{03}C_3}{(A_{03} + x_n)[A_{03}(1 - C_3) + x_n]}.$$

The value of K_R and y can then be determined by

$$K_R = x + \frac{(ee_2 - ee_1)A_{01}A_{02}C_1C_2}{(1 - ee_2)A_{02}C_2 \ln \frac{A_{01} + x}{A_{01}(1 - C_1) + x} - (1 - ee_1)A_{01}C_1 \ln \frac{A_{02} + x}{A_{02}(1 - C_2) + x}}$$

and

$$y = \frac{2\frac{(K_R - x) \ln \frac{A_{01} + x}{A_{01}(1 - C_1) + x}}{A_{01}C} + (1 + ee_1)}{(1 - ee_1)}.$$

A computer program of the numerical analysis is available upon request.

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