Classic and Multivariate Modeling Treatment of the Kinetics and Mechanism of Isomerization of 5-Cholesten-3-one Catalyzed by Sodium Ethoxide

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ABSTRACT: A classic kinetic methodology including the treatment of the steady-state method and a multivariate modeling kinetic treatment were applied to the kinetics and mechanism of the isomerization reaction of 5-cholesten-3-one to 4-cholesten-3-one catalyzed by EtO^- in ethanol absolute. The rate constants, thermodynamic parameters of activation, equilibrium constant, and the isomerization enthalpy were determined. The multivariate modeling kinetic treatment allows us to calculate the concentrations of the species, in which the 3,5-dienolate is included as a highly reactive intermediate species and was able to discriminate among several applicable mechanisms validating the one comprising two reversible steps. © 2005 Wiley Periodicals, Inc. Int J Chem Kinet 38: 38–47, 2006

INTRODUCTION

5-Cholesten-3-one (5-CHOL) is converted into its corresponding isomer 4-cholesten-3-one (4-CHOL) (Fig. 1) in absolute ethanol medium via a reversible process that can be catalyzed by acids, bases, and enzymes. Malhotra and Ringhold [1,2] studied the testosterone enolization reaction using isotope exchange techniques and observed the presence of 3,5-dienol thermodynamically more stable than the 2,4-dienol species (Fig. 1). Likewise, in the isomerization of unsaturated β-γ steroids, they detected the presence of the 3,5 species, excluding the possibility of direct protonation to the carbons involved.

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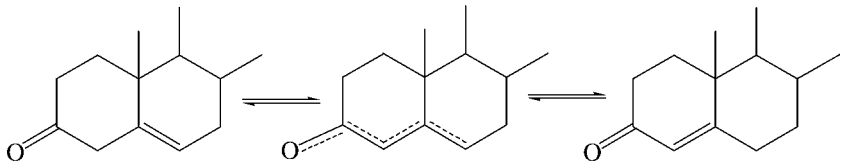

Figure 1 Chart describing the kinetic pathway proposed for the isomerization reaction of cholest-5-en-3-one (5-CHOL) to cholest-4-en-3-one (4-CHOL) considering the enolate ion (3,5-E^-)-like intermediate species.

In the present work, we performed an exhaustive study of the kinetics and mechanism of isomerization of 5-CHOL, catalyzed by ethoxide ions (EtO^-), in absolute ethanol medium applying a classic kinetic methodology and a multivariate modeling kinetic methodology. We structured the work according to the aims proposed: (a) determination of the experimental kinetic and thermodynamic parameters of activation by application of the classic kinetic treatment methodology; (b) confirmation of the validity of the mechanism considered appropriate after application of the steady-state method; (c) determination of the isomerization equilibrium constant from the kinetic data; (d) determination of the isomerization enthalpy; (e) application of a multivariate modeling kinetic methodology [3,4] to calculate the concentrations of the species (including those highly reactive intermediate species) and validation of the mechanism that best fitted the experimental kinetic data.

THEORETICAL ASPECTS

Classic Kinetic Treatment

The previous experimental evidence [1,2] permits us to propose the following reaction mechanism for the isomerization reaction of 5-cholesten-3-one (5-CHOL) to 4-cholesten-3-one (4-CHOL) catalyzed by EtO^- in ethanol absolute, comprising two reversible steps in which the 3,5-dienolate (3,5-E^-) is included as a highly reactive intermediate species.

$$\text{5-CHOL} + \text{EtO}^- \underset{k_{21}}{\overset{k_{12}}{\Leftrightarrow}} \text{3,5-E}^- + \text{EtOH} \underset{k_{32}}{\overset{k_{23}}{\Leftrightarrow}} \text{4-CHOL} + \text{EtO}^-$$

Scheme 1

The global reaction of isomerization catalyzed by the ions EtO^-, considering exclusively reactants, products, and catalyst, could be written as

$$\text{5-CHOL} \underset{k_{31}}{\overset{k_{13}}{\Leftrightarrow}} \text{4-CHOL}$$

Scheme 2

If the experimental kinetic data of absorbance, the kinetic treatment drives to the kinetic equation

$$\ln\frac{A_\infty - A_0}{A_\infty - A_t} = (k_{13} + k_{31})[\text{EtO}^-]\,t \tag{1}$$

and

$$k_{\text{exp}} = (k_{13} + k_{31})[\text{EtO}^-] = k_{\text{EtO}^-}[\text{EtO}^-] \tag{2}$$

where A_0, A_∞, and A_t, represent the initial and final absorbance values and the value at a given time; k_{13} and k_{31} are the kinetic constants of the direct and inverse process, respectively; k_{exp} is the second-order experimental constant, and k_{EtO^-} is the catalytic constant of the EtO^- ions.

When the steady-state criterion is applied (d[3,5-E^-]/d$t = 0$), one obtains the following expression for the concentration of 3,5-E^- species:

$$[\text{3,5-E}^-] = \frac{k_{12}[\text{5-CHOL}] + k_{32}[\text{4-CHOL}]}{k_{21} + k_{23}}[\text{EtO}^-] \tag{3}$$

Substituting (3) in the rate equation of formation of isomer [4-CHOL], one has

$$\frac{\text{d[4-CHOL]}}{\text{d}t} = \frac{k_{12}k_{23}[\text{CHOL}]_0 - (k_{12}k_{23} + k_{21}k_{32})[\text{4-CHOL}]}{k_{21} + k_{23}} \times [\text{EtO}^-] \tag{4}$$

where $[\text{CHOL}]_0 = [\text{5-CHOL}]_0 + [\text{4-CHOL}]_0$, since the starting 5-CHOL had approximately 3% of 4-CHOL. Integrating Eq. (4), one has

$$\frac{k_{12}k_{23}[\text{CHOL}]_0 - (k_{12}k_{23} + k_{21}k_{32})[\text{4-CHOL}]_0}{k_{12}k_{23}[\text{CHOL}]_0 - (k_{12}k_{23} + k_{21}k_{32})[\text{4-CHOL}]_t} = \frac{k_{12}k_{23} + k_{21}k_{32}}{k_{21} + k_{23}}[\text{EtO}^-]\,t \tag{5}$$

which, expressed as a function of absorbance (experimental variable being monitored), affords

$$\ln \frac{A_\infty - A_0}{A_\infty - A_t} = \frac{k_{12}k_{23} + k_{21}k_{32}}{k_{21} + k_{23}}[\mathrm{EtO}^-]\, t \qquad (6)$$

where

$$k_{\mathrm{exp}} = \frac{k_{12}k_{23} + k_{21}k_{32}}{k_{21} + k_{23}}[\mathrm{EtO}^-] = k_{\mathrm{EtO}^-}[\mathrm{EtO}^-] \qquad (7)$$

The relations (6) and (7) coincide with that of the experimental kinetic equation ((1) and (2)), showing that the mechanism proposed in Scheme 1 is valid if the experimental kinetic data fit to Eq. (1) satisfactorily.

Expressing Eq. (4) under initial conditions ($t \to 0$), one obtains

$$\left.\frac{\mathrm{d}[\text{4-CHOL}]}{\mathrm{d}t}\right|_{t\to 0} = \frac{k_{12}k_{23}[\mathrm{CHOL}]_0}{k_{21} + k_{23}}[\mathrm{EtO}^-] = k_0[\mathrm{CHOL}]_0[\mathrm{EtO}^-] \qquad (8)$$

and

$$k_0 = \frac{k_{12}k_{23}}{k_{21} + k_{23}} \qquad (9)$$

Considering Eqs. (7) and (9), k_0 and k_{EtO^-} can be related by the expression

$$k_{\mathrm{EtO}^-} = \frac{k_{12}k_{23} + k_{21}k_{32}}{k_{21} + k_{23}} = k_0 + \frac{k_{21}k_{32}}{k_{21} + k_{23}} \qquad (10)$$

Considering the expression of the equilibrium constant ($K_{\mathrm{eq}} = k_{12}k_{23}/k_{21}k_{32}$) after the multiplication of both factors by $k_{12}k_{23}$, one has

$$k_{\mathrm{EtO}^-} = k_0 + \frac{k_0}{K_{\mathrm{eq}}} \qquad (11)$$

that is

$$K_{\mathrm{eq}} = \frac{k_0}{k_{\mathrm{EtO}^-} - k_0} \qquad (12)$$

which allows one to obtain the equilibrium constant from exclusively kinetic parameters without having to perform a quantitative analysis of the equilibrium established between both isomers.

To determine k_0, it is possible to use

a. a *graphic method*, plotting the initial slopes ($t \to 0$) on the kinetic curves;
b. an *analytical method*, making use of the first derivative of the function A_t with respect to time in initial conditions ($t \to 0$).

The analytical expression of A_t obtained from Eqs. (1) and (2) is

$$A_t = A_\infty - (A_\infty - A_0)\, \mathrm{e}^{-k_{\mathrm{EtO}^-}[\mathrm{EtO}^-]t} \qquad (13)$$

after differentiation of the Eq. (13), transforming in function of the [4-CHOL], expressing the equation under initial conditions ($t \to 0$) and taking in account Eq. (8), one obtains

$$k_0 = \frac{(A_\infty - A_0)\, k_{\mathrm{EtO}^-}}{\varepsilon_{\text{4-CHOL}}[\mathrm{CHOL}]_0} \qquad (14)$$

where $\varepsilon_{\text{4-CHOL}}$ is the molar absorption coefficient of isomer 4-CHOL obtained at 242.0 nm from the Beer–Lambert law.

Multivariate Modeling Kinetic Treatment

Currently, many computational methodologies of multivariate modeling kinetic treatment kinetic and reaction mechanisms are available; they are based on different algorithms and mathematical strategies that evaluate the experimental kinetic data generated by different experimental techniques (partial least squares [5], neural networks (NN) [6,7], and multivariate curve resolution and similar MCR-ALS [8,9]). We have developed a technique based on the multivariate regression analysis whose origin was the KILET, a series of papers under the generic title "Computation in Kinetics" [10–13]. Later, we have designed at our laboratory* [14] the AGDC algorithm and later implemented in the corresponding multipurpose computational program, ANALKIN (AGDC) [4]. It has been successfully applied in many other different scientific fields [14,16] with different tasks, i.e. chemical kinetics (multivariate modeling); multicomponent mixtures resolution, both static (SMM) and dynamic (DMM); thermodynamic equilibrium constants (macro- and micro-), etc.

We consider the kinetic treatment on two types of mixture [3,4]: static multicomponent mixtures (SMM), whose quantitative composition we wish to know is invariable and constant (classical mixtures) and dynamic multicomponent mixtures (DMM), whose quantitative composition under study varies over time, that is, all the components play the role of reagents and/or products of a chemical reaction induced by the addition of the reagents and whose kinetic processes are evaluated with a view to determine the concentrations of the all species, permitting the validation of the proposed

Web site: http://web.usal.es/jlgh93.

model of the reaction (multivariate modeling kinetic). We performed the treatment by kinetic methodologies and later numerical computational analysis using a multivariate nonlinear regression technique, based in all cases on the use of the AGDC (controlled descent general algorithm) mathematical unconstrained optimization algorithm. It is a robust algorithm that can be used for such purposes, both in a regression technique and in any other different ones as long as the aim is to search for the minimum of a function in the *hyperspace* defined by the parameters to be optimized. This package of calculation programs has been assigned the generic name of ANALKIN (AGDC) [4] because it is applied to chemical systems for ANALytic purposes, via the use of KINetic techniques, using the AGDC mathematical unconstrained optimization algorithm. The generic expression of a DMM according to the IUPAC recommendations [19] will be

$$0 = \sum_j \nu_{j,r} \mathrm{B}_j \quad r = [1, N_r] \tag{15}$$

where the stoichiometric coefficients $\nu_{j,r}$ are less than 0 for the chemical species acting as reagents and $\nu_{j,r} > 0$ for those acting as products in the rth reaction considered. The differential rate equation for the reactant $B_{j,i}$ is

$$-\mathrm{d}[B_{j,i}]/\mathrm{d}t = k_r |\nu_{Bj,r}| \Pi_j [B_{j,i}]|\nu_{Bj,r}| \tag{16}$$

that represents a set of N_r differential equations composed of as many equations as species whose concentrations we might wish to know. The mathematical optimization consisting of the minimization of the numerical function of the sum of quadratic deviations (SQD), extended for a N_d pair of data and N_c species, is given by the developed expression

$$\mathrm{SQD} = \sum_{i=1}^{N_d} \left\{ \sum_{j=1}^{N_c} \varepsilon_j [B_{j,i}] - (A_{j,i})_{\mathrm{exp}} \right\}^2 \tag{17}$$

where

$$(A_i)_{\mathrm{cal}} = \sum_{j=1}^{N_c} \varepsilon_j [B_{j,i}] \tag{18}$$

$(A_{j,i})_{\mathrm{exp}}$ represents the experimental values of the monitored total absorbance, $A_{j,i,\lambda}$ represents the absorbance of each of the j species present in the mixture at time I, $[B_{j,i}]$ represents the concentrations of the N_c species, and ε_j is the molar absorption coefficient of each species. The solution of this type of differential equation is sometimes difficult, owing both to the characteristics of the systems and to the values taken by the rate constants, occasionally leading to "stiff" problems. The proposed treatment uses the Gear algorithm to solve the sets of differential equations and affords excellent results even in the case of complex systems with notable "stiff" characteristics. Next, the actual optimization process is to begin; this is carried out by application of the AGDC algorithm. It consists of the minimization of SQD through the development of an iterative process in which the vector of movement is determined and at all times is subject to strict control. A rigorous analysis is made of its elements, being suitably corrected in the event of detecting any errors, thereby ensuring that the minimum will be reached.

ANALKIN (AGDC) can be represented schematically, step by step, thus:

1. Generate the model
   - 1.1 Input data (matrix of $\nu_{j,r}$ number of reactions, species, experimental data, vector of ε_j, etc.
   - 1.2 Input of the initial estimates of the concentrations of the species to optimize ($[B_{j,i}]$), $\boldsymbol{X}^{(m)}$. Let $m = 1$ (m = number of the iteration).
2. Establish the rate differential equation system and its solution (the Gear algorithm) [28] obtaining $[B_{j,i}]_{\mathrm{cal}}$
3. Calculate the absorbance $(A_i)_{\mathrm{cal}}$
4. Determinate of the $\mathrm{SQD}^{(m)}$ function (Eq. (17)).
5. AGDC algorithm
   - 5.1. Compute partial numerical derivatives of $(A_i)_{\mathrm{cal}}$ with respect to the parameters to be determined $\boldsymbol{X}^{(m)}$, by the method of central-differences
   - 5.2. Compute the $\boldsymbol{g}^{(m)}$ and $\boldsymbol{H}^{(m)}$ (gradient vector and Hessian matrix)
   - 5.3. Compute $[\boldsymbol{H}^{(m)}]^{-1}$ by the Gauss elimination method and improvement by successive approximations method.
   - 5.4. Calculate the components of the vector of movement ($\boldsymbol{p}^{(m)} = -(\boldsymbol{H}^{(m)})^{-1}\, \boldsymbol{g}^{(m)}$)
   - 5.5. Control and correction of the vector of movement $\boldsymbol{p}^{(m)}$
     - 5.5.1 Direction of $\boldsymbol{p}^{(m)}$
       - 5.5.1.1 If $\boldsymbol{H}^{(m)}$ is singular, set $\boldsymbol{p}^{(m)} = -\boldsymbol{g}^{(m)}$, and go to 5.5.2
       - 5.5.1.2 If $\boldsymbol{p}^{(m)}\, \boldsymbol{g}^{(m)} < \varepsilon(\varepsilon$ = scalar close to zero), set $\boldsymbol{p}^{(m)} = -\boldsymbol{g}^{(m)}$ and go to 5.5.2.
       - 5.5.1.3 If $\boldsymbol{p}^{(m)}\, \boldsymbol{g}^{(m)} > 0$ set $\boldsymbol{p}^{(m)} = -\boldsymbol{p}^{(m)}$
     - 5.5.2. Length of $\boldsymbol{p}^{(m)}$
       - 5.5.2.1 Compute the scalar ($\alpha^{(m)}$) by the method of Hartley
       - 5.5.2.2 $\boldsymbol{X}^{(m+1)} = \boldsymbol{X}^{(m)} + \alpha^{(m)}\, \boldsymbol{p}^{(m)}$ ($[B_{j,i}]$ optimized concentrations)
       - 5.5.2.3 If the Goldstein–Armijo criterion is satisfied go to 5.6.
       - 5.5.2.4 $\alpha^{(m)} = \alpha^{(m)}/2$ and go to 5.5.2.2

5.6. Calculate $|SQD^{(m)}-SQD^{(m-1)}|$
5.7. If convergence is not attained ($|SQD^{(m)}-SQD^{(m-1)}| > CC$), set $m = (m+1)$ and go to 2.
6. Statistic residual analysis (SAR).
7. New validation of a different model, then go to 1
8. END

ANALKIN (AGDC) is written in C^{++} language using JAVA applications to facilitate the utilization for the user not expert. The computational application comprises a main program and a series of subroutines. At the same time, a previous and simpler version is available (vs 1.0), written in FORTRAN 77 and for its compilation the SVS $\mathbf{C^3}$ (Silicon Valley Systems) compiler was used. The source program is constituted by 10100 lines (approximately), and the executable application has a size of 330 KB and can be executed in DOS with minimum ×486 processors up to Pentium IV and even on computers endowed with multiprocessors.

EXPERIMENTAL

All chemicals were of analytical reagent grade, LiCl (Fluka). EtONa was prepared after controlled reaction of solid Na (Panreac) with ethanol absolute immediately before to perform the experiments: 5-cholesten-3-one and 4-cholesten-3-one (approximately 97%; SIGMA, St. Louis, MO).

The experimental measurements were performed on a SHIMADZU 240 diode array spectrophotometer, whose cell was thermostated by exterior circulation forced into the cell-holder from a cryostatic bath (±0.1°C).

Several series of ten replicated kinetic experiments were performed at eight different temperatures (between 15.0°C and 37.0°C) and at different concentrations of EtONa (3.00×10^{-4} M to 4.80×10^{-3} mol dm^{-3}). All kinetic experiments were carried out directly in the cell of the spectrophotometer. The kinetics starts at the moment when to an initial solution of 5-CHOL (range of concentrations between 6.10×10^{-5} and 6.50×10^{-5} mol dm^{-3}), recently prepared, was added the exact volume of EtONa solution (using a micropipette) recently prepared by reaction of solid Na with ethanol absolute.

RESULTS AND DISCUSSION

The kinetic experiments were evaluated from experimental data of absorbance/time based on the large difference in absorption between the two isomers in the UV spectrum at 242.0 nm. This technique allows one to collect a number of very precise experimental data which enables both the classic and multivariate modeling kinetic treatments. We checked the correct fulfillment of the Beer–Lambert law by both isomers, obtaining the molar absorption coefficients ($\varepsilon_{4\text{-CHOL}} = 1.64 \times 10^4$ $mol^{-1}\,dm^3\,cm^{-1}$; $\varepsilon_{5\text{-CHOL}} = 2.36 \times 10^2$ mol^{-1} dm^3 cm^{-1}) as shown in Figs. 3 and 4.

Taking into account the presence in the isomerization of ionic species (EtO^- and Na^+), it was first necessary to evaluate the influence of ionic strength on the reaction rate. We carried out several kinetic experiments previously adding different concentrations of inert electrolyte within a concentration range of 10^{-4} and 0.3 mol dm^{-3}. Since the solvent was ethanol absolute, LiCl was employed owing to its high solubility in this medium. No significant variation was observed in the k_{exp} values in such a broad range of ionic strength such that it was not necessary to add inert electrolyte or control the ionic strength in the kinetic experiments.

Several examples of kinetic experiments performed at constant temperature (26.2°C) where the absorbance is monitored with time, are represented in Fig. 2. The obtained kinetic data satisfied Eq. (1), significantly fitting the straight lines (Fig. 5) from whose slope we obtained k_{exp} confirming the assumed first order with respect to both isomers and EtO^-. The value of the catalytic constants k_{EtO^-} has been determined according Eq. (2) from the data in Table I, plotting the values of k_{exp} against the corresponding values of [EtO^-] (Fig. 6) and obtaining the catalytic constants k_{EtO^-} from the slope of the fitted straight line. The values of k_{EtO^-} are obtained from the experiments performed at the eight working temperatures (Table II) that permit us to determine the values of the thermodynamic parameters of activation, according to the Arrhenius equation (ln k_{EtO^-} versus $1/T$). These are $A = 5.44 \times$

Table I Values of k_{exp} Obtained from Several Kinetic Experiments from Eqs. (1) and (2) Carried Out at Different EtO^- Concentrations

[EtO^-] ($\times 10^3$ mol dm^{-3})	k_{exp} (min^{-1})
3.20	0.535
2.82	0.491
2.60	0.428
2.08	0.333
1.72	0.290
1.46	0.249
1.41	0.243
1.12	0.194
0.771	0.126

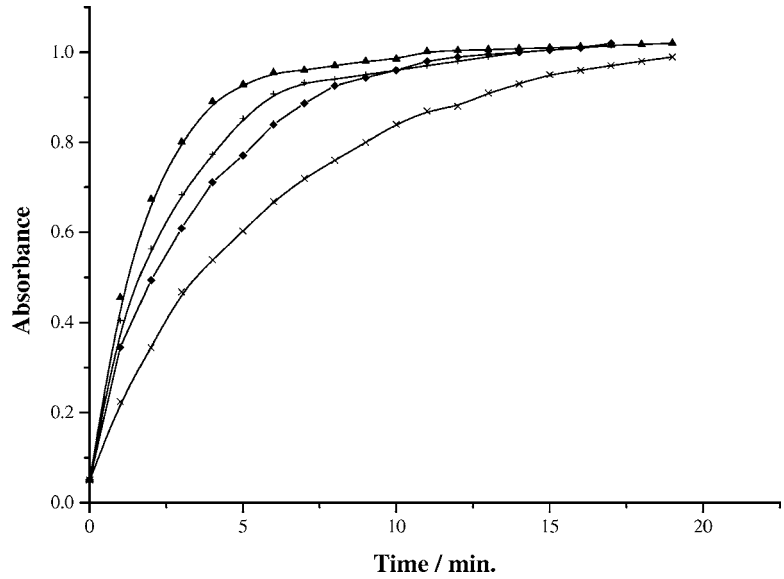

Figure 2 Plot of values of absorbances versus time corresponding to several kinetic experiments performed at 26.2°C, $[CHOL]_0 = 6.50 \times 10^{-5}$ mol dm^{-3} and EtO^- concentrations in the range 0.77×10^{-3} mol dm^{-3} – 3.20×10^{-3} mol dm^{-3}, as shown in Table I.

10^{11} min^{-1}, $E_{EtO^-} = 54.1$ kJ mol^{-1}; $\Delta H^{\neq} = 52.4$ kJ mol^{-1}; $\Delta S^{\neq} = -63.2$ kJ mol^{-1}, and $\Delta G^{\neq} = 71.3$ kJ mol^{-1}.

Determination of k_0 was accomplished using both the graphic method of initial tangent plots and the analytical method, obtaining concordant results. From these results, we obtained the values of the isomerization equilibrium constants (K_{eq}) at different temperatures (Table II). It should be noted that the values of K_{eq} were obtained with exclusively kinetic parameters (Eq. (12)) without using any data from the analysis of the equilibrium concentrations.

With the K_{eq} values obtained at different temperatures at constant pressure, we determined the isomerization enthalpy (ΔH_{isom}) from the slope of the line corresponding to the van't Hoff equation (17) when ln K_{eq} is plotted against $1/T$ (Fig. 7). The value obtained was -14.0 kJ mol^{-1}, which magnitude is acceptable

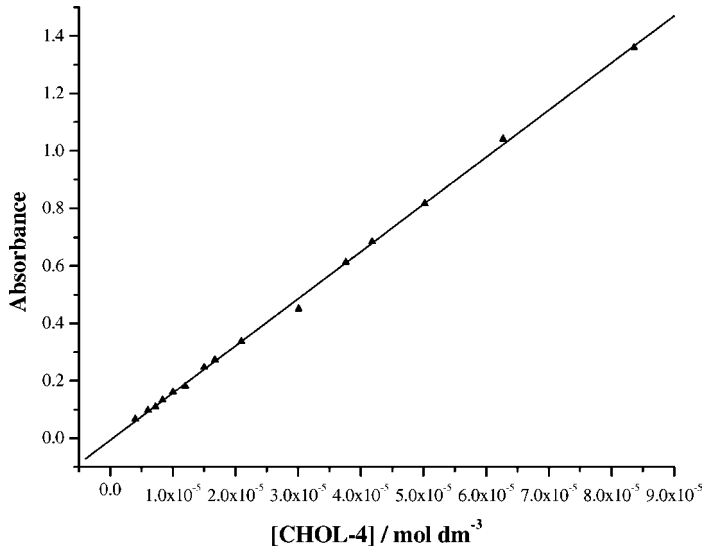

Figure 3 Lambert–Beer law for the isomer 4-CHOL plotting absorbance (0.0667–1.3595) vs. concentrations (4.01×10^{-5} – 8.36×10^{-5} mol dm^{-3}). From the slope, one obtains the value of the molar absorption coefficient of 1.64×10^5 mol^{-1} dm^{-3} cm^{-1}.

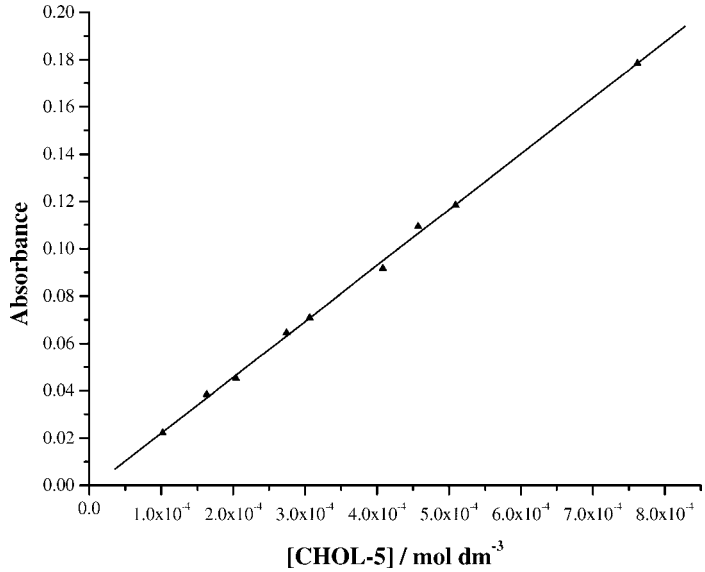

Figure 4 Lambert–Beer law for the isomer 5-CHOL plotting absorbance (0.0221–0.17849) vs. concentrations ($1.02 \times 10^{-4} - 7.62 \times 10^{-4}$ mol dm^{-3}). From the slope, one obtains the value of the molar absorption coefficient of 2.36×10^4 mol^{-1} dm^{-3} cm^{-1}.

since it is in agreement with the values reported by C. K. Ingold [18], experimentally obtained by comparison of hydrogenation heats of several compounds having conjugated and nonconjugated double bonds. The negative value shows that the enthalpy of 4-CHOL isomer is lower than that of 5-CHOL in agreement with the known greater stability of the conjugated isomer 4-CHOL.

Having checked that the experimental kinetic data fitted equation (1) satisfactorily, and bearing in mind that this equation is identical to Eq. (6), obtained from the mathematical treatment of the reaction mechanism, we can confirm the validity of the mechanism proposed in Scheme 1. It thus may be concluded that the dienolate species is highly reactive (hypothesis of steady state), and hence its concentration will always be very small

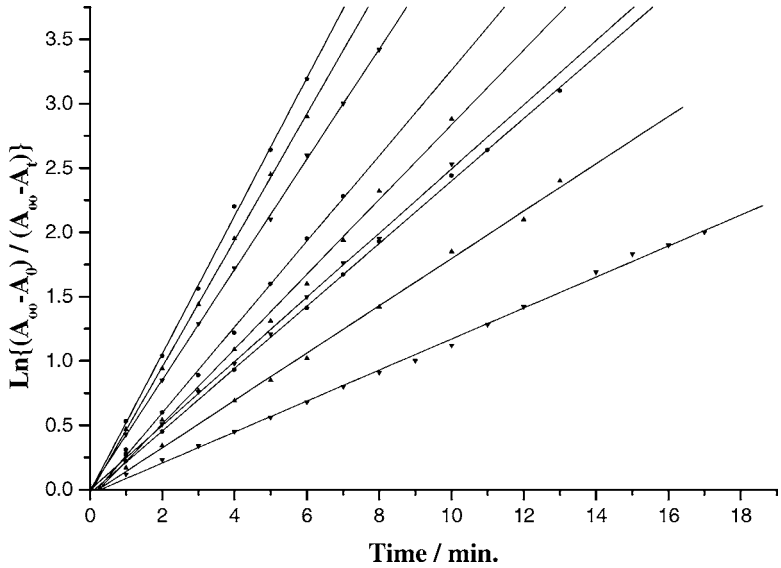

Figure 5 Plot for the determination of k_{exp} from the slopes of the straight lines corresponding to nine kinetic experiments performed at 26.2°C at different EtO^- concentrations in the range $0.77 \times 10^{-3} - 3.20 \times 10^{-3}$ mol dm^{-3}, as shown in Table I.

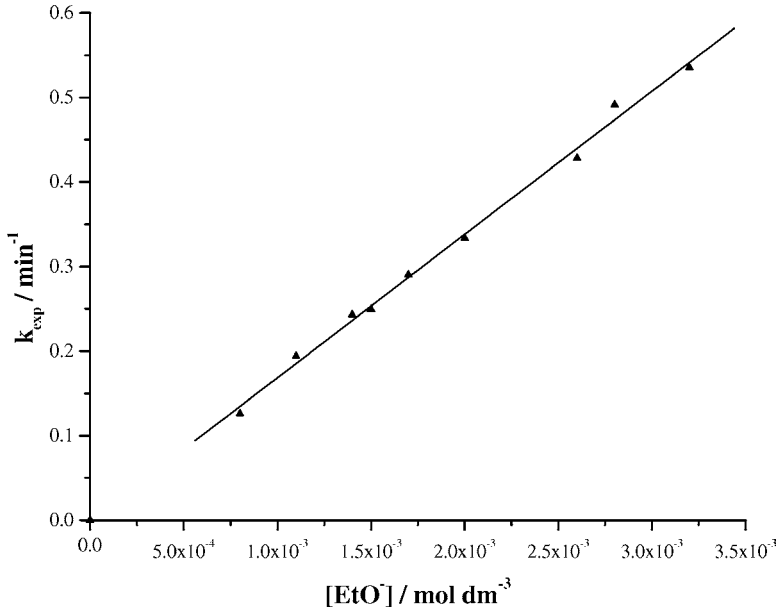

Figure 6 Plot of values of k_{exp} obtained from Fig. 5 vs. [EtO$^-$] according to Eq. (2) determining k_{EtO-} from the slope of the straight line.

Table II Values of k_0, k_{EtO^-}, and K_{eq} Obtained at Several Temperatures. Values of K_{eq} and T Are Plotted (Fig. 7) for the Determination of ΔH_{isom} According to the van't Hoff Equation

T (K)	k_{EtO^-} ($\times 10^{-2}$ mol)	k_o ($\times 10^{-2}$ mol)	K_{eq}
288.0	0.744	0.664	8.25
291.0	1.05	0.932	7.88
293.0	1.15	1.02	7.58
295.5	1.42	1.25	7.20
299.2	1.71	1.48	6.60
303.0	2.32	2.01	6.25
306.0	3.04	2.60	5.87
311.0	3.88	3.27	5.39

and negligible with respect to the concentration of the two isomers, although it is not possible to quantify the value of that concentration.

We have applied to the isomerization reaction the multivariate modeling kinetic treatment since it provides an valuable kinetic information that is complementary with that obtained after the application of the classic kinetic method. The multivariate modeling kinetic treatment was carried out applying ANALKIN (AGDC)† [3,4] that provides the values of the concentrations of the three species (5-CHOL, 4-CHOL, and 3,5-E$^-$) and simultaneously allows the validation of the postulated reaction mechanism.

That is very important because of 3,5-dienolate species is unstable in solution, and it is impossible to experiment with it and know its physical chemical properties and characteristics (i.e., the value of molar absorption coefficient). We performed a broad range of experiments, and those corresponding to 26.2°C from Figs. 5 and 6 are shown in Table III. Experiments 1–4 corresponded to the extreme values (greater and lower) of the initial estimates ([5-CHOL]$_{EST}$, [4-CHOL]$_{EST}$, and [3,5-E]$^-_{EST}$), determining the corresponding optimized equilibrium concentrations ([5-CHOL]$_{EQ}$, [4-CHOL]$_{EQ}$, and [3,5-E]$^-_{EQ}$). In fact, the calculated values are acceptable since the sum is equal to that of the initial concentration [CHOL]$_0 = 6.50 \times 10^{-5}$ mol dm^{-3}; they have satisfactory statistical parameters from residuals analysis, standard deviation (SD = 1.0×10^{-2}), variance etc. and acceptable values of K_{eq} at 26.2°C (Table II). They are coincident in both experiments, that is, they are independent of the values of the initial estimates. This treatment allows us to calculate the concentration of 3,5-dienolate and, as postulated in the classic treatment, the role of that species as an active species was confirmed being much lower than the two isomers. Experiment 5 corresponded to the same starting conditions as in experiment 1, except that the initial estimate for [3,5-E$^-$]$_{INIT}$ was zero. This value is modified progressively until the correct equilibrium value corresponding to those of experiment 1

†ANALKIN (AGDC) because it is applied to chemical systems for ANALytic purposes (i.e. determination of concentrations), via the use of KINetic techniques, using the AGDC mathematical unconstrained optimization algorithm.

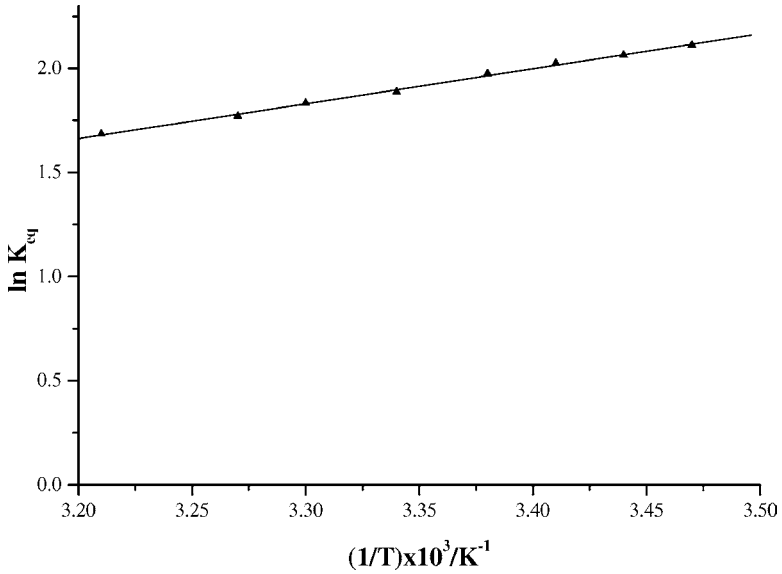

Figure 7 Plot of ln K_{eq} versus $1/T$ (K^{-1}) from the data in Table II. From the slope, one obtains ΔH_{isom} according to the van't Hoff equation.

is obtained again. This points to the necessary involvement of the 3,5-dienolate anion, eventhough its concentration may be lower than that of the most abundant isomer in the equilibrium. In experiments 6 and 7, we kept respectively the same initial estimates for both isomers as were in the experiments 1 and 4, but we did not assign any value to [3,5-E^-]$_{INIT}$, that is, we considered that the dienolate specie does not participate in the reaction mechanism (according to Scheme 2). One obtains a very important conclusion: the results obtained in experiments 6 and 7 are unacceptable, since the equilibrium concentrations were very different from the ones obtained in experiments 1–5, and it was even necessary to stop the process on detecting signs of divergence (negative sign). Obviously, the values of the statistical parameters of residuals analysis were unacceptable (SD = 2.0 and 3.0), and the value of K_{eq} is negative. This again shows the necessary presence of the 3,5-dienolate anion in the reaction mechanism that permits to discard the mechanism of Scheme 2.

To confirm the validity and exclusivity of the reaction mechanism proposed in Scheme 1 (double reversible process and four rate constants), it was necessary to check the validity of all those in which the three species are involved with all the possibilities of transformation among them. These are (a) lineal mechanisms and (b) cyclic mechanisms. In both cases, it is necessary to consider the complete set of possibilities of transformation between the three species combining reversible and irreversible processes and both senses of transformation in those irreversible. That is, (1) both

Table III Experiments 1–5 to the Reaction Mechanism Comprising Three Species (5-CHOL, 4-CHOL, and 3,5-E^-). Experiments 6 and 7 Correspond to the Reaction Mechanism of Two Species (5-CHOL and 4-CHOL)

Experiment	[5-CHOL]$_{EST}$ ($\times 10^7$ mol dm^{-3})	[4-CHOL]$_{EST}$ ($\times 10^7$ mol dm^{-3})	[3,5-E^-]$_{EST}$ ($\times 10^7$ mol dm^{-3})	[5-CHOL]$_{EQ}$ ($\times 10^6$ mol dm^{-3})	[4-CHOL]$_{EQ}$ ($\times 10^5$ mol dm^{-3})	[3,5-E^-]$_{EQ}$ ($\times 10^7$ mol dm^{-3})	SD ($\times 10^2$)	K_{eq}
1	10.0	100.0	1.0	8.549	5.633	1.138	1.0	6.60
2	100.0	100.0	100.0	8.544	5.634	1.137	2.0	6.59
3	10.0	10.0	10.0	8.546	5.635	1.139	2.0	6.59
4	1.0	1.0	1.0	8.545	5.633	1.137	1.0	6.61
5	10.0	100.0	0.0	8.556	5.634	1.138	2.0	6.59
6	10.0	100.0		2.113	7.427		20.0	35.3
7	1.0	1.0		−4.773	39.42		300.0	−82.6

processes are irreversible with two rate constants; (2) the first process is irreversible and the second one reversible, with three rate constants; and (3) the first process is reversible and the second one irreversible, with three rate constants. Though the number of cases is very high, this does not represent a great disadvantage to apply ANALKIN (AGDC) since this one prepared to automatically generate the model (see steps 1 and 7 of the program) and later to evaluate its validity. The treatment of all these cases led to unacceptable results (in fact, in some cases divergence occurred) for both the parameters optimized and for the parameters and errors provided by statistical analysis of the residuals (SD). These results allow us to discard the validity of these possibilities (linear and cyclic mechanisms) and definitively confirm that the one proposed in Scheme 1 is the only one that fits the experimental kinetic data.

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