BBA 66466

STUDIES ON $\Delta^{5\rightarrow4}$-3-OXOSTEROID ISOMERASES

III. EFFECT OF SOLVENT ON THE ENZYMATIC PROTON TRANSFER REACTION

HADASSA WEINTRAUB[a], ETIENNE-EMILE BAULIEU[a] AND ANNETTE ALFSEN[b]

[a]*Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroides de l'Institut National de la Santé et de la Recherche Médicale, Département de Chimie Biologique, Hôpital de Bicêtre, 94-Bicêtre, and* [b]*Laboratoire des Etats Liés Moléculaires, Equipe de Recherches du Centre National de la Recherche Scientifique, Département de Chimie Biologique, Faculté de Médecine-75, Paris (France)*

(Received September 17th, 1971)

SUMMARY

The kinetics of the isomerization of different substrates and the binding of inhibitors to *Pseudomonas testosteroni* $\Delta^{5\rightarrow4}$-3-oxosteroid isomerase have been determined by the calculation of the parameters, K_m, K_i and k_{cat} (the catalytic constant) in mixtures of water and several organic solvents (*i.e.* methanol, ethanol, propanol and butanol isomers, and dioxane) at low concentrations. The partition coefficient of steroids between isooctane and an aqueous phase containing increasing concentrations of organic solvent has been determined. In order to correlate the free energy change of partition to the free energy change of binding of the same steroid to the enzymatic protein, thermodynamic studies on the different steps of the reaction have been performed with increasing concentrations of alcohol.

With the straight chain aliphatic alcohols, the main effect is a decrease in affinity of substrates and inhibitors for the enzyme, and a decrease of k_{cat} with increasing concentrations of the alcohol. The inhibition is competitive only with dioxane, where k_{cat} is unchanged. A linear relationship between the logarithm of the partition coefficient of a given steroid and the logarithm of $1/K_m$ and $1/K_i$ has been found.

With branched alcohols, no change of K_m is observed and k_{cat} increases with chain branching.

The relation between the kinetic parameters and the dielectric constant (ε) of the reaction medium has been studied.

The changes in entropy and enthalpy for the activation step with increasing concentrations of organic solvent (methanol and ethanol) suggest the participation of liquid water structure in the enzymatic reaction.

INTRODUCTION

Studies on protein–steroid interaction have been performed to gain insight into

the highly specific mechanism of steroid hormone activity. In the plasma as well as in the target organ cells, steroid hormones are bound to specific proteins by non-covalent interactions[1,2]. Furthermore it has been observed that such interactions of bovine serum albumin with testosterone induce small but definite conformation changes in the protein molecule[3]. However, albumin is not directly involved in hormone action and binds steroids and compounds of all sorts and therefore cannot be taken as a relevant model for specific interaction.

The $\Delta^{5\to4}$-3-oxosteroid isomerase inducible in *Pseudomonas testosteroni*[4] can be obtained in pure and crystalline form. It does not need any known coenzyme. The reaction is an intramolecular proton transfer from the C-4β to the C-6β position of the steroid substrate[5]. From enzymatic kinetics, precise evaluation of the specific interaction can be obtained by varying conditions such as concentration of reactants, temperature, medium, *etc.*

A general equation for describing the enzymatic process of isomerization has been used:

$$E_i + S_i \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} E_iS_i \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} E_iP_i \underset{k'_3}{\overset{k_3}{\rightleftharpoons}} P_i + E_i \tag{1}$$

where E_i is the initial amount of enzyme in a solvent medium (i) which can be varied by changing the amount and/or the nature of the organic solvent added to the aqueous medium.

S_i is a substrate in the same condition of solvent medium, and P_i is the resulting product of the reaction. E_iP_i is the complex before the desorption step.

According to the usual simplification the overall velocity of formation of P_i is:

$$v_i = k_3(E_iS_i) \tag{2}$$

k_3 is the rate of transformation of E_iS_i into $P_i + E_i$ through $E_i{}^*P_i{}^*$ (activated complex). It has been demonstrated[5] that k_3 is the rate limiting step $k_3 \ll k'_1$.

On the other hand the formation of E_iS_i is described by the equilibrium:

$$(E_iS_i) = \frac{k_1(S_i)\cdot(E_i)}{k_1'} \tag{3}$$

The Michaelis constant K_m is equal to

$$K_m = \frac{k'_1 + k_3}{k_1} \tag{4}$$

The affinity of the substrate for the enzyme is approximated by k_1/k'_1 equal to $K_m{}^{-1}$, and k_3 is the catalytic constant k_{cat} (ref. 6,7).

For inhibitors the process is described by:

$$E + I \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EI;\ K_i{}^{-1} \tag{5}$$

the dissociation constant of the enzyme–inhibitor complex is $K_i = k'_2/k'_1$.

The affinity parameters, $1/K_m$ and $1/K_i$, measure the free energies involved in the binding of the substrate or the inhibitor at specific sites. The catalytic constant (k_{cat}) approximated by k_3 gives a picture of the second part of the reaction, expressing the rate of formation of the product at substrate saturation. It reflects the mechanism

of the transformation of the substrate into the product through intermediate reactions which are not experimentally observed.

Steroids are rather rigid hydrocarbon molecules with few polar groups. Such molecules are of low solubility in water. With the isomerase system, in order to be able to work with sufficiently high concentrations of substrate or inhibitor, it is necessary to introduce small amounts of organic solvent (alcohols or dioxane) in the assay medium to solubilize the steroids. Hence studies have been carried out on the effect of different organic solvents on the enzymatic parameters, under the same conditions as they have been studied for their interactions with pure water[8]. In this work are reported the effects of dioxane and different alcohols from C_1 to C_4 at increasing mole fractions from $5.5 \cdot 10^{-3}$ to $9.0 \cdot 10^{-2}$, on enzymatic parameters in the presence of the different substrates and inhibitors.

Data were obtained at different temperatures and thermodynamic constants are calculated for the different steps of the enzymatic process.

EXPERIMENTAL PROCEDURE

The purification of isomerase has been described in previous papers[9,10]. The purity of enzyme is tested by its specific activity (60 000 units/mg) and its ultraviolet spectrum (Fig. 1).

The substrates are: Δ^5-androstene-3,17-dione (5-androstenedione), Δ^5-estrene-3,17-dione (5-estrenedione) and Δ^5-pregnene-3,17-dione (5-pregnenedione). The competitive inhibitors are: estradiol, its isomer 17α-estradiol and estradiol 17-acetate. Steroids (95% pure) are given by Roussel-UCLAF. Crystallographic studies of these steroids in different alcohol media at increasing concentrations do not show any dimerization phenomenon.

Organic solvents are reagents grade products (Merck). Methanol is redistilled before utilization. Ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, *sec.*-butanol, *tert.*-butanol and dioxane are used without any further purifications.

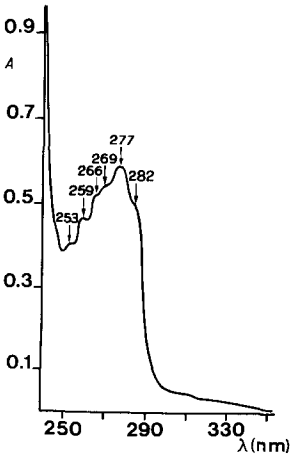


Fig. 1. Ultraviolet spectrum of pure isomerase, performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°.

Kinetic measurements

Principles and methods of the kinetic studies have been described[10]. The data were plotted according to LINEWEAVER AND BURK[11] and DIXON[12]. k_{cat} is expressed in min^{-1} or in sec^{-1}. A Radiometer pH meter Model 25 (precision ± 0.05 pH unit) is used to determine the pH of the solution before and immediately after reaction. The organic solvent increases somewhat the pH of phosphate buffer[13]. The pH changes are small, and since the kinetic parameters K_m, k_{cat} and K_i of 5-androstenedione isomerization are not changed between pH 6 and 9, these changes can be considered as negligible[10].

The effect of different organic solvents studied is not due to irreversible inactivation of isomerase; initial enzyme activity is restored by dilution of the solution incubated with organic solvents at 25° up to 1 h. Isomerization rates are followed within the first 5 min.

The temperature is checked during the kinetic measurements with a digital thermometer (Limited Systems Corp., Dayton-Ohio). The precision is $\pm 0.05°$.

The transition temperature of isomerase has been measured after incubation of the enzyme at different concentrations of organic solvent at various temperatures, between 15 and 55°, for 10 min before measuring its activity at 25° where it is stable; the activity remains constant up to 40°.

Partition coefficients

The principles and the techniques of measurements of partition coefficient (K_p) of steroids between an aqueous phase and isooctane have been described[9]. As in previous studies Δ^4-3-oxosteroid partition coefficients have been measured. The partial isomerization of Δ^5-3-oxosteroids occurring during equilibration of the phases did not allow the use of substrates for such measurements. There is a small difference between the solubility of Δ^4- and Δ^5-steroids but the partition coefficient of both types of compounds varies in the same direction when the polarity of the aqueous phase changes, *i.e.* when different concentrations of alcohols or dioxane are added to the aqueous phase. These measurements of K_p app. give only apparent values for the partition coefficients, since there is in fact a ternary mixture. However, by using isooctane instead of octanol[14], the solubility of the alcohol in the apolar phase can be neglected. Furthermore, it is only the variation of K_p app. relative to the variation of K_m which is used.

Thermodynamic studies

Kinetic measurements for the determination of K_i and k_{cat} have been performed at different temperatures, in order to determine the variation of free energy, enthalpy and entropy in the different steps of the reaction with increasing concentrations of organic solvents. In the formation of enzyme–inhibitor complex (EI), Arrhenius plots, log $1/K_i$ *vs.* $1/T$, at three different concentrations in methanol have been determined, and are linear between 16 and 37°. From the slope of the straight lines equal to $\Delta H°/2.3R$, $\Delta H°$ values can be calculated ($\Delta G° = -2.3\ RT \log K_i$). In the activation step of the reaction, Arrhenius plots at six different ethanol concentrations, have been determined; they are linear between 15 and 37°. From the slope of the straight lines equal to $-(\Delta H^* + RT)/2.3R$, ΔH^* can be calculated.

ΔG^* is calculated from the absolute reaction rate theory[15]:

$$k_{cat} = \frac{k_B \times T}{h} \times K^* \tag{6}$$

K^* is the equilibrium constant for the equilibrium between the activated complex (ES^*) and the unactivated complex (ES), k_B is the Boltzman constant, h is the Planck constant.

The Van't Hoff equation can be applied to this equilibrium:

$$\Delta G^* = -2.3\, RT \log K^* \tag{7}$$

ΔS° and ΔS^* are calculated from equations:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \text{ or } \Delta G^* = \Delta H^* - T\Delta S^* \tag{8}$$

For the compensation phenomenon EXNER[16] uses the logarithm of either rate or equilibrium constants at two different temperatures, to test for a linearity relation between enthalpy and entropy changes, on the basis that these quantities (ΔH and ΔS) are derived from experimental rate constants k, or equilibrium constants K.

From the equation:

$$K = e^{\Delta S/R} \cdot e^{-\Delta H/RT} \tag{9}$$

one can calculate ΔH and ΔS values, from measurements of K at two temperatures T_1 and T_2 with $T_2 > T_1$:

$$\Delta H = \frac{RT_1T_2}{T_2 - T_1} (\ln K_2 - \ln K_1) \tag{10}$$

$$\Delta S = \frac{RT_2}{(T_2 - T_1)} (\ln K_2 - T_1/T_2 \ln K_1) \tag{11}$$

K_1 and K_2 are the kinetic parameters at the two selected temperatures.

When a linear relationship is observed, one can write

$$\Delta H = \alpha + T\Delta S \tag{12}$$

and for two selected temperatures T_1 and T_2

$$\ln K_2 = \frac{(T_2 - T_1)}{(T_1 - T_c)} \frac{\alpha}{T_2 R} + \frac{(T_2 - T_c)}{(T_1 - T_c)} \frac{T_1}{T_2} \ln K_1 \tag{13}$$

can be derived from Eqns. 11 and 12.

According to RITCHIE AND SAGER[17] and LUMRY AND RAJENDER[18] if

$$\Delta H = \alpha + T\Delta S \tag{14}$$

it can easily be shown that ΔG must be a linear function of ΔH

$$\ln k(T) = -\frac{\alpha}{T_c R} + \frac{\Delta H}{R} \left(\frac{1}{T_c} - \frac{1}{T} \right) \tag{15}$$

$$\Delta G(T) = \alpha \frac{T}{T_c} + \Delta H(T) \left(1 - \frac{1}{T_c} \right) \tag{16}$$

The linear relationship between ΔH and ΔS has been called compensation law[19,20].

The proportionality constant T_c obtained from the slopes of both Lumry's and Exner's type of representations is called the compensation temperature.

For all the plots used in the determination of the different parameters in this study, the slopes of the straight lines fitted to the data have been obtained from weighted least squares treatment of the data.

RESULTS

Effect of different organic solvents on the kinetic parameters of the reaction

Effect of increasing concentrations of each alcohol

There is a decrease in the initial rate of isomerization of the three substrates when the alcohol concentration is increased, but there is an exception for methanol with 5-estrenedione and 5-pregnenedione, since the rates pass through a maximum as already described[9] (Figs. 2 and 3).

K_m and k_{cat} values give more information on the isomerization and they have been calculated for each alcohol. The data are reported in Tables I, II, III and IV.

One can conclude that with all the 3 substrates and with any one of the organic solvents used, the parameters vary in the same direction. K_m increases, and k_{cat}

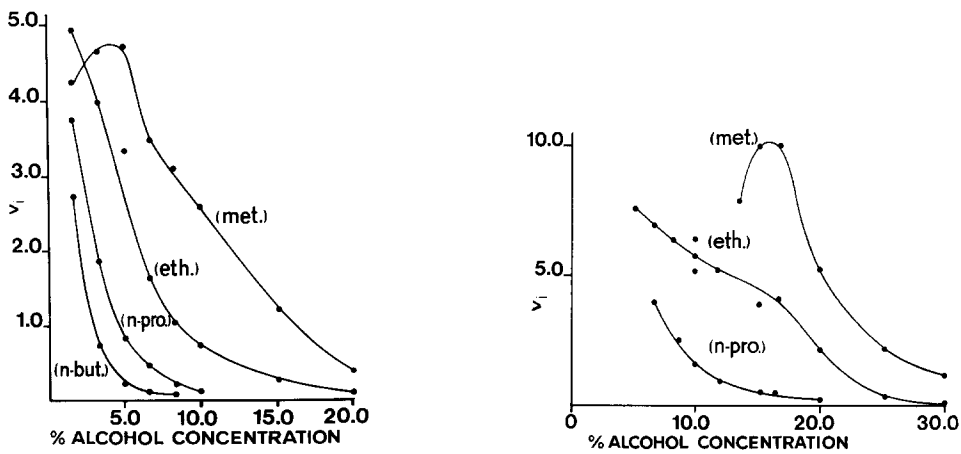


Fig. 2. Effect of methanol (met.), ethanol (eth.), *n*-propanol (*n*-pro.) and *n*-butanol (*n*-but.) on the rate of isomerization of 5-estrenedione. Cells contain 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 and increasing concentrations of different alcohols (v/v) in a final volume of 3 ml. Pure alcohol is added to cells in place of an equivalent volume of buffer. Blank cells contain all reactants but substrate. 5-Estrenedione is added as an alcoholic solution to give the final concentration of $6.1 \cdot 10^{-5}$ M. Reactions are initiated by addition of 10 or 25 μl of enzyme diluted in the same buffer and rate of formation of the 4-estrenedione is followed at 248 nm at 25°.

Fig. 3. Effect of methanol (met.), ethanol (eth.), *n*-propanol (*n*-pro.) on the rate of isomerization of 5-pregnenedione. Cells contain 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0, and different alcohols (v/v) in a final volume of 3 ml. Pure alcohol is added to cells in place of an equivalent volume of buffer. Blank cells contain all reactants but substrate 5-pregnenedione is added as an alcoholic solution to give the final concentration $2.5 \cdot 10^{-5}$ M. Reactions are initiated by addition of 10 or 25 μl of enzyme diluted in the same buffer and rate of formation of the 4-pregnenedione is followed at 248 nm at 25°.

TABLE I

EFFECT OF METHANOL ON THE KINETIC PARAMETERS FOR ISOMERIZATION OF 5-ANDROSTENEDIONE

ε is the dielectric constant at 25° of the methanol–water mixture, expressed in Debye units. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7 at 25°, with 5-androstenedione as substrate.

Methanol		$k_{cat} \times 10^{-6}$ *at 25° (min^{-1})*	$K_m \times 10^4$ *(M)*	K_p *at 25°*
% (v/v)	$\varepsilon_{25°}$			
1.23	78.0	22.0	1.25	10
3.3	77.2	14.7	3.0	7.0
6.6	76.1	9.55	3.70	5.87
10.0	75.0	6.8	7.15	5.10
13.3	74.0	5.95	9.10	4.3
15.0	73.35	4.48	12.5	4.0

TABLE II

EFFECT OF METHANOL ON COMPETITIVE INHIBITION OF ISOMERASE

Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7 at 25° with Δ^5-androstenedione as substrate, and the three inhibitors are estradiol, estradiol 17-acetate, 17α-estradiol.

Methanol (%, v/v)	$K_i \times 10^6$ *(M)*		
	Estradiol	*Estradiol 17-acetate*	*17α-Estradiol*
3.3	5.25	0.10	2.2
5.0	10.0	0.35	4.3
10.0	20.0	1.0	
15.0	50.0		30.0
16.6	65.0	3.75	56.0

TABLE III

EFFECT OF ETHANOL ON THE KINETIC PARAMETERS FOR ISOMERIZATION OF 5-ANDROSTENEDIONE

ε is the dielectric constant at 25° of the ethanol–water mixture expressed in Debye units. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25 and 37° and 5-androstenedione as substrate.

Ethanol (%, v/v)	$\varepsilon_{25°}$	$k_{cat} \times 10^{-4}$ *at 25° (sec^{-1})*	$K_m \times 10^4$ *(M) at 25°*	K_p *at 25°*
1.85	77.7	17.3	1.7	11.3
3.3	77.05	13.2	2.0	7.45
5.0	76.2	13.2	3.6	6.72
6.6	75.5	10.7	5.0	
8.5	74.5	9.85	9.0	
10.0	73.9	6.87	12.5	5.35
15.0	71.35	4.17	20.0	4.20

TABLE IV

EFFECT OF *n*-PROPANOL AND ISOPROPANOL ON THE ENZYMATIC ISOMERIZATION OF 5-PREGNENEDIONE

ε is the dielectric constant at 25° of *n*-propanol and isopropanol–water mixtures, expressed in Debye units. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°, and 5-pregnenedione as substrate.

Solvent (%, *v/v*)	$K_m \times 10^4$ (*M*)		$k_{cat} \times 10^{-6}$ (min^{-1})		$\varepsilon_{25}°$	
	n-Propanol	*Isopropanol*	*n-Propanol*	*Isopropanol*	*n-Propanol*	*Isopropanol*
10	1.0	1	2.46	5.35	73.1	72.75
20	5.0	5	0.265	2.35	67.45	66.8
30	10.0	12.0	0.0294	0.735	61.5	60.5

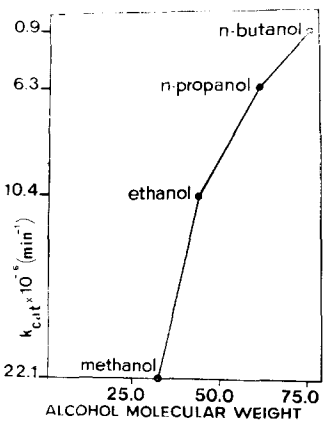


Fig. 4. Plot of k_{cat} of isomerization of 5-androstenedione at 0.304 M of each alcohol *vs.* the molecular weight of the alcohol (methanol is taken as reference). Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°.

TABLE V

VARIATION OF K_m AND k_{cat} AT A CONCENTRATION 0.304 M OF EACH ALCOHOL

E_c: efficiency coefficient. It is equal to the ratio of k_{cat} in methanol (taken as a standard) on the k_{cat} value for each alcohol, reported to one carbon unit. Experiments were performed in 0.03 M potassium phosphate buffer, 0.1 M KCl at 25°, 5-androstenedione is the substrate.

Alcohol	$K_m \times 10^4$ (*M*)	$k_{cat} \times 10^{-6}$ (min^{-1})	K_p	E_c
Methanol	2.56	22.1	10.0	1
Ethanol	1.70	10.4	7.10	
n-Propanol	4.35	6.3	7.30	
Isopropanol	4.20	10.3	7.10	
n-Butanol	3.12	0.92	7.8	6
Isobutanol	2.85	1.26	8.0	
sec.-Butanol	3.12	2.50	7.70	
tert.-Butanol	3.3	7.35	7.9	$\ll 1$

TABLE VI

VARIATION OF K_m AND k_{cat} AT A CONCENTRATION 0.304 M IN THE BUTANOL SERIES

Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl at 25°, 5-pregnenedione is the substrate.

Alcohol	$K_m \times 10^5$ *(M)*	$k_{cat} \times 10^{-6}$ *(min^{-1})*	K_p
n-Butanol	2.0	3.96	90.0
Isobutanol	2.5	5.45	99.0
sec.-Butanol	2.8	6.17	90.0
tert.-Butanol	2.0	9.1	90.0

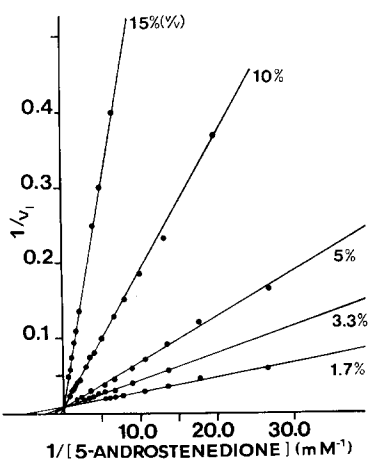
decreases with increasing concentrations of alcohol. Therefore the inhibition by these alcohols looks like a mixed type. The same phenomenon is observed for K_i values for estradiol, 17α-estradiol and estradiol 17-acetate, with methanol, ethanol and dioxane.

Effect of increasing the length of straight chain alcohols

Fig. 4 shows the effect on k_{cat} of methanol C_1, ethanol C_2, *n*-propanol C_3 and *n*-butanol C_4 at the same concentration (0.304 M). There is a decrease of k_{cat}. K_m shows no significant change (Table V).

Effect of branched alcohols

Experiments were performed in the propanol and butanol series. Table V gives the kinetic parameters with 5-androstenedione and all alcohols used at 0.304 M. Table VI gives the results with 5-pregnenedione and Table VII with 5-estrenedione, both with the series of butanol.



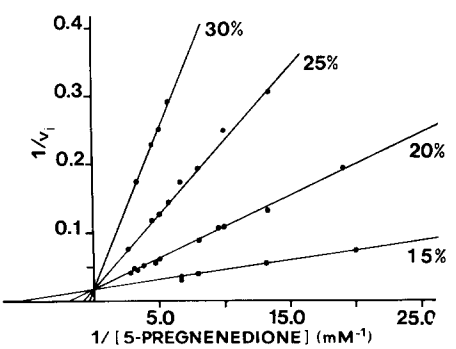


Fig. 5. Lineweaver–Burk plots of the isomerization of 5-androstenedione in dioxane, in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°. Line slopes are obtained from weighted least-squares treatment of the data.

Fig. 6. Lineweaver–Burk plots for isomerization of 5-pregnenedione with increasing concentration of dioxane, in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°. Line slopes are obtained from weighted least-squares treatment of the data.

TABLE VII

VARIATION OF K_m AND k_{cat} AT A CONCENTRATION 0.304 M IN THE BUTANOL SERIES

Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°, 5-estrenedione is the substrate.

Alcohol	$K_m \times 10^4$ *(M)*	$k_{cat} \times 10^{-6}$ *(min^{-1})*
n-Butanol	5.0	1.47
Isobutanol	5.0	1.47
sec.-Butanol	6.0	2.20
tert.-Butanol	6.0	9.2

The data show that K_m remains unchanged while K_p and k_{cat} increase from *n*-butanol to *tert.*-butanol whatever the substrate. For each alcohol if an efficiency coefficient E_c is calculated for enzyme inhibition on the basis of the decrease of k_{cat}, it is observed that with the straight chain alcohols, one carbon in the C_4 *n*-butanol is 6 times more efficient than one carbon for the C_1 methanol. However, with the branched alcohol series, one carbon in the C_4 *tert.*-butanol has approximately the same efficiency as the carbon of the C_1 methanol.

Effect of dioxane

Effect of dioxane on substrate binding. The high solubility in dioxane of 5-androstenedione (150 mg/ml) and 5-pregnenedione (130 mg/ml) has made possible the saturation of isomerase with substrate. Figs. 5 and 6 show the variations of $1/v$ *vs.* $1/[S]$ at different dioxane concentrations with 5-androstenedione and 5-pregnenedione as substrates. K_m is increasing and k_{cat} is constant with increasing concentrations of dioxane. In contrast to the alcohols studied dioxane acts as a competitive inhibitor.

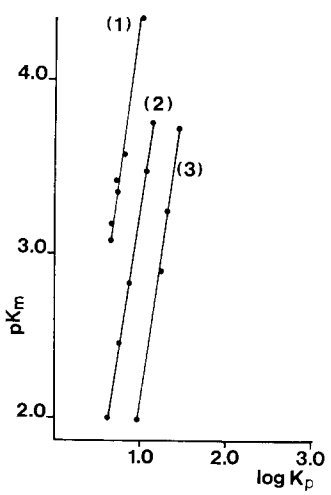


Fig. 7. Plot of pK_m (expressed as log $1/K_m$) of isomerase *vs.* log K_p (K_p is the partition coefficient between isooctane and an aqueous phase containing different concentrations of the various organic solvents). Curve 1, methanol and 4-androstenedione; 2, ethanol and 4-androstenedione; 3, dioxane and 4-pregnenedione. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°.

Effect of dioxane in presence of competitive inhibitors. Table VII shows the variations of K_i, which increases with increasing concentration of dioxane, for the three inhibitors.

Relation between the kinetic parameters and the partition coefficients

Fig. 7 shows a linear relationship between log K_p and log $1/K_m$ in different organic solvents: methanol, ethanol and dioxane. This relationship can be quantitatively described by the following equation:

$$\log 1/K_m = a \log K_p \text{ app.} + b \tag{17}$$

Since ΔG_m° for K_m is equal to:

$$\Delta G^\circ_m = -2.3\, RT \log K_m \tag{18}$$

then

$$-2.3\, RT \log 1/K_m = 3(-2.3\, RT \log K_p) - RTb \tag{19}$$

$$-\Delta G^\circ_m = 3 \times \Delta G_p - \text{constant} \tag{20}$$

from the data a is equal to 3.0 ± 0.2 in Eqn. 17 and is independent of the nature of the solvent and the structure of the steroid. For the same solvent, the b value is dependent on the steroid structure. b decreases with decreasing polarity of the substrate.

Relationship between dielectric constant and kinetic parameters

The dielectric constants ε of the different organic solvent–water mixtures have been taken from [21] and are given in Tables I, III, IV and VIII.

Fig. 8 shows a linear relationship between $1/\varepsilon$ *vs.* log K_m in dioxane with 5-androstenedione and 5-pregnenedione. Fig. 9 shows the variation of log K_i *vs.* $1/\varepsilon$ with methanol, for the three inhibitors.

There is also a direct relation between the dipole moments of these molecules and their affinity values: $1/K_i$ increases from estradiol to 17α-estradiol and estradiol 17-acetate. The dipole moment (μ) values in dioxane expressed in Debye units (D) (ref. 22) vary in the same direction:

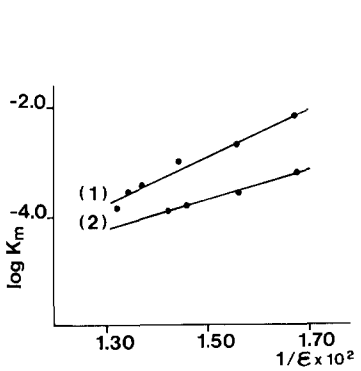
Estradiol (2.33 D) < 17α-estradiol (2.56 D) < estradiol 17-acetate (2.61 D).

TABLE VIII

EFFECT OF DIOXANE ON COMPETITIVE INHIBITION OF ISOMERASE

ε is the dielectric constant at 25° of dioxane–water mixture, expressed in Debye units. Experiments are performed in 0.03 M phosphate potassium buffer, 0.1 M KCl, pH 7.0 at 25°, with 5-pregnenedione as substrate.

Dioxane		$K_i \times 10^4$ *(M)*		
% (v/v)	ε_{25}°	*Estradiol*	*17α-Estradiol*	*Estradiol 17-acetate*
10	68.7	1.8	0.15	0.012
15	64.1	4.0	1.0	0.045
20	59.9	8.8	1.6	1.0
30	50.6	20.0		



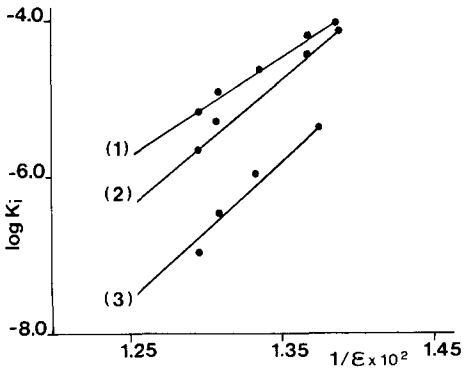


Fig. 8. Plot of log K_m *vs.* $1/\varepsilon$ (ε is the dielectric constant) in increasing concentration of dioxane. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°. Curve 1, with 5-androstenedione; 2, with 5-pregnenedione.

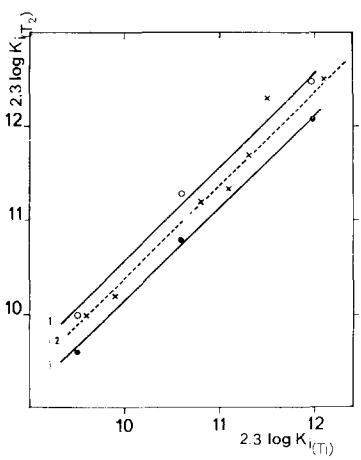
Fig. 9. Plot of log K_i *vs.* $1/\varepsilon$ (ε is the dielectric constant) at increasing concentration of methanol. 5-Androstenedione is the substrate. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0 at 25°. Curve 1, estradiol; 2, 17α-estradiol; 3, estradiol 17-acetate.

Thermodynamic data for the sequential formation of complex and product

In order to correlate the variations of kinetic parameters in different organic solvents and in the same solvent at increasing concentrations, with variations of enthalpies, entropies and free energies, experiments at different temperatures have been performed.

Formation of the complex EI

Table IX gives the thermodynamic parameters for the complex formation *EI*



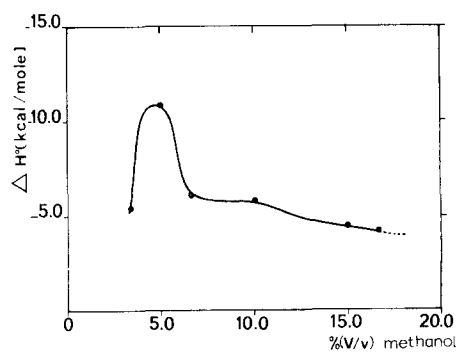


Fig. 10 Variation of 2.3 log $K_{i(T_1)}$ *vs.* 2.3 log $K_{i(T_2)}$ (Exner plots), at different methanol concentrations. 5-Androstenedione is the substrate and estradiol the competitive inhibitor. 1, 16.6° and 37°; 2, 25° and 37°; 3, 16.6° and 25°.

Fig. 11. Variation of $\Delta H°$ *vs.* methanol concentration. 5-Androstenedione is the substrate, estradiol the competitive inhibitor. Experiments are performed in 0.03 M potassium phosphate buffer 0.1 M KCl, pH 7.0, at two temperatures 25° and 37°. K_i is the parameter selected for $\Delta H°$ calculation (from Arrhenius plots and Exner's equations).

TABLE IX

THERMODYNAMIC PARAMETERS, ΔG° ΔH°, AND ΔS° FOR THE COMPLEX FORMATION ISOMERASE–ESTRADIOL AT DIFFERENT METHANOL CONCENTRATIONS

Experiments are performed in 0.03 M potassium phosphate buffer and 0.1 M KCl, pH 7.0, with 5-androstenedione as substrate and estradiol as competitive inhibitor. K_i is expressed in M, and obtained from Dixon plots. ΔH° are obtained from Arrhenius plots[b]. All the ΔH° and ΔS° values are calculated from Exner's equations[a]

$$\Delta H^\circ = \frac{RT_1T_2}{T_1 - T_2}(\ln K_2 - \ln K_1)$$

$$\Delta S^\circ = \frac{RT_2}{T_2 - T_1}(\ln K_2 - \frac{T_1}{T_2}\ln K_1)$$

Methanol (%, v/v)	$K_i \times 10^6$ *16.6°*	$K_i \times 10^6$ *25°*	$K_i \times 10^6$ *37°*	ΔH° *(cal/mole)*	ΔG° *16.6° (cal/mole)*	ΔS° *16.6° (e.u.)*	ΔG° *25° (cal/mole)*	ΔS° *25° (e.u)*	ΔG° *37° (cal/mole)*	ΔS° *37° (e.u.)*
3.3	7.0	5.25	3.8	− 5400[b]	−6700	+5	−7150	+ 0	−7650	+ 7
5.0		10.0	5.0	−10850[a]			−6800	+14	−7540	+11
6.6		12.35	8.0	− 6100[a]			−6650	+ 2	−7180	+ 3
10.0	25.0	20.0	12.5	− 5850[b]	−6040	+1	−6380	+ 2	−6870	+ 3
15.0		50.0	37.0	− 4500[a]			−5850	+ 5	−6250	+ 6
16.6	73.5	65.0	44.0	− 4220[b]	−5400	+4	−5680	+ 5	−6150	+ 6

TABLE X

THERMODYNAMIC PARAMETERS ΔG^*, ΔH^* AND ΔS^* FOR THE ACTIVATION STEP, IN THE ISOMERIZATION OF Δ^5-ANDROSTENEDIONE AT DIFFERENT ETHANOL CONCENTRATIONS

Experiments are performed in 0.03 M potassium phosphate buffer and 0.1 M KCl, pH 7.0, with 5-androstenedione as substrate. ΔH^* are obtained from Arrhenius plots. ΔG^* from absolute reaction rates equation and k_{cat} is expressed in sec^{-1}.

Ethanol (%, v/v)	$k_{cat} \times 10^{-4}$ *15° (sec^{-1})*	$k_{cat} \times 10^{-4}$ *25° (sec^{-1})*	$k_{cat} \times 10^{-5}$ *37° (sec^{-1})*	ΔH^* *37° (cal/mole)*	ΔG^* *37° (cal/mole)*	ΔS^* *37° (e.u.)*	ΔH^* *25° (cal/mole)*	ΔG^* *25° (cal/mole)*	ΔS^* *25° (e.u.)*	ΔH^* *15° (cal/mole)*	ΔG^* *15° (cal/mole)*	ΔS^* *15° (e.u.)*
1.85		17.3	1.92	910.0	+7750	−22	920	7560	−22			
3.3	10.2	13.2	1.73	2 385	+7800	−18	2 400	7720	−18	2 425	7620	−18
5.0	7.05	13.2	1.55	4 485	+7900	−11	4 600	7720	−11	4 625	7800	−11
6.6	3.25	10.7	1.55	6 385	+7900	− 5	6 400	7850	− 5	6 425	8250	− 6
8.35	3.06	9.85	1.35	7 385	+7885	− 2	7 400	7900	− 2	7 425	8300	− 3
10.0	1.485	6.87	1.23	9 385	+8000	+ 4	9 400	8140	+ 4	9 425	8700	+ 2
15.0	0.447	4.17	0.81	14 585	+8300	+20	14 600	8400	+21	14 625	9400	+18

at different methanol concentrations expressed in % (v/v), obtained from experiments at three different temperatures, with estradiol as inhibitor and 5-androstenedione as substrate. At fixed cosolvent concentrations, the $\Delta S°$ values are the same at three different temperatures, in the error limit which is of ± 2 e.u. Fig. 10 shows Exner's plots which are linear for the equilibrium data. The average T_c value is equal to zero, calculated from the Exner's plot for the three pairs of temperatures 288.9–298°K, 288.9–310°K and 298–310°K, the slope being equal to 1.0 ± 0.05.

But compensation plots are not horizontal lines for the complex formation step. Therefore, Hinshelwood's classification[23] corresponding to $T_c = 0$ cannot be applied. Table IX shows that the entropy variation has a maximum value at 5% (v/v) methanol, at the three temperatures studied.

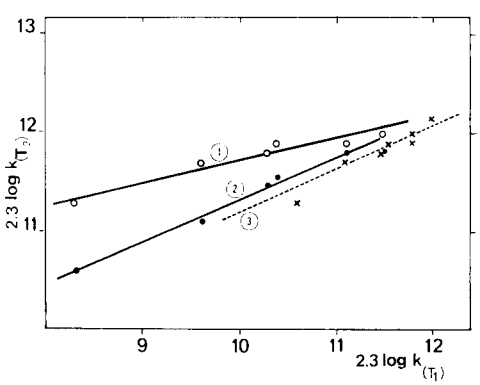
Fig. 11 shows the variation of $\Delta H°$ with increasing concentrations of methanol, for the complex formation step. A maximum value of $\Delta H°$ is observed at 5% (v/v) methanol, corresponding to a mole fraction of $2.2 \cdot 10^{-2}$. In the complex formation step the compensation phenomenon cannot be analyzed in the present experimental conditions, where substrate and inhibitor are present together.

Activation step

Table X gives the thermodynamic parameters for the activation process at different ethanol concentrations, at three different temperatures with 5-androstenedione as substrate.

Fig. 12 shows Exner's plots which are linear for the activation step. Fig. 13 shows the compensation plots for the activation step. The average T_c value obtained from both types of representations calculated by the weighted least square method is 314 ± 10°K.

The average experimental temperature, which has been called Error slope[20,24],



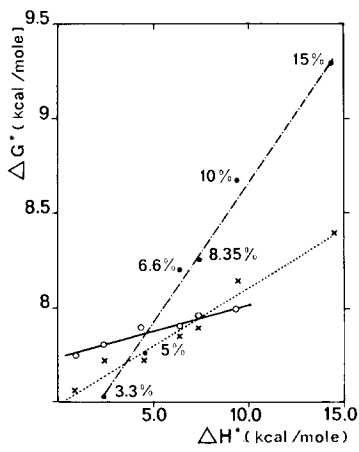


Fig. 12. Variation of 2.3 log $k_{(T_1)}$ *vs.* 2.3 log $k_{(T_2)}$ at different ethanol concentrations with 5-androstenedione as substrate. k_{cat} is the kinetic parameter selected. 1, 15° and 37°; 2, 15° and 25°; 3, 25° and 37°.

Fig. 13. Variation of ΔG^* *vs.* ΔH^* at different ethanol concentrations. 5-Androstenedione is the substrate. Experiments are performed in 0.03 M potassium phosphate buffer, 0.1 M KCl, pH 7.0. k_{cat} is the kinetic parameter selected for ΔG^* and ΔH^* calculations. Line slopes are obtained from a weighted least-squares treatment of the data at three pairs of temperatures: ●, 37°; ○, 25°; ×, 15°. Percentage in volumes of ethanol are only indicated on curve ●—·—● at 37°.

is equal to 298.6°K. It is significantly different from the slope giving the T_c. It can be concluded that the compensation phenomenon is real for the activation step. According to Hinshelwood's classification[23] for the mutual relation of activation parameters the present data correspond to the Case 3a, where T_c is higher than the highest experimental temperature. The slope is positive and smaller than T_2/T_1. The reactivity is getting smaller with increasing temperatures in the same range of alcohol concentration from 3.3 to 15.0% (v/v). Table X shows that at 15° the variation of k_{cat} (3.3%)/k_{cat} (15.0%) is equal to 22.8, a very large effect. It is 4.15 at 25° and 2.35 at 37°. For the complex formation step, the reactivity remains constant for the 3 temperatures in the range of methanol concentrations from 3.3 to 16.6% (v/v).

K_i (3.3%)/K_i (16.6%) = 10.5 at 15°, 12.4 at 25° and 11.6 at 37°.

DISCUSSION

As a solvent, water plays a unique role in biological systems. A biological interaction between two molecules (enzymatic or not) is at least a ternary system involving water molecules. Therefore enzymatic isomerizations of different steroids substrates and the effect of some competitive inhibitors have been studied in aqueous medium modified by addition of organic solvents at low mole fraction.

These organic solvents produce several changes in the physical properties of the reaction medium[8]. From the data obtained, some hypotheses can be formed on the implication of the solvent in both steps of the enzymatic reaction defined by K_m, K_i and k_{cat}.

It should be noticed that the solubility of steroids is increased by the addition of organic solvent in the aqueous solution, but all the data obtained cannot be explained by such an increased solubility, since in that case $1/K_m$ should increase, and not decrease as it is observed with the increasing concentrations of the alcohol in the reaction medium.

The dielectric constant decreases as well as the affinity of steroids for the enzyme, when the organic solvent concentration is increased. In agreement with the data on uncharged molecules involving dipole moments of the reactants discussed by BELL[25], it is conceivable that electrostatic interaction can be involved in the enzymatic reaction. The dipole moments of the substrate or inhibitor could play a role in their orientation at the active site, and in fact a relationship between the dipole moment of steroids with various substituants at the C-17 position and their affinity for the enzyme is observed. The decrease of affinity of the steroid for the enzyme with the decrease of the dielectric constant is in agreement with the data obtained for the partition coefficient K_p, which also decreases with increasing concentration of alcohol or dioxane in the aqueous phase.

But the change of the dielectric constant and the electrostatic interactions which are involved in the binding step cannot explain all effects of alcohols on the enzymatic reaction, particularly on the activation step. There is a striking effect of the chain branching of the alcohol on the k_{cat} of the reaction, K_m being not affected. In the propanol series, the *n*-isomer is a much better inhibitor than isopropanol, in spite of a higher dielectric constant. In the butanol series, the *tert.*-butanol which produces the greatest dielectric constant decrease is the poorest inhibitor.

It appears from the data that the alcohols can affect more specifically the rate

of the proton transfer as approximated by k_{cat}. In order to compare alcohols of different chain length, results have been calculated on the basis of one carbon unit.

Per unit the decrease of k_{cat} is more pronounced from the C_1 to the C_4 aliphatic alcohols: it is known that methanol < ethanol < *n*-propanol < *tert.*-butanol is the order of increasing proton accepting facility[26]. However, the efficiency of a carbon unit in the butanol series is much lower for *tert.*-butanol than for the normal butanol (Tables VI, VII). So that the effect of chain branching on k_{cat} is the opposite of the effect of increasing chain length in normal alcohols. This might be related to the effects of the same branched alcohol on the water structure[8]. Therefore the participation of alcohol on water structure appears to be an important effect in the present study in competition with the corresponding effect of the steroids. The correlation between log K_p and log $1/K_m$ which shows a small change in K_p relative to that in K_m, with a high value of the slope (approx. 3.0), indicates that the free energy change of the transfer of the steroid molecule from the water to the highly specific hydrophobic enzymatic site is not a good measure of what goes on in the Michaelis complex formation step. Therefore, K_m must consist of at least one more step and the energy of substrate desolvation from water is only a small part of the total free energy change of the binding step. Additional free energy change could be due to some conformational change of the protein induced by the steroid binding. The positive entropy change calculated for the binding of substrate[10] and inhibitor (Table IX) to the enzyme can be explained by the desolvation of steroid molecule with destruction of an ordered layer of water around these molecules, contributing to the free energy change. The effect of dioxane, the only organic solvent studied which seems to act as a competitive inhibitor on the enzymatic reaction, can be related to the similarity between the structure of this molecule and the steroid molecule. Dioxane is a cyclic ether with two fixed polar sites; moreover dioxane is characterized by strong solute–solute interaction.

The participation of the solvent in the enzymatic reaction can be suggested also from the thermodynamic data for K_m and k_{cat}, but the mechanism of the participation must be very different for the two steps.

The variation of ΔH° in function of methanol concentration shows a maximum near a mole fraction of $2.2 \cdot 10^{-2}$, this phenomenon could be related to water participation in the enzymatic binding step, which is probably too complex to show the compensation phenomenon in presence of competitive inhibitors.

The striking difference between the two steps of the enzymatic reaction what concerns the effect of temperature on reactivity[23], very large on k_{cat}, is in agreement with a real compensation phenomenon in the activation step. According to LUMRY AND RAJENDER[18] such a linear relationship between enthalpy and entropy suggests a manifestation of the same property of water involved in the process under investigation.

ACKNOWLEDGMENTS

We thank Professor Rufus Lumry and Doctor Felix Franks for making unpublished material available to us and for many important discussions during the course of this work. We thank also Mrs. L. Pénasse, P. Poirier and G. Nominé who

have provided bacteria and steroids. Technical help provided by S. Calvo is acknowledged.

Partial support has been obtained from la Délégation Générale à la Recherche Scientifique et Technique, the Ford Foundation, Roussel-UCLAF and the Centre National de la Recherche Scientifique.

REFERENCES

1 U. WESTPHAL, in C. A. VILLE AND L. L. ENGEL, *Mechanism of Action of Steroid Hormones*, Pergamon Press, New York, 1961, p. 33.
2 E. E. BAULIEU, A. ALBERGA, H. ROCHEFORT, J. P. RAYNAUD, I. JUNG AND H. RICHARD-FOY, in G. RASPÉ, *Advances in Biosciences*, Vol. 2, Pergamon Press, Vieweg, 1968, p. 241.
3 A. ALFSEN, *C. R. Trav. Lab. Carlsberg*, 33 (1963) 415.
4 P. TALALAY AND J. BOYER, *Biochim. Biophys. Acta*, 105 (1965) 389.
5 S. K. MALHOTRA AND H. J. RINGOLD, *J. Am. Chem. Soc.*, 87 (1965) 3228.
6 M. L. BENDER AND F. J. KEZDY, *J. Am. Chem. Soc.*, 86 (1964) 3704.
7 B. ZERNER AND M. L. BENDER, *J. Am. Chem. Soc.*, 86 (1966) 3669.
8 F. FRANKS AND D. J. D. IVES, *Quart. Rev.*, 20 (1966) 1.
9 F. FALCOZ-KELLY, E. E. BAULIEU AND A. ALFSEN, *Biochemistry*, 7 (1968) 4119.
10 H. WEINTRAUB, A. ALFSEN AND E. E. BAULIEU, *Eur. J. Biochem.*, 12 (1970) 217.
11 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
12 M. DIXON, *Biochem. J.*, 55 (1953) 161.
13 H. P. MARSHALL AND E. GRUNWALD, *J. Chem. Phys.*, 21 (1953) 2143.
14 T. FUJITA, J. ISAWA AND C. HANSCH, *J. Am. Chem. Soc.*, 87 (1964) 15175.
15 H. EYRING, *J. Chem. Phys.*, 3 (1935) 107.
16 O. EXNER, *Collect. Czechoslov. Chem. Commun.*, 29 (1964) 1094.
17 C. D. RITCHIE AND W. F. SAGER, *Prog. Phys. Org. Chem.*, 2 (1964) 323.
18 R. LUMRY AND S. RAJENDER, *Biopolymers*, 9 (1970) 1125.
19 S. ROGINSKY AND L. ROSENKEWISCH, *Z. Physik. Chem.*, 103 (1930) 47.
20 R. A. FAIRCLOUGH AND C. N. HINSHELWOOD, *J. Chem. Soc.*, (1937) 538.
21 G. AKERLÖF, *J. Am. Chem. Soc.*, 54 (1932) 4125.
22 W. NEUDERT AND H. RÖPKE, *Atlas of Steroid Spectra*, Springer-Verlag, Berlin and Heidelberg, 1965.
23 D. A. BLACKADDER AND C. N. HINSHELWOOD, *J. Chem. Soc.*, (1958) 2720.
24 R. F. BROWN, *J. Org. Chem.*, 27 (1962) 3015.
25 R. P. BELL, *J. Chem. Soc.*, (1943) 629.
26 W. GERRARD AND E. D. MACKLEN, *Chem. Rev.*, 59 (1959) 1105.