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The Effects of Temperature and Substrates on Component Reactions of the Hepatic Microsomal Mixed-Function Oxidase

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SUMMARY

The effects of temperature and substrates on components of the hepatic microsomal mixed-function oxidase have been investigated. Aminopyrine caused a 50–100% increase in cytochrome *c* reductase activity without affecting the apparent activation energy; other substrates of the mixed-function oxidase were without effect. Solubilization of the reductase by deoxycholate did not alter the apparent activation energy or remove the positive modifier action of aminopyrine. Substrates causing the type I spectral change caused a positive modifier effect on NADPH–cytochrome P-450 reductase activity without altering the apparent activation energy (12.7 ± 0.3 kcal/mole). Aniline, which causes a type II spectral change, exerted an apparent negative modifier effect, but also did not alter the apparent activation energy. Benzopyrene, which only causes a type I spectral change in microsomes of polycyclic hydrocarbon-treated animals, exerted a positive modifier action on P-450 reductase only with these microsomes.

The rate of microsomal oxidation of aminopyrine (7 nmoles/min/mg at 37°) was the same as the rate of reduction of cytochrome P-450 in the presence of aminopyrine. The rates of oxidation of ethylmorphine (a type I substrate) and aniline were about 50% and 10% of that of aminopyrine, respectively. The activation energies for the three mixed-function oxidations were 12.8 ± 0.5 kcal/mole (aminopyrine), 16.9 ± 0.8 kcal/mole (ethylmorphine), and 20.9 ± 0.8 kcal/mole (aniline). These observations suggest that different steps are rate-limiting in the oxidations of aminopyrine, aniline, and ethylmorphine.

INTRODUCTION

Like many other enzymes, the hepatic microsomal mixed-function oxidase can act on a large number of substrates. However, substrates of the microsomal oxidase, unlike those of most of these other enzymes, often bear no chemical, structural, or pharmacological similarities to each other. To date, over 300 organic compounds are known to be metabolized by the enzyme system.

The mixed-function oxidase system requires NADPH as a source of reducing equivalents (1–4), reputedly for "activation" of molecular oxygen (5); the exact nature of this activated oxygen is unknown. While it was assumed early that the flavoprotein enzyme NADPH–cytochrome *c* reductase was responsible for transfer of reducing equivalents to the terminal oxidase, cytochrome P-450, it was studies with partially resolved mixed-function oxidase systems (6, 7) and with antibodies to NADPH–cytochrome *c* reductase (8–10) which definitely established the role of this enzyme in the mixed-function oxidase system. Because of these studies, a

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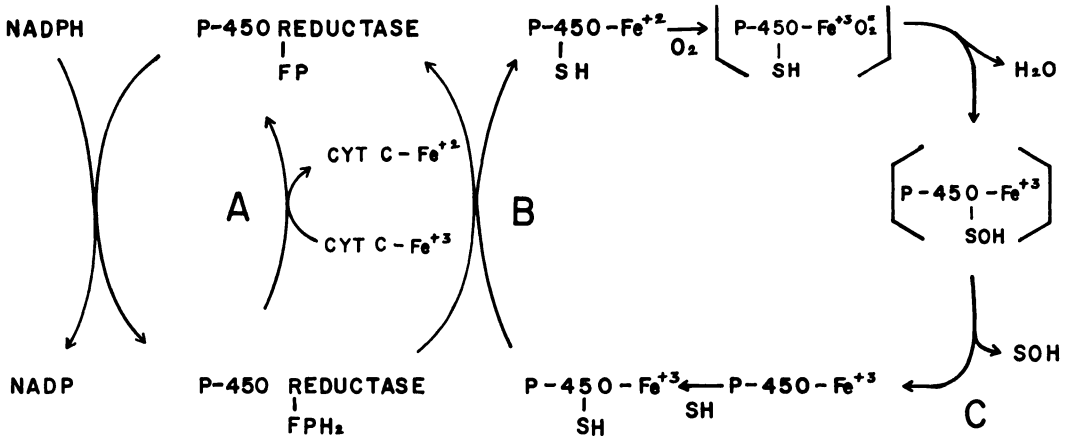

FIG. 1. *Scheme of hepatic microsomal mixed-function oxidase*

more accurate name for the reductase is probably NADPH–cytochrome P-450 reductase. However, both names will be used in this paper, depending upon the hemoprotein acceptor used.

The sequence of events encountered in the mixed-function oxidase reaction appears to be as shown in Fig. 1. The hemoprotein cytochrome P-450 is written as P-450, and SH is the substrate to be oxidized. Substrates interact with the ferri form of the hemoprotein (11, 12). Cytochrome P-450 can be reduced enzymatically in the absence of exogenous substrates, but addition of type I substrates to the microsomal suspension stimulates the rate of reduction of the hemoprotein (13, 14); the presence of type II substrates causes an apparent decrease in the rate (13). *A* in Fig. 1 is a point at which the reductase can be measured, uncoupled from the microsomal electron transport chain, using exogenous cytochrome *c* as the electron acceptor. *B* is a point in the chain where the microsomal reductase–hemoprotein coupled reaction can be measured (by appearance of reduced cytochrome P-450 in the CO complex). *C* indicates a point where the over-all coupled enzyme system can be measured; the rate of product formation from substrates like aminopyrine, ethylmorphine, or aniline is determined.

The purpose of this study was to learn something about component interaction in the microsomal mixed-function oxidase system, using temperature change as a tool, and to determine potential rate-limiting steps in the reaction. A preliminary report has appeared (15).

MATERIALS AND METHODS

The microsomes were prepared by fractional centrifugation as described earlier (11) from 0.25 M sucrose, and were washed once in 0.15 M KCl. After protein determination by a biuret method, they were diluted to 6 mg of protein per milliliter in 0.15 M KCl–50 mM Tris, pH 7.6, for use. Fresh preparations were used daily. Animals treated with 3-methylcholanthrene were given 20 mg of the compound per kilogram intraperitoneally in 0.1 ml of corn oil (Mazola) daily for 3 days, and were killed on the fifth day. Methylcholanthrene was obtained from Eastman Organic Chemicals; phenobarbital, from Mallinckrodt; aminopyrine, from Matheson, Coleman, and Bell; benzo[*a*]pyrene, from Aldrich Chemical Company; NADPH, NADP, and horse heart cytochrome *c*, from Sigma Chemical Company; and aniline, from Fisher Scientific Company. Aniline was redistilled under vacuum before use.

Cytochrome *c* reductase was isolated as described by Omura and Takesue (16), with the following modification. Lyophilized trypsin (Worthington, 220 units/mg) was dissolved in 2 mM HCl and was diluted to 0.03 mg/ml in a 20 mg/ml microsomal suspension in 0.1 M potassium phosphate buffer, pH 7.6. The preparation was put under N_2 after bubbling with N_2 to remove O_2, and was placed

for 1 hr at 37° in a water bath. The digest was centrifuged at 150,000 × *g* in a refrigerated centrifuge for 1 hr, and the supernatant solution was concentrated to 2 ml under vacuum. It was then passed through a 10-cm Sephadex G-100 column. Apparently no heme was released along with the flavoprotein. The fractions were collected and tested for cytochrome *c* reductase activity as described below. The fraction possessing maximal activity was lyophilized, and this crude, heme-free preparation was used for studies on the isolated enzyme.

Cytochrome *c* reductase was also isolated by solubilization of the reductase with deoxycholate in glycerol, as described by Lu and Coon (17). However, centrifugation was carried out at 179,000 × *g* (40,000 rpm, Spinco) for 90 min instead of at 105,000 × *g* for 2 hr; this sedimented the microsomes, leaving a clear, almost heme-free, yellow supernatant fluid, which, when passed through a DEAE-cellulose column (6, 17), proved to be cytochrome *c* reductase.

Cytochrome *c* reductase activity was measured by the appearance of absorption at 550 nm relative to the 441 nm isosbestic point, in an Aminco-Chance dual-wavelength spectrophotometer. The medium used contained 50 mM Tris-Cl or potassium phosphate buffer, pH 7.6, and 20 μM cytochrome *c* (Sigma, type IV). The reaction was started by adding 25 μl of 5% NADPH (final concentration, 0.33 mM), and the rate of reduction was taken from the initial part of the tracing (within the first 4 sec), extrapolating back to the point of addition of the reducing agent.

Cytochrome P-450 reductase was measured as described before (15), from the initial rate of formation of the reduced CO complex in an anaerobic medium. The reaction was started by adding 25 μl of 5% NADPH (final concentration, 0.33 mM), and the rate was taken from the initial 1–5 sec of reduction. Aminopyrine and ethylmorphine demethylase activity were measured by formaldehyde production, and aniline hydroxylase, by *p*-aminophenol production, as described previously (11). Temperatures were controlled with a Lauda K-21R water bath, and were measured in the sample with the expanded-scale YSI telethermometer (46TUC), using a YSI needle probe (No. 514) with a temperature correction table (Yellow Springs Instrument Company).

Results are given as means ± standard errors, with the number of different preparations tested as *n*. Significance of differences is expressed as *p* values, obtained with Student's *t* test. Energies of activation were obtained from the slopes of lines in Arrhenius plots, which are equal to $E/2.3R$, where E is the activation energy and $R = 1.986$ cal/mole-degree.

RESULTS

Studies at A. The influence of substrate concentration on the Arrhenius plot of microsomal NADPH–cytochrome *c* reductase is shown in Fig. 2. It was suggested by Gibson (18) that subsaturating concentrations of substrate could markedly affect the observed energy of activation, giving a substrate-dependent *apparent* activation energy (μ):

$$\mu = E + \left(\frac{K_m}{S + K_m}\right)\Delta H$$

Under these conditions, "μ will differ from the true energy of activation (E) by an amount which will vary with the concentration of substrate, being greatest at low substrate levels and falling to zero as the concentration is made very large" (18). The data of Laidler and Hoare (19) support this equation; when their observed activation energies for urease at different urea concentrations are plotted against the urea level, the result is a curve which decreases with increasing urea concentration in the shape of an inverted rectangular hyperbola, as would be predicted from the above equation. However, results obtained with microsomal cytochrome *c* reductase were quite different. Figure 3 is a plot of the apparent activation energies (μ) obtained at different concentrations of cytochrome *c*; the apparent activation energy values *increased* with the concentration of electron acceptor, forming a positive rectangular hyperbola. The energy of activation for cytochrome *c* reduction (E), determined from V_{max} values obtained from Lineweaver-Burk plots at the different temperatures (×, Fig. 2), was 14.1 kcal/mole.

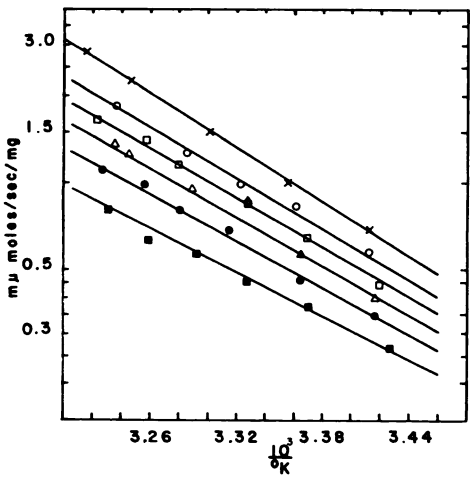

FIG. 2. *Effect of cytochrome c concentration on energy of activation of microsomal NADPH–cytochrome c reductase*

The medium used is described under MATERIALS AND METHODS; it contained 0.2 mg of microsomal protein per milliliter. The figure shows an Arrhenius plot of NADPH–cytochrome *c* reductase in media containing different cytochrome *c* concentrations. ■, 2.24 μM, E = 11.6 kcal/mole; ●, 4.48 μM, E = 12.3 kcal/mole; △, 6.72 μM, E = 12.8 kcal/mole; □, 11.2 μM, E = 13.2 kcal/mole; ○, 19.2 μM, E = 13.4 kcal/mole; ×, V_{max}, E = 14.1 kcal/mole.

Using fixed concentrations of cytochrome *c* (20 μM), attempts were made to determine whether substrates of the mixed-function oxidase would affect microsomal NADPH–cytochrome *c* reductase. The addition of 8 mM aminopyrine (a type I substrate[2] to the assay medium caused an increase in the rate of cytochrome *c* reductase activity at all temperatures (Fig. 4). The magnitude of this positive modifier effect ranged from a 50% to a 100% increase, and did not change the slope of the Arrhenius plot (Fig. 4). Another type I substrate, ethylmorphine, the type II compound aniline, and benzopyrene all were without effect on the microsomal cytochrome *c* reductase.

Prior treatment of the rats with 3-methylcholanthrene did not alter either the microsomal cytochrome *c* reductase activity or the slope of the Arrhenius plot (Table 1). In microsomes of the 3-methylcholanthrene-treated animals aminopyrine retained its positive modifier action on the cytochrome *c* reductase, and ethylmorphine and aniline were still without effect. Although in these microsomes benzopyrene becomes a type I substrate (22), and its rate of oxidation is reportedly elevated 10–40-fold (23), the presence of 4 μM or 40 μM benzopyrene in the medium had no effect on the cytochrome *c* reductase.

Studies on membrane-free cytochrome c reductase. It is implied in the scheme shown in Fig. 1 that substrates of the mixed-function oxidase interact with the hemoprotein moiety and not with the reductase. However, as shown in Fig. 4, aminopyrine does stimulate cytochrome *c* reductase activity. In order to determine whether this effect is mediated via cytochrome P-450, is a membrane phenomenon, or is due to a direct effect on the enzyme, cytochrome *c* reductase was isolated by the tryptic digestion procedure of Omura and Takesue (16). Removal by this procedure altered the properties of the enzyme considerably (Table 1); the apparent activation energy was considerably elevated, and the enzyme lost its sensitivity to positive modifier action by aminopyrine. Other substrates of the mixed-function oxidase were also without effect on this enzyme.

Since tryptic digestion could have caused a modification of the cytochrome *c* reductase characteristics by partial digestion of the enzyme, a less drastic method was also tried; Lu and Coon (17) reported that, when isolated from the microsomes by deoxycholate, cytochrome *c* reductase retained cytochrome-P-450 reductase activity in their partially resolved oxidase system. The results of studies on the deoxycholate-solubilized enzyme are shown in Table 1; the enzyme resembled the membrane-bound cytochrome

[2] Type I spectral changes are characterized by the loss of light absorption at 420 nm and an increase in absorption at 390 nm in difference spectra when the substrate is added. Compounds causing this spectral change are designated type I compounds (20). Type II spectral changes are characterized by a loss of light absorption at 390 nm and an increase in absorption between 425 and 435 nm. Compounds causing this spectral change are designated type II compounds (20). This type of spectral change should not be confused with the modified type II spectral change, which appears to be a reversal of the type I spectral change (21).

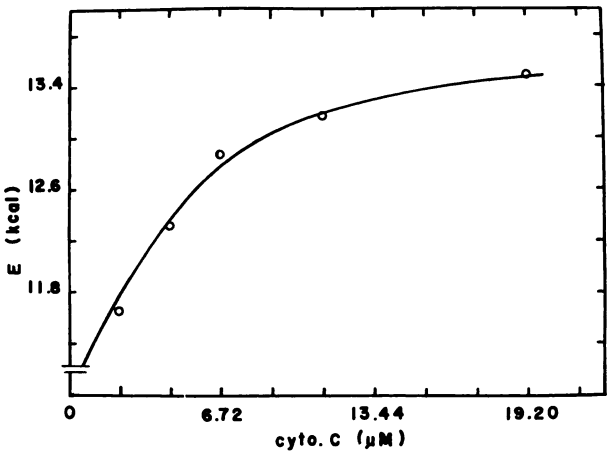

FIG. 3. *Substrate dependence plot of energy of activation*

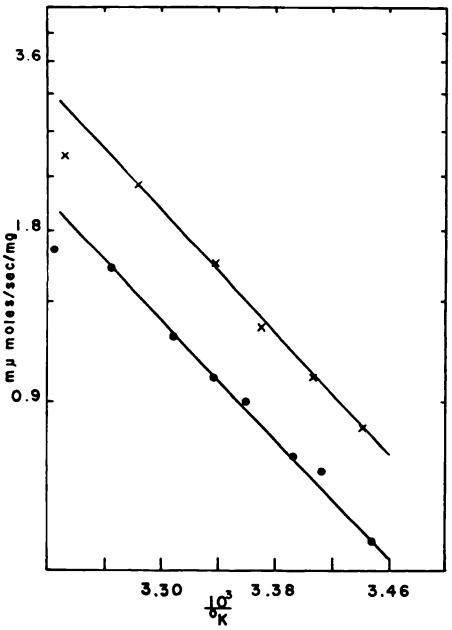

FIG. 4. *Arrhenius plot of temperature effect on microsomal NADPH–cytochrome c reductase of an untreated male rat in the presence and absence of aminopyrine*

The concentration of microsomes used was 0.11 mg of protein per milliliter in the medium described in the text. Absorption was measured at 440–541 nm; the amount of cytochrome *c* reduced was determined from the extinction coefficient 19.6 mM^{-1} cm^{-1}. ●, no added substrates of the mixed-function oxidase; ×, with 8 mM aminopyrine.

P-450 in its sensitivity to stimulation by aminopyrine and exhibited the same apparent activation energy. As with the membrane-bound enzyme, ethylmorphine, aniline, and benzopyrene were without effect.

When incubated with 8 mM aminopyrine and an NADPH-generating system, the deoxycholate-solubilized enzyme exhibited a weak ability to oxidize the analgesic, yielding about 1.5 nmoles of formaldehyde per minute per milligram of partially purified enzyme in the absence of exogenous cytochrome *c*; in the presence of exogenous cytochrome *c* (20 mM), the rate of formaldehyde generation was about 3.8 nmoles/min/mg of enzyme. The increase in demethylase activity with added cytochrome *c* in the medium is the opposite of the effect of this hemoprotein on microsomal aminopyrine demethylation (2). The contribution of the reductase to microsomal aminopyrine demethylation is negligible in view of the amount of the reductase in the microsomes.

Studies on NADPH–cytochrome P-450 reductase. The effect of temperature on the membrane-coupled cytochrome P-450 reductase reaction (*B* in Fig. 1) is shown in Fig. 5. Reductase activities were measured at the different temperatures within 5 sec after NADPH addition. In the presence of the type I substrate aminopyrine (×, Fig. 5), the rate of reduction was virtually doubled at the different temperatures, displacing

TABLE 1

Effects of modifiers and prior treatment on NADPH–cytochrome c reductase and NADPH–cytochrome P-450 reductase

Electron acceptor	Treatment	Modifier action	Apparent activation energy	Rate at 37°[a]
			kcal/mole	
20 μM cytochrome *c*	Microsomes	+[b]	12.4 ± 0.6 (5)[c]	108
20 μM cytochrome *c*	Trypsin-solubilized	None	16.6 (2)	
20 μM cytochrome *c*	Deoxycholate-solubilized	+[b]	12.9 ± 0.3 (3)	
20 μM cytochrome *c*	3-Methylcholanthrene, rat microsomes	+[b]	10.3 ± 0.7 (5)	104
Cytochrome P-450	Microsomes	+[b], −[e]	12.7 ± 0.3 (5)	3.2
Cytochrome P-450	3-Methylcholanthrene, rat microsomes	+[f], −[e]	13.1 ± 1.1 (4)	3.0

[a] Nanomoles reduced per minute per milligram of microsomal protein.
[b] Effect of 8 mM aminopyrine.
[c] Numbers in parentheses indicate number of preparations tested.
[d] Effects of 8 mM aminopyrine and 8 mM ethylmorphine.
[e] Effect of 8 mM aniline.
[f] Effects of 8 mM aminopyrine, 8 mM ethylmorphine, and 4 μM benzopyrene.

the Arrhenius plot upward. Ethylmorphine, another type I substrate, also exerted this positive modifier effect, and to the same extent. Benzopyrene, which only causes a type I spectral change in microsomes of polycyclic hydrocarbon-treated animals, was without effect. In the presence of high concentrations of aniline (3–8 mM), the apparent rate and extent of reduction of cytochrome P-450 were markedly diminished; at low concentrations of aniline, those used for K_m determination, no apparent diminution in the rate or extent of cytochrome P-450 reduction was observed. Although the type I and type II substrates exert positive and negative modifier effects on NADPH–cytochrome P-450 reductase, displacing the Arrhenius plot up or down, none of them altered the slope of the plot. The apparent energy of activation obtained for reduction of cytochrome P-450 was 12.7 ± 0.3 kcal/mole (Table 1).

The initial rate of cytochrome P-450 reductase was generally about 3–5% of the rate of cytochomre *c* reductase in the usual assays (Table 1); at a comparable hemoprotein concentrations (approximately 1 μM), cytochrome *c* reductase activity was lower, but still about 6 times faster. Unlike measurements of NADPH–cytochrome *c* reductase activities, no control can be exerted over the concentration of the endogenous hemoprotein acceptor, cytochrome P-450. The concentration of this hemoprotein per milligram of microsomal protein varied during

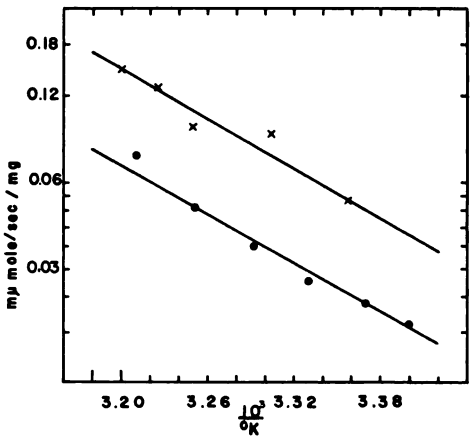

FIG. 5. *Arrhenius plots of NADPH–cytochrome P-450 reductase in the presence and absence of aminopyrine*

The media were bubbled for 3 min with deoxygenated carbon monoxide, and the reactions were started with NADPH as described under MATERIALS AND METHODS. The microsomal protein concentration was 1.0 mg/ml. Cytochrome P-450 content in microsomes was 0.97 nmole/mg of protein. ●, no substrate; ×, 5 mM aminopyrine.

the course of this study (2.5 years) from 0.36 to 1.28 nmoles in untreated rats. However, attempts to plot the observed activation energy against the cytochrome P-450 concentration (as in Fig. 3) were unsuccessful, since the values obtained were very similar.

Prior treatment of animals with substrates of the mixed-function oxidase elevates the level of the enzyme system between 3- and 5-fold. Prior treatment with polycyclic hydrocarbons like 3-methylcholanthrene elevates the level of benzopyrene hydroxylase 10–40-fold (24) while causing only a 3–5-fold increase in cytochrome P-450 (25). Data from three laboratories (20, 26, 27) have tended to suggest formation of a new hemoprotein terminal oxidase, after 3-methylcholanthrene induction, which has been named cytochrome P_1-450 (20). Since recent studies have shown the new hemoprotein to be convertible to the usual cytochrome P-450 with substrates (22), it was of interest to study the effect of induction with 3-methylcholanthrene on the coupled reductase (*B* in Fig. 1). As with microsomes of untreated rats, all of the type I compounds tested exerted a positive modifier effect on NADPH–cytochrome P-450 reductase (Table 1). In addition, benzopyrene, which is a type I compound with these microsomes (22), also behaved as a positive modifier, stimulating the reductase activity 30–80%. Aniline was a negative modifier of cytochrome P-450 reductase in these microsomes, as in control rat liver microsomes. None of the substrates altered the slope of the Arrhenius plot, yielding an apparent activation energy of 13.1 ± 1.1 kcal/mole (Table 1).

Studies on the mixed-function oxidase reaction In Fig. 6 is shown the effect of temperature on the over-all reaction (*C* in Fig. 1), the oxidation of three substrates of the enzyme system. Two of the substrates, aminopyrine and ethylmorphine, are type I substrates, and one, aniline, is a type II substrate. All of the value points are V_{max} values extrapolated from Lineweaver-Burk plots at the different temperatures. Although the oxidations of aminopyrine (×) and ethylmorphine (△) are both *N*-demethylation reactions, the shapes of the two curves suggest different temperature sensitivities.

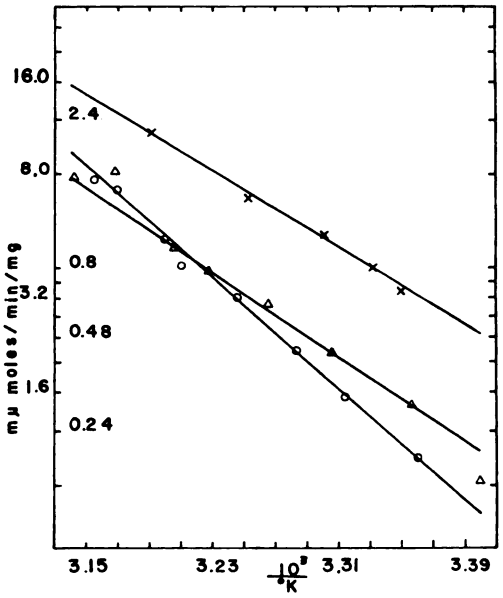

FIG. 6. *Effect of temperature on hepatic microsomal mixed-function oxidase*

The concentration of microsomal protein used was 1 mg/ml. Activity is expressed as nanomoles of product formed per minute per milligram of protein. Assay times were 2.5–7 min for aminopyrine and ethylmorphine, and 5–15 min for aniline (11). The figure shows an Arrhenius plot of V_{max} for product formation from aniline (○, right of ordinate), ethylmorphine (△), and aminopyrine (×).

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The curve for aniline hydroxylase (○) also differed from the other two curves. At 37° the rate of aminopyrine demethylation was about twice as fast as ethylmorphine demethylation, and about equal to the rate of cytochrome P-450 reduction in the presence of a positive modifier (7 nmoles/min/mg of microsomes). Aniline hydroxylation at this temperature was about 7–10% of that of aminopyrine demethylation (Fig. 6).

Typical Arrhenius plots of the three mixed-function oxidations are shown in Fig. 6. As seen from the different slopes of the lines, the activation energies for the three reactions are quite different. The energy of activation for aminopyrine demethylation

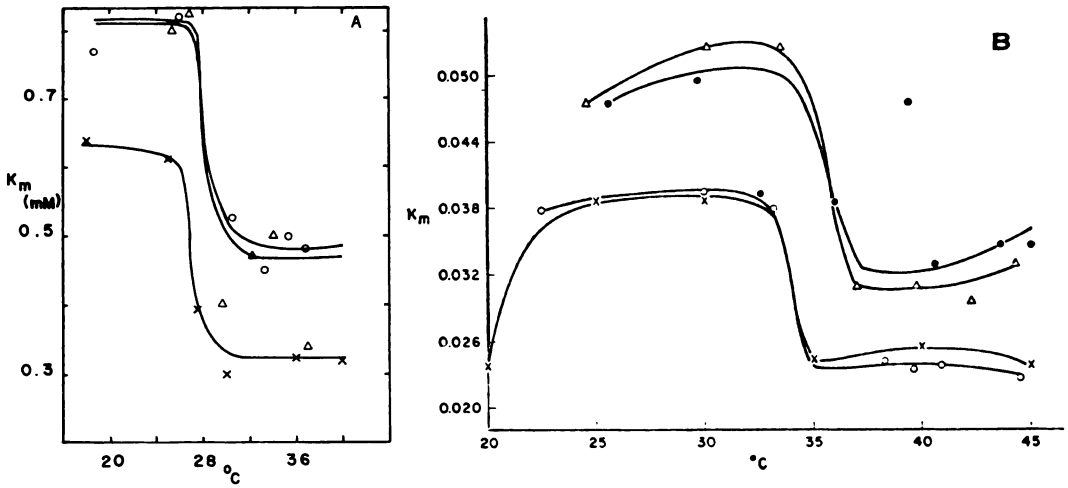

FIG. 7. *Effect of temperature on Michaelis constant (K_m) obtained with aniline and aminopyrine*
All assays were performed with 1 mg of microsomal protein per milliliter (11). A. Aminopyrine demethylase, three different preparations. B. Aniline hydroxylase, four different preparations.

was 12.8 ± 0.6 kcal/mole (n = 4); for ethylmorphine demethylation, 16.9 ± 0.8 kcal/mole (n = 9); and for aniline hydroxylation, 20.9 ± 0.8 kcal/mole (n = 5). Further differences in the three reactions were indicated by plots of their Michaelis constants (K_m) at different temperatures (Fig. 7). The aminopyrine demethylase (Fig. 7A) and aniline hydroxylase (Fig. 7B) reactions both exhibited a critical temperature effect on substrate affinity. The midpoint temperatures differed markedly for the two reactions; it was 27–29° for aminopyrine demethylase (Fig. 7A) and 34–36° for aniline hydroxylase (Fig. 7B). The magnitude of the K_m change with temperature was about 2-fold. In addition, the enzymes both revealed two K_m ranges, one high and one low; the critical temperatures of the high and low K_m ranges were the same. The ranges for aniline were 0.032–0.052 mM and 0.025–0.038 mM; for aminopyrine the ranges were 0.33–0.63 mM and 0.48–0.83 mM. The significance of the two K_m ranges is not yet apparent, but they do not appear to be due to seasonal variation or age of the animal. Ethylmorphine demethylase did not respond in the same manner; little or no change in K_m was observed with temperature.

The rate of aminopyrine demethylase activity in liver microsomes was not altered by prior treatment of the animals with 3-methylcholanthrene, nor was aniline hydroxylase activity appreciably elevated in either of two experiments. The energies of activation of the two reactions were likewise unaffected by the polycyclic hydrocarbon treatment; for aminopyrine demethylation 11.8 kcal/mole, and for aniline hydroxylation 22.2 kcal/mole, were obtained.

DISCUSSION

The function of NADPH–cytochrome *c* reductase was examined when divorced from the microsomal electron transport chain by providing an exogenous electron acceptor, cytochrome *c*, and in a reaction coupled to the endogenous electron acceptor, cytochrome P-450. One of the type I substrates of the mixed-function oxidase, aminopyrine, was capable of stimulating the microsomal reduction of exogenous cytochrome *c* as well as reduction of cytochrome P-450. The stimulation of cytochrome *c* reduction was not mediated via cytochrome P-450, as electron flow to this latter hemoprotein is many times slower (Table 1); the positive modifier action was due to a direct effect on NADPH–cytochrome *c* reductase because it was retained by the heme-free, deoxycholate-solubilized enzyme. No other tested substrates of the mixed-function oxidase exerted a modifier

effect on cytochrome *c* reduction. Solubilization by tryptic digestion is evidently a much harsher procedure, because it abolished the positive modifier effect of aminopyrine on NADPH–cytochrome *c* reductase and increased the apparent activation energy of that enzyme (Table 1).

The apparent energy of activation of the deoxycholate-solubilized cytochrome *c* reductase was the same as that of the membrane-bound microsomal cytochrome *c* reductase ($p > 0.5$). The energy of activation of the microsomal cytochrome *c* reductase was found to be 14.1 kcal/mole.

Unlike cytochrome *c* reductase activity, NADPH–cytochrome P-450 reductase activity appears to be stimulated by all substrates capable of causing the type I spectral change; benzopyrene, which only causes a type I spectral change in microsomes of animals previously treated with polycyclic hydrocarbons (22), exerted a positive modifier effect on NADPH–cytochrome P-450 reductase only with microsomes of treated animals. This would indicate that prior treatment of the animals with 3-methylcholanthrene in some way altered the NADPH–cytochrome P-450 reductase, at least with respect to its interaction with the substrate-bound terminal oxidase. Whatever the nature of this alteration, no change was observed in the apparent activation energy of the NADPH–cytochrome P-450 reductase ($p > 0.7$, Table 1).

It is not yet clear whether another component, often designated *x* (e.g., ref. 28), exists between the flavoprotein and cytochrome P-450 (Fig. 1). The presence of another component has often been suggested from the effects of inhibitors and substrates of the microsomal mixed-function oxidase on microsomal lipid peroxidase (29). Although the more rapid rate of reduction of cytochrome *c* than of cytochrome P-450 by the flavoprotein could be due to differences in ease of enzymatic reduction or to differences in accessibility of the hemoproteins to the reductase (cytochrome P-450 being held in place in the membrane), it is possible that component *x* acts as a link between the two other components; component *x* would serve to transfer electrons to cytochrome P-450, but at a slower rate than the reductase (Table 1) can function. In support of this possibility would be the observation of Glazer *et al.* (9) that an amount of antibody to NADPH–cytochrome *c* reductase capable of 90% inhibition of cytochrome *c* reduction was needed before inhibition of cytochrome P-450 reduction could be observed (15 times higher antibody concentration). Further evidence would be the positive modifier action of all type I substrates tested on NADPH–cytochrome P-450 reductase activity but not on NADPH–cytochrome *c* reductase activity (Table 1).

Earlier it was suggested that the rate-limiting step in the mixed-function oxidase reaction was NADPH–cytochrome P-450 reductase (15), based upon similarities in the energy of activation of this reaction and the aminopyrine demethylase reaction. This possibility is strengthened by the present observation that the rates of aminopyrine demethylase and cytochrome P-450 reductase were the same (7 nmoles/min/mg of microsomes at 37°); the similarity in activation energies was confirmed (12.8 ± 0.6 kcal/mole vs. 12.7 ± 0.3 kcal/mole; $p > 0.8$). However, these similarities did not extend to all substrates tested. Aminopyrine and ethylmorphine were both *N*-demethylated, both caused the type I spectral change, and both increased the rate of cytochrome P-450 reductase to the same extent; however, the rate of ethylmorphine demethylase was half that of aminopyrine demethylase. The two demethylase reactions also differed greatly in activation energies (12.8 ± 0.6 kcal/mole vs. 16.9 ± 0.9 kcal/mole; $p < 0.005$). The aniline hydroxylase reaction differed even more from the other two reactions; its rate (0.6 nmole/min/mg of microsomes) was about one-fifth that of NADPH–cytochrome P-450 reductase (no modifier effect), and about one-tenth that of aminopyrine demethylation. The energy of activation of the aniline hydroxylase reaction (20.9 ± 0.8 kcal/mole) differed quite significantly from those for aminopyrine demethylation and ethylmorphine demethylation ($p < 0.001$ and 0.005, respectively).

It is possible that the differences in activation energies for the three mixed-function oxidations may be indicative of different rate-limiting steps. Although the rate of

aminopyrine demethylation was equal to the rate of cytochrome P-450 reduction in the presence of the positive modifier, the rate of ethylmorphine demethylation was half that of hemoprotein reduction in the presence of ethylmorphine; the reductase may not be rate-limiting in this case, and the higher activation energy could be an expression of the new limiting step. A more extreme example is that of aniline hydroxylase, whose rate of oxidation is many times slower than the rate of reduction of the hemoprotein cytochrome P-450; the reduction of the hemoprotein is clearly not limiting in this reaction. Since aniline can bind to cytochrome P-450, it is possible (30) that it slows its own metabolism by interfering with oxygen activation (5). This explanation would be in agreement with the often observed decrease in NADPH and oxygen consumption by liver microsomes in the presence of aniline.[3] Slowing down a step in a reaction by poisoning it is just one means of changing a rate-limiting step. This method was used by Hadidian and Hoagland (31) to study the activation energies of different steps in the respiratory chain; they used NaCN to slow cytochrome oxidase and make it rate-limiting.

However attractive the above idea may be, an alternative explanation may exist. As shown by Sizer and Gould (32) with milk aldehyde dehydrogenase, differences in activation energies and rates of metabolism can be obtained with a purified enzyme, using substrates of dissimilar structure.

The linearity of the Arrhenius plots in Fig. 6 would tend to obviate the possibility that more than one nonspecific enzyme exists in liver microsomes. The lack of a break in the lines indicates that the microsomal mixed-function oxidase does not involve two or more independent, concurrent enzymes oxidizing the same substrate; were this the case, we should obtain a curve with an upward concavity. It is not expected that two or more different enzymes capable of metabolizing the same substrate, and yielding the same product, would have similar activation energies (33).

The significance of the findings in Fig. 7A and B is not known. The effect of temperature on the K_m for the substrates aminopyrine and aniline was very small, barely halving the K_m values at higher temperatures. The responses did not fit the linear increase plot of pK_m with reciprocal temperature used by Massey (21) to obtain heats of formation (ΔH) of the enzyme-substrate complex of aldehyde oxidase. Similarly, no reason is as yet available for the opposite effect of cytochrome *c* concentration from that predicted by Gibson's equation (18), on apparent activation energy for the cytochrome *c* reductase reaction.

[3] J. B. Schenkman and R. W. Estabrook, unpublished observations.

REFERENCES

1. J. R. Cooper and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **114**, 409 (1955).
2. B. N. La Du, L. Gaudette, N. Trousof and B. B. Brodie, *J. Biol. Chem.* **214**, 741 (1955).
3. B. B. Brodie, *J. Pharm. Pharmacol.* **8**, 1 (1956).
4. C. Mitoma, H. S. Posner, H. C. Reitz and S. Udenfriend, *Arch. Biochem. Biophys.* **61**, 431 (1956).
5. J. R. Gillette, B. B. Brodie and B. N. La Du, *J. Pharmacol. Exp. Ther.* **115**, 532 (1957).
6. A. Y. H. Lu, K. W. Junk and M. J. Coon, *J. Biol. Chem.* **244**, 3714 (1969).
7. T. Omura, E. Sanders, R. N. Estabrook, D. Y. Cooper and O. Rosenthal, *Arch. Biochem. Biophys.* **117**, 660 (1966).
8. T. Omura, S. Takesue and N. Oshino, *Int. Symp. Microsomes and Drug Oxidations* (1969) [J. B. Schenkman, *Science* **168**, 612 (1970)].
9. R. I. Glazer, J. B. Schenkman and A. C. Sartorelli, *Mol. Pharmacol.* **7**, 683 (1971).
10. B. S. S. Masters, J. Baron, W. F. Taylor, E. L. Isaacson and J. Lospalluto, *J. Biol. Chem.* **246**, 4141 (1971).
11. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113 (1967).
12. Y. Imai and R. Sato, *Eur. J. Biochem.* **1**, 419 (1967).
13. J. B. Schenkman, *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1624 (1968).
14. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. Biophys. Res. Commun.* **31**, 558 (1968).
15. J. B. Schenkman and D. L. Cinti, *Biochem. Pharmacol.* **19**, 2396 (1970).
16. T. Omura and S. Takesue, *J. Biochem. (Tokyo)* **67**, 249 (1970).
17. A. Y. H. Lu and M. J. Coon, *J. Biol. Chem.* **243**, 1331 (1968).

18. K. D. Gibson, *Biochim. Biophys. Acta* **10**, 221 (1953).
19. K. J. Laidler and J. P. Hoare, *J. Amer. Chem. Soc.* **72**, 2489 (1950).
20. K. Bidleman and G. J. Mannering, *Mol. Pharmacol.* **6**, 697 (1970).
21. V. Massey, *Biochem. J.* **53**, 72 (1952).
22. J. B. Schenkman, H. Greim, M. Zange and H. Remmer, *Biochim. Biophys. Acta* **171**, 23 (1969).
23. A. H. Conney, E. C. Miller and J. A. Miller, *J. Biol. Chem.* **228**, 753 (1957).
24. A. H. Conney and A. G. Gelman, *J. Biol. Chem.* **238**, 3682 (1963).
25. H. Remmer, H. Greim, J. B. Schenkman and R. W. Estabrook, *Methods Enzymol.* **10**, 703 (1967).
26. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. Biophys. Res. Commun.* **29**, 521 (1967).
27. A. Hildebrandt, H. Remmer and R. W. Estabrook, *Biochem. Biophys. Res. Commun.* **30**, 607 (1968).
28. R. W. Estabrook, A. Shigematsu and J. B. Schenkman, *Advan. Enzymol.* **8**, 121 (1970).
29. S. Orrenius, *J. Cell Biol.* **26**, 713 (1965).
30. J. B. Schenkman, *Biochemistry* **9**, 2081 (1970).
31. Z. Hadidian and H. Hoagland, *J. Gen. Physiol.* **24**, 339 (1940).
32. I. W. Sizer and B. S. Gould, *Enzymologia* **8**, 75 (1940).
33. M. Dixon and E. C. Webb, "Enzymes," p. 158. Academic Press, New York, 1964.