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# THE EFFECT OF TEMPERATURE ON MONOXYGENASE REACTIONS IN THE MICROSOMAL MEMBRANE *

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## Summary

The effect of temperature on the rates of monoxygenase reactions was studied with microsomes prepared from phenobarbital pretreated rats. The rates of the *N*-demethylation of ethylmorphine, benzphethamine, aminopyrine, and *p*-nitroanisole were studied. Breaks at temperatures around 24°C were observed in the Arrhenius plots of all these reactions. The energy of activation of these reactions has values of 10—12 and 19—21 kcal per mol at temperature ranges above and below the break temperature, respectively. The break, however, was not observed if 30% glycerol was added to the microsomes. The Arrhenius plot of the microsomal NADPH-cytochrome *c* reductase activity also did not show any break. The implications of these observations in relationship to the fluidity of the membrane, the translational mobility of membrane enzymes, and the rate of monoxygenase reactions are discussed.

## Introduction

The endoplasmic reticulum of liver cells contains a monoxygenase enzyme system which catalyzes the biotransformation of steroids, drugs, carcinogens and various other compounds [1,2]. In this system, the reducing equivalents of NADPH are transferred through NADPH-cytochrome *P*-450 reductase to cytochrome *P*-450, where molecular oxygen is activated for the oxygenation of substrates. In rat liver microsomes, the number of cytochrome *P*-450 molecules is 10—25-times greater than that of the reductase [3]. Previously we have proposed that cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase are not rigidly organized in the membrane and they possess lateral mobility [4,5]. If such a mobility is important in catalysis, then the fluidity of the lipid in the

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membrane may affect the rates of the monoxygenase reactions. In a closely related system, the importance of the translational diffusion of cytochrome $b_5$ and cytochrome $b_5$ reductase in their catalysis as well as the temperature effect on these processes have been clearly demonstrated [6—8].

The fluidity of the membrane changes dramatically in the temperature range where a crystalline-liquid crystalline phase transition or phase separation occurs [9—20]. Such a change is known to affect the rotational and translational mobilities of membrane components [15—20]. The influence of temperature-induced phase changes on the kinetics of many membrane-associated enzyme systems has been reviewed by Raison [21]. An understanding of the interrelationship between membrane structure and the activities of many membrane enzyme systems is beginning to evolve [18,20—23].

The effect of temperature on the rates of the cytochrome *P*-450-catalyzed demethylations of ethylmorphine and aminopyrine have been studied [24—26]. These authors were presumably interested in the energy of activation and the rate-limiting step of the reaction at physiological temperatures and a break in the Arrhenius plot was not observed. On the other hand, breaks have been reported for the oxidation of lipophilic substrates such as octadecane and 7-ethoxycoumarin which proceed at rather slow rates [27,28]. It is not known whether the oxygenation of these substrates involves the same rate-limiting step as that of the more efficient monoxygenase reactions. We have studied the effect of temperature on several efficient monoxygenase reactions with hepatic microsomes. This manuscript reports the observation of breaks in the Arrhenius plots of these reactions. During the preparation of this work, a more detailed report on the effect of temperature on the microsomal dealkylations of 7-ethoxycoumarin and *p*-nitroanisole [29] has appeared.

## Materials and Methods

*Preparation of microsomes*

Unless stated otherwise, microsomes were prepared from male Long-Evans rats (body weight 75—125 g) which had received a daily injection of phenobarbital (75 mg/kg body weight) for 4 days. The livers from 40—60 animals were pooled and homogenized in a 0.05 M Tris · HCl buffer, pH 7.4, containing 1.15% KCl. The postmitochondrial supernatant was centrifuged at 105 000 × *g* for 90 min. The microsomal pellet was washed once with a solution containing 1.15% KCl and 10 mM EDTA and resuspended in 0.25 M sucrose. The cytochrome *P*-450 content of the microsomes was usually between 2.6—3.1 nmol per mg protein. The microsomal suspension was rapidly frozen in a dry ice/acetone bath and stored at −90°C. Freshly thawed microsomes were used for the experiments and the demethylase activities were found not to be different from the freshly prepared microsomes.

*Assay of demethylase activities*

Each set of experiments was carried out at eight different temperatures on the same day. Equal amounts of the freshly thawed microsomes (20—30 mg/ml) were placed in 8—10 tubes, and the microsomes were stored in ice under $N_2$ until they were ready to be used for assays at different temperatures. No changes in the catalytic activity and cytochrome *P*-450 content of the micro-

somes due to this storage were observed. The demethylase activities were assayed by procedures similar to those described previously [30]. The incubation mixture contained 50 mM Tris · HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 10 mM isocitrate, 0.4 unit isocitrate dehydrogenase, microsomes corresponding to 1.5—2.5 mg protein and various concentrations of ethylmorphine, benzphetamine, aminopyrine and *p*-nitroanisole. After preincubation at a specified temperature for 2—3 min, the reaction was initiated by the addition of NADPH to a concentration of 0.5 mM in a final volume of 2 ml. The temperature of the shaking water bath was maintained constant by a circulating water bath equipped with a suction and ejection pump. After different incubation periods, the reactions were stopped by the addition of 0.25 ml each of a 25% $ZnSO_4$ solution and a saturated $Ba(OH)_2$ solution. The amount of formaldehyde formed was measured by the method of Nash [31], and the velocity of the reaction was expressed in nmol HCHO formed per min per mg microsomal protein. When different substrate concentrations were used for the determination of $K_m$ and $V$ values, selected incubation periods were used for each temperature. As the reaction temperature was varied from 38 to 13° C, incubation times were increased from 4 to 30 min, so as to keep the amounts of the product formed within a range for accurate measurements. Under these conditions, the reaction was linear with the time of incubation and the results are highly reproducible. In all the double-reciprocal and Arrhenius plots, the intercepts and slopes of the lines were determined by linear regression analysis. The entire study consisted of more than three thousand determinations with different microsomal preparations, and was carried out in a period of one and a half years.

*Other assays*

Protein was determined by the method of Lowry et al. [32]. The NADPH-cytochrome *c* reductase activity was assayed by the method of Masters et al. [33]. Cytochrome *P*-450 concentration was measured with a Cary 17 recording spectrophotometer according to the method of Omura and Sato [34] using an extinction coefficient of 91 $cm^{-1} \cdot mM^{-1}$ for $A_{450-490\,nm}$.

*Chemicals*

Isocitrate dehydrogenase, DL-isocitric acid, NADP, NADPH and bovine serum albumin were purchased from Sigma Chemical Company. *p*-Nitroanisole was supplied by Eastman Organic Company. 3-Methylcholanthrene was obtained from Mann Research Laboratories. Chemicals received as gifts were: ethylmorphine · HCl from Merck and Company, aminopyrine from Sterling-Wintrop Research Institute, benzphetamine · HCl from Upjohn Company, and phenobarbital (sodium salt) from Lilly Laboratories.

## Results

*Determination of V and $K_m$*

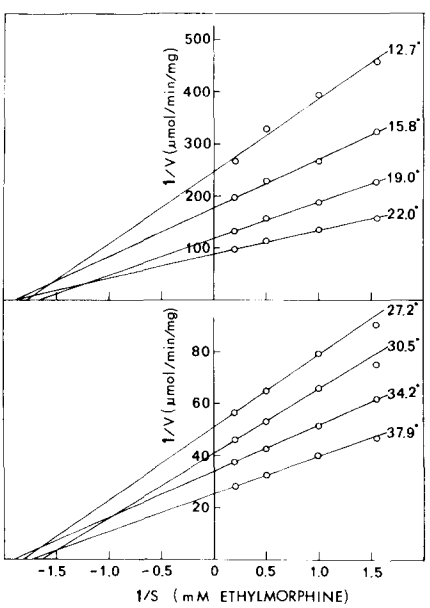
Representative data of the ethylmorphine demethylase reaction are given in double-reciprocal plots as shown in Fig. 1 *. At each temperature, the reaction

* The results obtained with the lowest substrate concentration at 27.2 and 30.5° C were deemphasized. When these values were used in linear regression analysis, they had very little effect on the $V$ values and did not affect the conclusion of the experiment.

rates were determined at four ethylmorphine concentrations, each in duplicate. The differences between the duplicates were usually very low (below 2—3% for the data in Fig. 1). The conditions used for the assay were similar to those of Franklin and Estabrook [35], and the observed $K_m$, 0.51—0.62 mM, were slightly higher than the 0.38 mM of ethylmorphine reported by these authors. Since the present study tends to yield more accurate results for the values of $V$ than for $K_m$, the presently observed $K_m$ values represent a scattering of data rather than any temperature-depending change. On the other hand the $V$ was highly temperature-dependent, with values ranging from 4.1—39.4 nmol per min per mg microsomal protein in the temperature range of 12.7—37.9°C. The rate observed at 25°C was higher than that reported by Franklin and Estabrook [35], possibly due to the high cytochrome *P*-450 content of our microsomes.

*Arrhenius plots of ethylmorphine demethylation*

The $V$ values obtained from Fig. 1 are shown in an Arrhenius plot as set A in Fig. 2. A linear plot was not observed. Rather, the data can best be fitted by two straight lines of different slopes. The activation energies for reactions carried out above and below the break temperature (where the two lines intersect) at 25.2°C, were 11.8 and 19.5 kcal per mol, respectively. A similar break was



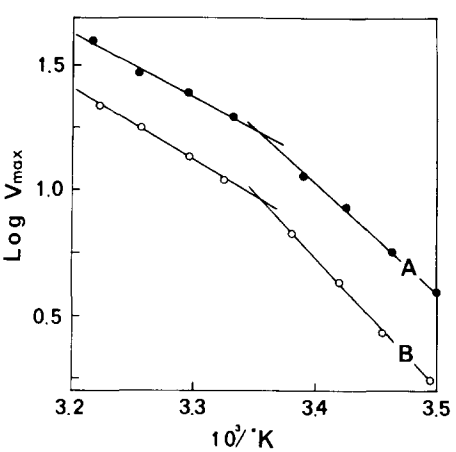




Fig. 1. Double-reciprocal plots of the ethylmorphine demethylase reaction. The reaction mixture contained 1.5 mg microsomal protein and 0.65, 1.00, 2.00, or 5.00 mM of ethylmorphine. The incubation periods were: 4 min at 37.9°C, 5 min at 34.2°C, 6 min at 30.5°C, 7 min at 27.2°C, 11 min at 22.0°C, 15 min at 19.0°C, 20 min at 15.8°C and 25 min at 12.7°C.

Fig. 2. Arrhenius plots of the ethylmorphine demethylase reaction. The $V$ values (in nmol HCHO/min per mg) calculated from Fig. 1 were used for the plot in set A. For set B, the conditions for the assays were similar to those in Fig. 1, except 2.5 mg of microsomal protein were used and the reaction was initiated by NADP. When the assay was initiated with NADP, a lag period before the linear range of the reaction was observed. Corrections were made for the lag periods in the calculation of the rates of the reactions at each temperature.

observed when the assay was initiated with NADP. * For example, data set B in Fig. 2 showed activation energies of 13.5 and 23.6 kcal/mol for temperatures above and below the break at 25.0° C. A similar break in the Arrhenius plot of the ethylmorphine demethylase activity of microsomes from control rats (which had received no phenobarbital treatment) was also observed, except that the energies of activation at temperatures above and below the break temperature (24—25° C) were 11—13 and 16—18 kcal per mol, respectively (data not shown).

The microsomes used in most of the experiments had been stored frozen at —90° C. When freshly prepared microsomes were used for the study, results similar to those in Fig. 2 were obtained (data not shown), indicating that the presently observed break in the Arrhenius plot is not an artifact caused by the freezing and thawing of the microsomes.

*The effect of substrate concentration on the activation energy of ethylmorphine demethylase*

In Fig. 3, the observed rates of ethylmorphine demethylase were shown in Arrhenius plots for each substrate concentration. With all four concentrations used, the break and apparent activation energies were very close to those shown in Fig. 2. Comparable results were also observed with similar sets of experiments. This analysis indicates that the activation energy of microsomal catalyzed ethylmorphine demethylation is apparently independent of the substrate concentration, at least within the concentration range used in this study.

*Temperature-dependence of the microsomal-catalyzed demethylations of benzphetamine, aminopyrine and p-nitroanisole*

Instead of the $V$ values, the rates of the reactions at selected substrate concentrations were used in this study. As shown in Fig. 4, the *N*-demethylations of benzphetamine and aminopyrine as well as the *O*-demethylation of *p*-nitroanisole all showed breaks in the Arrhenius plots. The results of this series of studies together with those obtained with ethylmorphine are summarized in Table I. In all the reactions, breaks at temperatures around 24° C in the Arrhenius plots were observed and the energy of activation at temperatures above and below the break had values of 10—12 and 19—21 kcal per mol, respectively.

*The effect of temperature on NADPH-cytochrome c reductase activity*

The effect of temperature on the activity of this flavoprotein is shown in Fig. 5. The Arrhenius plot showed a straight line corresponding to an energy of activation of 9—10 kcal per mol in the temperature range of 13—37° C.

*The effect of glycerol on the ethylmorphine demethylase reaction*

The approach of Duppel and Ullrich [29] was used to assess the role of membrane on the energy of activation of the microsomal demethylase reaction.

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* The rate of the reaction was slower than that of the NADPH-initiated reaction, possibly due to the inhibitory action of NADP on the NADPH-dependent demethylation reactions [36]. Results similar to those observed in set B could be obtained if the assay was initiated with a mixture of NADPH and NADP.

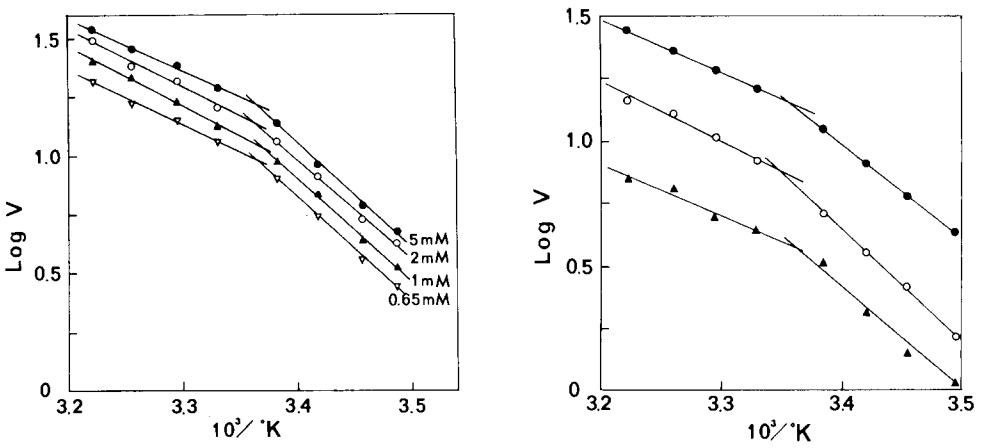


Fig. 3. Effect of substrate concentration on the activation energy. Assay conditions were similar to those in Fig. 1, except 2.0 mg microsomal protein were used per incubation.

Fig. 4. Arrhenius plots of the microsomal-catalyzed demethylations of benzphetamine, aminopyrine and *p*-nitroanisole. Assay conditions were similar to those in Fig. 1, except that 2.0 mg of microsomal protein and 0.5 mM of benzphetamine, (●———●); or 1 mM of aminopyrine (○———○) were used. For the *O*-demethylase assay, 2.5 mg microsomal protein and slightly longer incubation periods were used. *p*-Nitroanisole (▲———▲) was added in 20 μl of acetone to a final concentration of 1 mM.

In this experiment, microsomes were preincubated with 30% glycerol at 30°C for 30 min and the ethylmorphine demethylase activities were assayed in the presence of 30% glycerol at different temperatures. No break in the Arrhenius plot was observed over the entire temperature range examined, in agreement with the results of Duppel and Ullrich [29] observed with 7-ethoxycoumarin. The observed energy of activation, 15.2 kcal/mol, was slightly higher than the value of 12.6 kcal/mol for 7-ethoxycoumarin dealkylation [29].

## Discussion

The present results clearly demonstrate the existence of a break in the Arrhenius plot of the oxidative *N*-demethylation of ethylmorphine, benzphetamine,

TABLE I

ENERGY OF ACTIVATION OF MICROSOMAL DEMETHYLASE REACTIONS

The assay conditions were similar to those described in the legends of Fig. 1—4. With ethylmorphine as a substrate, *V* values were used for the Arrhenius plot. With other substrates, the observed velocity at selected substrate concentrations, i.e., benzphetamine at 0.2 or 0.5 mM, aminopyrine at 1.0 or 2.0 mM, and *p*-nitroanisole at 0.5 or 1.0 mM, were used. Since the substrate concentration did not affect the activation energy of the reactions, results were summarized and expressed as mean ± S.D.

| Substrate | Number of experiments | Break temperature (°C) | $E_a$ (38—25°C) (kcal/mol) | $E_a$ (23—12°C) (kcal/mol) |
|---|---|---|---|---|
| Ethylmorphine | 14 | 24.4 ± 1.4 | 12.3 ± 2.1 | 21.5 ± 1.9 |
| Benzphetamine | 6 | 23.9 ± 1.2 | 10.1 ± 0.7 | 18.9 ± 1.4 |
| Aminopyrine | 4 | 24.0 ± 1.8 | 11.7 ± 1.2 | 20.6 ± 0.8 |
| *p*-Nitroanisole | 6 | 23.9 ± 1.6 | 9.7 ± 0.3 | 19.8 ± 1.5 |

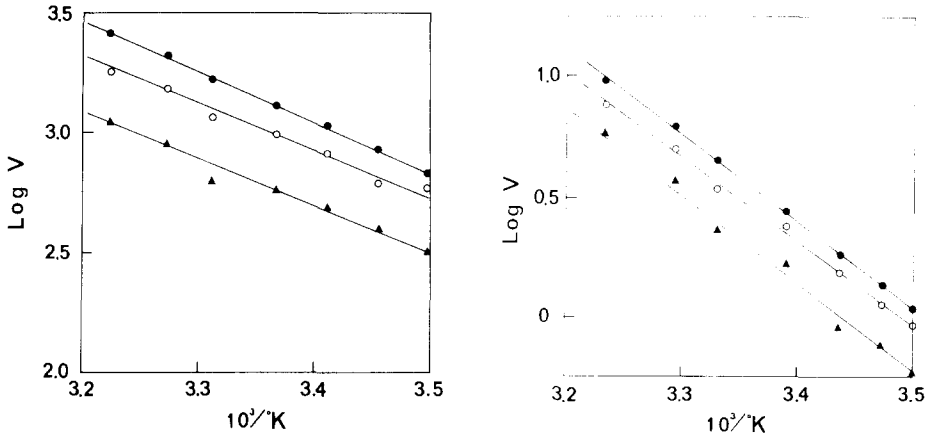


Fig. 5. Arrhenius plot of the NADPH-cytochrome *c* reductase reaction. The reaction mixture contained 0.1 M phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM KCl, 0.2 mM NADPH, 20 μg of microsomal proteins per ml, and 40 μM (●); 20 μM (○); or 10 μM (▲), cytochrome *c*. The absorbance change at 550 nm was measured with a Cary 16 recording spectrophotometer. The velocity was expressed as nmol of cytochrome *c* reduced/min per mg microsomal protein. Each point represents the average of three determinations.

Fig. 6. Effect of glycerol on the energy of activation of the ethylmorphine demethylation. Assay conditions were similar to those for Fig. 1, except that the microsomes were preincubated with 30% glycerol at 30°C for 30 min and the assay was carried out in the presence of 30% glycerol. The reaction mixture contained 2 mg of microsomal protein and 4 mM (●); 2 mM (○); or 1 mM (▲), of ethylmorphine. Each point represents the average of three determinations.

and aminopyrine catalyzed by liver microsomes. These three water-soluble substrates provide an advantage over hydrophobic substrates in avoiding problems such as solvent effects and unknown effective concentration of substrates in the assay. The results obtained with *p*-nitroanisole indicate that the drastic change in the energy of activation around 24°C can also be observed in the *O*-demethylase reaction with a hydrophobic substrate. The present results differ from those of Schenkman [25] and Holtzman and Carr [26], but are consistent with the results of Annayev et al. obtained with octadecane [27], and of Duppel and Ullrich obtained with 7-ethoxycoumarin and *p*-nitroanisole [28,29]. It has been suggested by Gibson [37] that the substrate concentration could affect the apparent energy of activation. However, the data in Fig. 3 do not show a substrate-dependent change of the apparent activation energy.

Duppel and Ullrich [29] have postulated that the break in the Arrhenius plot obtained with the dealkylations of 7-ethoxycoumarin and *p*-nitroanisole is due to a phase transition of membrane phospholipids and is not the result of a direct regulatory effect of the membrane on either cytochrome *P*-450 or the reductase [29]. The absence of a break in the Arrhenius plots of the NADPH-cytochrome *c* reductase activity and the ethylmorphine demethylase activity in the presence of 30% glycerol as reported here is consistant with this postulation. We have suggested previously that cytochrome *P*-450 and its reductase are not rigidly associated in the membrane and they possess lateral mobility [4,5]. In agreement with the interpretation of Duppel and Ullrich [29], the presently observed break in the Arrhenius plots of demethylation reactions may be attributed to the change of lateral mobility of the monoxygenase enzymes caused by a phase separation or a phase transition of the phospholipids sur-

rounding these enzymes. Microsomal membrane contains a mixture of many phospholipids possessing hydrocarbon chains with high degrees of unsaturation [38] and a thermally induced phase transition above 5°C was not observed [39, 40]. Stier and Sackmann [40] and Stier [41], however, have suggested that the NADPH-cytochrome *P*-450 reductase is enclosed in a halo of phospholipids which differs from the bulk of lipids in the membrane and undergoes a phase transition. The presence of 30% glycerol would change the water structure, weaken the hydrophobic interactions of membrane lipids, and alter the membrane structure. It is conceivable that under these conditions the phospholipid matrix surrounding the monoxygenase enzymes may not show a drastic structural change in the temperature range studied and hence a break in the Arrhenius plot was not observed.

The oxidative demethylation of ethylmorphine or benzphetamine, at a rate of 30—40 nmol per min per mg of microsomal protein at 37°C, represents some of the most efficient monoxygenase reactions. It has been suggested that the rate-limiting step of these reactions is the transfer of the first electron [2,42, 43] or the second electron [44] to cytochrome *P*-450 in the catalytic cycle. If such a step is also rate-limiting for other microsomal monoxygenase reactions, it is not known why the metabolism of some substrates, such as *p*-nitroanisole (Fig. 4) and 7-ethoxycoumarin [29] are much slower. Cytochrome *P*-450 is known to exist in multiple forms [45—47]. It is possible that only a fraction of the cytochrome *P*-450 molecules can catalyze the demethylation of a certain substrate. It is also possible that there is more than one slow step in the monoxygenase reaction and different rate-limiting steps may be involved in the oxygenation of different substrates. The observation of the kinetic isotope effect in the oxidation of certain substrates [48—50] but not others [51] is consistent with this concept.

The rate of cytochrome *P*-450 reduction has been studied by many investigators and biphasic reduction kinetics has been reported [24—26,42,52]. Estabrook et al. [52] have observed a break in the Arrhenius plot of the "slow phase" but not the "fast phase" of the NADPH-dependent reduction of cytochrome *P*-450 in microsomes. This result may not be incompatible with the interpretation presented above if (a) the rate-limiting step of the monoxygenase reaction is the transfer of the second electron to cytochrome *P*-450 [44], or (b) the rate of cytochrome *P*-450 reduction measured under anaerobic conditions in the presence of CO does not reflect the rate of electron transfer during active oxygenation reactions. The concept that the lateral mobility of cytochrome *P*-450 and the reductase may be a rate-limiting step of monoxygenase reactions remains to be substantiated further. At the present stage of our understanding of the mechanisms of monoxygenase reactions, alternative interpretations of the present results cannot be ruled out. For example, it is possible that different rate-limiting steps may be involved under different experimental conditions and the change of temperature may cause a shift in the rate-limiting step of the monoxygenase reactions.

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