Biochemical Pharmacology, Vol. 19, pp. 2396–2400. Pergamon Press. 1970. Printed in Great Britain

The rate-limiting step in aminopyrine demethylase of rat liver microsomes

(*Received* 4 *November* 1969; *accepted* 3 *February* 1970)

MANY studies have been performed to learn the mechanism of action of the hepatic microsomal mixed function oxidase and to discern whether a single or multiple enzyme is responsible for the metabolism of the many substrates of the enzyme system.

In studies on the mechanism of action of the enzyme system previous work[1] pointed out that substrates of the enzyme system cause two types of spectral changes. From the similarity of the dissociation constant for the spectral change with the Michaelis constant, it was suggested that one of the spectral changes (type I) was an expression of the enzyme–substrate complex between the terminal oxidase (cytochrome P-450) and substrate. It was reported that the magnitude of the type I spectral change correlated better with the velocity of enzymatic drug oxidation by liver microsomes than did the absolute amount of cytochrome P-450.[2]

In a report on studies to determine the mechanism of action of the drug oxidase system, it was shown[3] that substrates causing the type I spectral change doubled the rate of reduction of the terminal oxidase. Davies *et al.*[4] found that the rate of NADPH–cytochrome P-450 reduction, measured in liver microsomes of different species, more closely paralleled the rate of ethylmorphine *N*-demethylation than did the magnitude of the type I spectral change.

These latter studies suggest that the reduction of cytochrome P-450 is the rate-limiting step in the hepatic microsomal mixed function oxidase reaction. The purpose of this communication is to show data indicating that only one aminopyrine demethylase is present in liver microsomes of normal male rats, and that the NADPH–cytochrome P-450 is the rate-limiting step in the reaction.

Microsomes were prepared from the livers of adult male rats (150–200 g) as previously described[1] from 0·25 M sucrose homogenates, and were washed in 0·15 M KCl. Aminopyrine demethylase activity was determined from formaldehyde production as described elsewhere.[5]

NADPH–cytochrome P-450 reductase activity was determined in an Aminco-Chance dual wavelength recording spectrophotometer, in the dual wavelength mode, by measuring the difference in absorption between 450 and 465 nmoles. Samples were bubbled with oxygen-free carbon monoxide (prepared by action of concentrated sulfuric acid on formic acid)* for 3 min and were equilibrated at each temperature for 10 min before injection† of 25 μl of 4% NADPH in 1% $KHCO_3$; the microsomes were suspended in 50 mM Tris (pH 7·6) containing 8 mM isocitrate and isocitric dehydrogenase (15 μg/ml, Sigma type IV) and 1 mM $MgCl_2$.

The aminopyrine demethylase reaction has previously been reported to be linear with time up to 7 min,[5] hence this time was chosen as the limit for assays; the reaction was linear for longer periods at temperatures lower than 37°, but was still linear up to 5 min at 45°.

Figure 1 shows substrate dependence plots for aminopyrine demethylase at different temperatures

* Carbon monoxide generated by this procedure is bubbled through sintered glass gassing tubes into a deoxygenating medium containing 2 g vanadyl sulfate in 200 ml of 2 M HCl over heavily amalgamated zinc. Vanadium IV (blue) is reduced to vanadium II (purple) which reacts avidly with oxygen, regenerating vanadium IV. If the system becomes brown-black, a few milliliters of concentrated HCl will return the vanadite ion to vanadyl ion.

† Aminco anaerobic cells with rapid mixing plunger attachment (Al-65085) were tested for use in measuring the P-450 reductase, but were discarded when it was found that the plunger shaft provides a channel through which air readily leaks into the cuvette. In addition, the plunger-stop clip is made of iron, which rusts readily. In place of this apparatus, a thunburg-type cuvette with sidearm (no. 190, Hellma Cells, Inc., Jamaica, N.Y.) was fitted with a rubber stopper through which two 22 gauge, 3 in. needles were pushed. Carbon monoxide was bubbled through one needle, exiting through the sidearm, and a 0·25-ml syringe was attached to the other needle; medium was drawn up into this second syringe in order to expel all air and provide a column of medium through the needle shaft. After gassing the cuvette contents, the sidearm was sealed, the carbon monoxide was shut off, and the gassing plug was replaced with another 0·25-ml syringe containing 0·025 ml of 4% NADPH. The reaction was begun by drawing medium up into both syringes and depressing both plungers simultaneously; this also provided good mixing action.

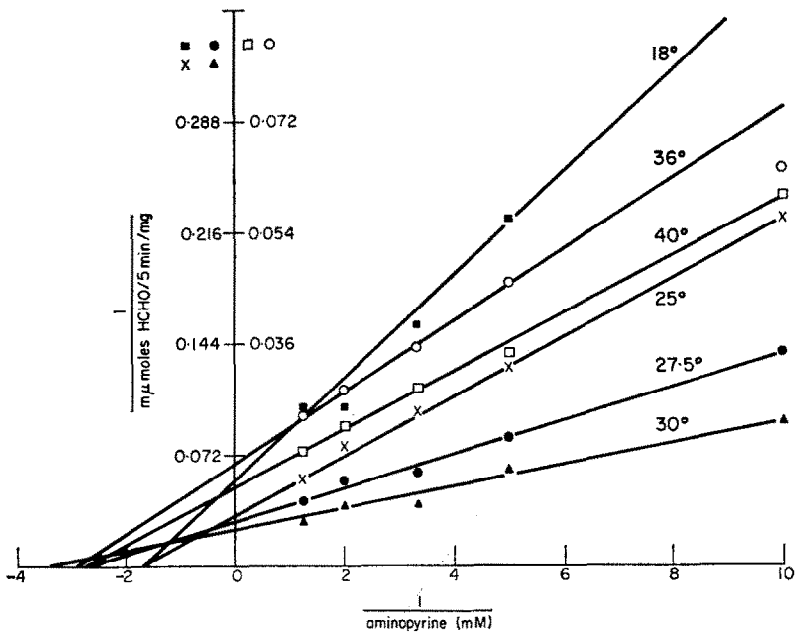

FIG. 1. Substrate dependence of aminopyrine demethylase at different temperatures. The reaction contained 1 mg of microsomal protein per ml. After 10 min preincubation of medium to generate NADPH at 37°, 2·5 ml of incubation medium was preincubated in a Dubnoff shaker for 10 min. For temperature equilibration, microsomes were temperature equilibrated for the last 3 min, then added to the medium to start the reaction. The reaction was terminated at 5 min and the HCHO concentration was determined.

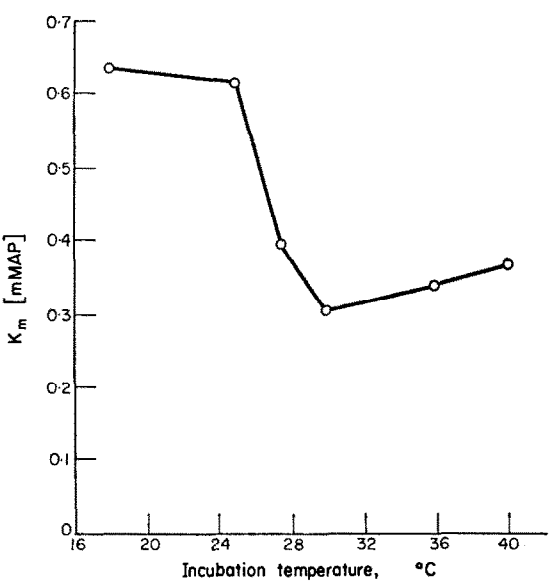

FIG. 2. Variation of K_m with temperature. Data were obtained from Lineweaver–Burk plots of reactions using 1 mg of microsomal protein per ml. Conditions as in Fig. 1.

(incubation time, 5 min). In agreement with the report of Dewaide and Henderson,[6] the reaction has a strong temperature dependence. However, in addition to affecting the velocity of the demethylase reaction, temperature also influenced the substrate affinity (Fig. 1). The Lineweaver–Burk plot of substrate dependence shows, in addition to increasing V_{max} with increasing temperature, a decrease in the K_m with increasing temperature; the decrease was fairly sudden between 25° and 30°, a minimum appearing at the latter temperature. With further increase in temperature, there was a slight rise in the K_m for aminopyrine (Fig. 2), giving the plot a backward "S" shape. The effect of temperature on the K_m is a reversible phenomenon; preincubation of microsomes at 37° and assaying the aminopyrine demethylase activity at 25° yielded the same K_m as preincubation at 25°. However, in the former case, V_{max} was a little decreased.

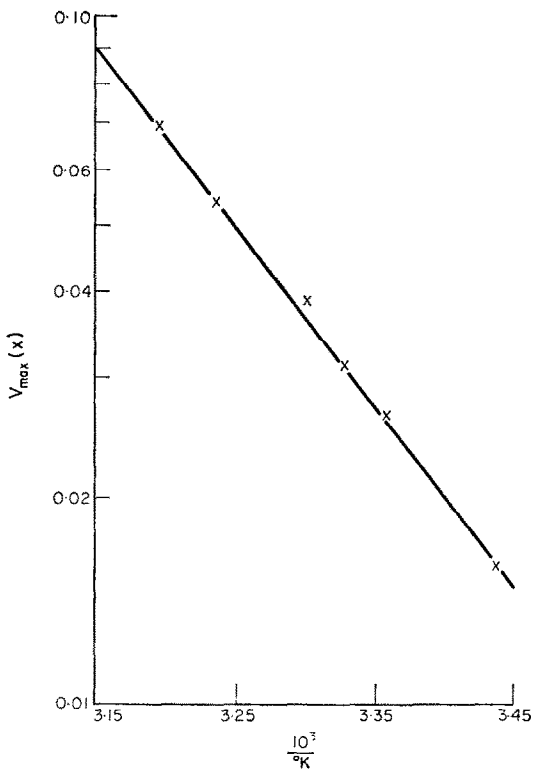

FIG. 3. Arrhenius plot of aminopyrine demethylase activity (V_{max}). Data were obtained from Lineweaver–Burk plots of aminopyrine demethylase activities. Extrapolated V_{max} values were plotted from reactions in which 1 mg of microsomal protein was contained per ml. Conditions as in Fig. 1.

In Fig. 3 is shown an Arrhenius plot of the extrapolated maximum velocities (V_{max}) of aminopyrine demethylase at different temperatures. The energy of activation of the enzyme–substrate complex, determined from Fig. 3, was 12,000 cal/mole. A similar plot of initial velocities of NADPH–cytochrome P-450 reduction at different temperatures is shown in Fig. 4; the energy of activation of the reductase reaction, obtained from Fig. 4, gave a value of 13,300 cal/mole. A similar value was obtained in the presence of 8 mM aminopyrine.

The data shown in this paper provide information about the hepatic microsomal mixed function oxidase system. In agreement with the previous report,[6] aminopyrine demethylase is temperature sensitive. The Q_{10} for the reaction is 2·0. The finding that the K_m for aminopyrine is temperature

sensitive indicates that between 25° and 30° there is a marked alteration in the substrate (aminopyrine) affinity. The significance of this observation is at once apparent; when reactions are run at lower temperatures, care must be taken to ensure that substrate saturating conditions are used. The reason for the sudden change in substrate affinity is not yet understood and is under investigation.

The similarity of the activation energy value for the aminopyrine demethylase reaction with that for NADPH–cytochrome P-450 reductase suggests a common rate-limiting step. The Q_{10} for the latter was 1·9. Since the latter measurement is done under anaerobic conditions, activation of oxygen, interaction of the activated oxygen with donor substrate, and release of product are all steps which

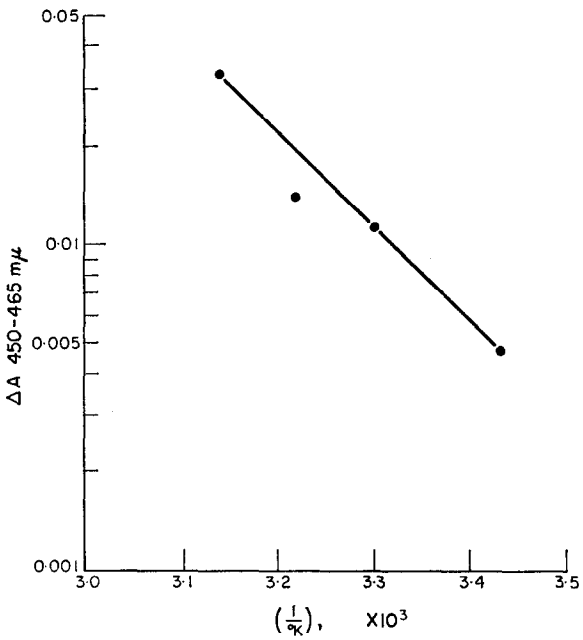

FIG. 4. Arrhenius plot of NADPH–cytochrome P-450 reductase activity (initial velocities). Data were obtained as described in Methods; 1 mg of microsomal protein was contained per ml.

may be eliminated from consideration as rate-limiting. This indicates that the suggestion by Davies *et al.*[4] that the cytochrome P-450 reductase reaction is rate-limiting may be correct. The addition of substrates of the mixed function oxidase to microsomes speeds up the rate of cytochrome P-450 reduction[3] by an alteration of the electronegativity of one ligand of the heme iron.[7] The addition of substrates of the enzyme system (hexobarbital and aminopyrine), while increasing the rate of reduction of cytochrome P-450,[3, 4] did not change the activation energy,* and therefore did not change the rate-limiting step. Neither did a new step become rate-limiting due to the changes in temperature, as evidenced by lack of a break in the plot in Fig. 2. This latter point could also be taken as evidence for the presence of only one enzyme responsible for demethylation of aminopyrine; were more than one demethylase to be functioning, such a plot would also be expected to show a break.

Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

JOHN B. SCHENKMAN
DOMINICK L. CINTI

* J. B. Schenkman and D. L. Cinti, unpublished observations.

REFERENCES

1. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3**, 113 (1967).
2. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3**, 516 (1967).
3. J. B. SCHENKMAN, *Hoppe-Seyler's Z. physiol. Chem.* **349**, 1624 (1968).
4. D. DAVIES, P. L. GIGON and J. R. GILLETTE, *Life Sci.* **8**, 85 (1969).
5. J. B. SCHENKMAN, J. A. BALL and R. W. ESTABROOK, *Biochem. Pharmac.* **16**, 1071 (1967).
6. J. H. DEWAIDE and P. TH. HENDERSON, *Biochem. Pharmac.* **17**, 1901 (1968).
7. J. B. SCHENKMAN and R. SATO, *Molec. Pharmac.* **4**, 613 (1967).

Biochemical Pharmacology, Vol. 19, pp. 2400–2403. Pergamon Press. 1970. Printed in Great Britain

An immunological study of an enzyme made by phage containing 5-iodo-2′-deoxyuridine-substituted deoxyribonucleic acid

(*Received* 9 *December* 1969; *accepted* 22 *January* 1970)

T4 PHAGE in which 5-iodo-2′-deoxyuridine (IUdR) has replaced more than 60 per cent of DNA-thymidine (60-heavy phage) are unable to induce normal levels of the enzyme dCMP hydroxymethylase in *E. coli*.[1] Since IUdR in DNA may alter the codon for dCMP hydroxymethylase, an attempt was made to determine if an altered enzyme fraction exists that is biologically inactive but immunologically related to the normal dCMP hydroxymethylase molecules. For this purpose the precipitin reaction between control and anti-dCMP hydroxymethylase mouse ascitic fluids and preparations of dCMP hydroxymethylase from *E. coli* B3 cells infected with normal or 60-heavy T4td8 phage was investigated.

MATERIALS AND METHODS

The dCMP hydroxymethylase for eliciting antibody production was prepared from *E. coli* B cells infected with the amber mutant T4N82 and purified 270-fold, according to the method of Mathews *et al.*[2] The purified enzyme (774 units/ml) in 0·05 ml portions was injected with 0·1 ml Freund's incomplete adjuvant intraperitoneally into mice weekly for 5 weeks, and Sarcoma 180 TG cells were injected simultaneously at the fifth week.[3] The immune ascitic fluid, collected 9 to 16 days later, was centrifuged (2000 *g* for 20 min) and the supernatant fraction was stored at − 20°.

The normal and 60-heavy phage were prepared and used for infection of *E. coli* B3 cells as described previously.[1] The dCMP hydroxymethylase preparations used in the precipitin reaction were only partially purified from the phage-infected cells by the method of Mathews *et al.*[2] This included treatment with streptomycin sulfate, ammonium sulfate fractionation, and treatment with Sephadex G25. This gave a 2- to 3-fold purification.

The general character of the precipitin reaction used in these experiments is demonstrated in the antibody titration. A series of dilutions of antibody was made. A solution of 3% gelatin (0·02 ml) and an appropriate volume of potassium phosphate buffer (50 mM, pH 8·0) were added such that the final volume, after addition of enzyme, was 0·6 ml. The reaction was initiated by the addition of a constant volume of enzyme to each of the tubes and mixing. The mixtures were incubated at 37° for 2 hr, at 4° for 3 days, and then centrifuged. The enzymic activity in the supernatant fluid and in the precipitate was determined.

The dCMP hydroxymethylase assay was performed on 0·2 ml of the supernatant fluid and 0·2 ml of a solution of the precipitate (dissolved in 0·01 ml 3% gelatin and 0·29 ml potassium phosphate buffer). The reaction mixture contained 1 μmole of tetrahydrofolic acid, 20·3 μmoles of β-mercapto-ethanol, 2 μmoles of ^{3}H-dCMP (50 mμc/μmole), and 120 μmoles of potassium phosphate, pH 8·0, in a total volume of 0·2 ml. The reaction vessel was incubated 30 min at 37° and terminated by the