Subramanian, A. R., Holleman, J. W., and Klotz, I. M. (1968), *Biochemistry 7*, 3859.

Toennies, G., and Kolb, J. J. (1951), *Anal. Chem. 23*, 823.

York, J. L., and Fan, C. C. (1971), *Biochemistry 10*, 1659.

Conversion of Retinyl Methyl Ether into Retinol in the Rat *in Vitro**

S. Narindrasorasak† and M. R. Lakshmanan‡

ABSTRACT: In rats retinyl methyl ether (RME) is converted into retinol by everted intestinal sacs, liver slices and liver homogenates. The RME cleavage enzyme of liver is localized in the microsomal fraction, and can be solubilized and stabilized by the preparation of an acetone powder. Mg^{2+} and EDTA have an additive stimulatory effect on the fresh microsomal enzyme but not on the acetone powder preparation. The K_m for RME was found to be 4×10^{-4} M from kinetic studies. Tetrahydropteridine is a required cofactor, also with a K_m of 4×10^{-4} M. The pteridine analog, tetrahydroquinazoline, inhibits the reaction by competing with tetrahydropteridine, and has a K_i of 4.25×10^{-4} M. Molecular oxygen is also required, and NADPH enhances the enzyme activity, presumably by reducing dihydropteridine. Thus, the microsomal enzyme which catalyzes the cleavage of RME to retinol appears to be a typical pteridine requiring monooxygenase.

Previous studies in this laboratory (Narindrasorasak *et al.*, 1971) and elsewhere (Thompson and Pitt, 1963) have shown that retinyl methyl ether (RME),[1] which has good growth-promoting activity (Isler *et al.*, 1949), is efficiently converted into retinol when fed to rats. Analysis of the kinetics of uptake, storage, and cleavage indicated that the intestine and liver were probably the major conversion sites (Narindrasorasak *et al.*, 1971). In this paper, we wish to report the oxidation of RME into retinol by liver slices, everted intestinal sacs, and a microsomal enzyme of rat liver. The enzyme has been characterized as a typical pteridine requiring monooxygenase.

Materials and Methods

Preparation of [15-^{3}H]RME. [$^3H_4$]LiAl (5 mCi, specific activity 1.94 mCi/mg) was dissolved in dry diethyl ether and cooled to −15°. *all-trans*-Retinal (5 mCi) in dry diethyl ether was added to the [$^3H_4$]LiAl solution drop by drop until the yellow color of retinal disappeared, which indicated the end point for the reduction of retinal to retinol. One drop of a saturated solution of nonradioactive $LiAlH_4$ in diethyl ether was then added to reduce the last traces of retinal. The reaction was stopped by the addition of distilled water. The ether layer was washed, dried over anhydrous Na_2SO_4, reduced in volume *in vacuo*, and finally dissolved in petroleum ether (bp 40–60°). The product had the characteristic fluorescence and absorption spectrum (λ_{max} at 325 nm) of retinol. The yield of [15-^{3}H]retinol was 22.7%. [15-^{3}H]Retinol was then mixed with 100 mg of nonradioactive retinol and methylated with dimethyl sulfate by the method described by Hanze *et al.* (1948). The yield of [15-^{3}H]RME[1] from labeled retinol was 12.5%.

Preparation of the Enzyme from Rat Liver. METHOD A. A normal male rat, fasted for 24 hr, was killed by a blow on the head. After perfusing with 0.16 M Krebs–Ringer phosphate buffer (pH 7.4), the liver was removed and homogenized with two volumes of 0.05 M potassium phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer together with four volumes of ice-cold 0.25 M sucrose. Cell debris and nuclei were removed by centrifugation at 400*g* for 5 min in a Sorvall RC-2 refrigerated centrifuge. The mitochondrial fraction was sedimented by centrifugation at 10,000*g* for 10 min, and the microsomes were then collected by centrifuging at 100,000*g* for 60 min in a Beckman Model L2-65 ultracentrifuge. The mitochondrial and microsomal fractions were resuspended in a volume of fresh 0.25 M sucrose equivalent to one-fifth the volume of the original homogenate. The crude homogenate and the mitochondrial, microsomal, and final supernatant fractions were then tested for enzyme activity.

METHOD B. The microsomal fraction obtained as described in method A was further homogenized with five volumes of cold acetone (−15°) for 2 min in a Waring blender and then quickly filtered through a sintered-glass funnel with suction. The residue was thoroughly washed with cold acetone, dried, and stored in a desiccator at −15°. Although the acetone powder was no more active than the original microsomal enzyme, it could be stored at −15° for 2 weeks without appreciable loss of activity. The acetone powder enzyme was used in the assay for cofactor requirements.

Measurement of Enzyme Activity. [15-^{3}H]RME (5 μg; specific activity 4×10^5 cpm/mg) was solubilized in 0.5 ml of propylene glycol and incubated in a 50-ml Raysorb flask with various subcellular fractions in a final volume of 10 ml of

* From the Biochemistry Department, Faculty of Science, Mahidol University, Bangkok, Thailand. *Received June 7, 1971.* This study was supported by grants from National Institutes of Health (5 ROI-AM 11367), the Rockefeller Foundation (GA-BMS-7006) and the Faculty of Graduate Studies, Mahidol University, Bangkok. Part of this work was conducted by S. N. in partial fulfillment of the requirements of a M.Sc. degree.

† Present address: St. Louis-Chiangmai Malnutrition Center, Chiangmai Medical School, Chiangmai, Thailand.

‡ Present address: Lipid Metabolism Laboratory, Veterans Administration Hospital, Madison, Wisconsin 53705. To whom correspondence should be addressed.

[1] Abbreviation used is: RME, retinyl methyl ether.

TABLE I: Conversion of Tritiated RME into Retinol by Liver Slices and Everted Intestinal Sacs.[a]

Expt No.	Tissue or Soln	RME in Tissue or Soln (cpm)	Retinol in Tissue or Soln (cpm)
1–3	Liver slices	2,240	470
4–11	Serosal fluid	3,250	540
	Intestinal tissue	1,560	50
	Medium	13,110	40

[a] Each value is the average of three liver slice experiments or of eight intestinal sacs. The initial radioactivity present in all cases was 20,000 cpm.

0.02 M potassium phosphate buffer (pH 7.4) in the presence of glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit), NADP (1 mM), NADH (1 mM), 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (0.125 mM), and reduced glutathione. The mixture was incubated in air with slow shaking in a Dubnoff shaker waterbath for 1 hr at 37°. After the addition of 10 ml of acetone, the mixture was extracted three times with 10 ml of diethyl ether. The concentrated lipid extract was analysed by thin-layer chromatography (tlc) (Narindrasorasak *et al.*, 1971) and the product was eluted, dissolved in 10 ml of toluene scintillation fluid, which contained 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per l. of toluene (Bray, 1960), and counted in a Packard TriCarb liquid scintillation spectrometer.

Studies with Liver Slices and Everted Intestinal Sacs. Normal male rats were fasted for 24 hr and killed by a blow on the head. The livers were perfused well with cold 0.9% NaCl, removed, chilled in ice, and sliced on a Stadie-Riggs tissue slicer. Slices weighing a total of about 2 g were added to separate 50-ml Ray-sorb flasks containing 50 μg of [^{3}H]RME (4×10^5 cpm/mg) and the appropriate cofactors, as mentioned above, in 10 ml of 0.16 M Kreb's–Ringer phosphate buffer (pH 7.4). After incubation in air at 37° for 2 hr, the tissues were washed with 0.9% NaCl and extracted by the method described by Glover *et al.* (1948).

Intestines were washed with cold 0.9% NaCl, cut into two halves, and everted. After being filled with 10 ml of 0.016 M Krebs–Ringer phosphate buffer (pH 7.4) and ligated, the everted sac was incubated at 37° for 2 hr with 50 μg of [^{3}H]-RME (4×10^5 cpm/mg) and the appropriate cofactors, mentioned above, in 20 ml of 0.016 M Krebs–Ringer phosphate (pH 7.4). The serosal fluid and incubation medium were separately extracted with acetone and ether, and the intestinal sac was extracted by the method of Glover *et al.* (1948).

Lipid extracts were analyzed and radioactive fractions were counted as described above.

Reagents. The nucleotide cofactors (NADH, NADP), and glucose 6-phosphate were obtained from Sigma Chemical Co., glucose-6-phosphate dehydrogenase from the Calbiochem Co., 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine from the Aldrich Chemical Co., and *all-trans*-retinol and -retinal from Distillation Products Industries, Rochester, N. Y. RME was a generous gift from Professor O. Isler, Hoffmann-LaRoche, Inc., Basel, Switzerland. [$^3H_4$]LiAl was obtained from the New England Nuclear Corp.

TABLE II: The Effect of Various Metal Ions on the Conversion of RME to Retinol by 9000*g* Supernatant Solutions of Rat Liver.[a]

Additions	Retinol Formed (cpm)
None	418
$+Mg^{2+}$	830
$+Mn^{2+}$	808
$+Fe^{2+}$	414
$+Fe^{3+}$	380
$+Cu^{2+}$	135
+EDTA	760
EDTA + Mg^{2+}	1167

[a] Whole homogenate or a 9000*g* supernatant fraction equivalent to that contained in 1.5 g of original liver was incubated at 37° for 1 hr with 50 μg of [^{3}H]RME (specific activity 4×10^5 cpm/mg) and cofactors, as described in the Materials and Methods section, in phosphate buffer (pH 7.4). Wherever indicated, metal ions and/or EDTA were added at a final concentration of 1 mM before incubation.

Results

Conversion of RME into Retinol by Liver Slices and Everted Intestinal Sacs. When liver slices were incubated with [^{3}H]-RME, 14% of the substrate was taken up by the tissue, of which about 17% was converted into retinol (Table I). In the experiments with everted intestinal sacs, over 25% of the RME added was found either in the serosal fluid or in the intestinal tissue (Table I). Of the radioactivity present in the serosal fluid, 14% was in retinol and the remainder in the methyl ether. Apparently both RME and retinol can be transported into the serosal fluid from intestinal tissue.

TABLE III: Intracellular Distribution of Enzyme Activity in Rat Liver.[a]

Source of Enzyme	Retinol Formed (cpm)
Whole homogenate	920
Mitochondria + microsomes + supernatant	1318
Mitochondria	522
Microsomes	1468
Supernatant	614
Mitochondria + microsomes	1404
Mitochondria + supernatant	316
Microsomes + supernatant	1526
Boiled microsomes + boiled supernatant	11
Boiled microsomes + supernatant	249
Microsomes + boiled supernatant	1429

[a] Various fractions, equivalent to those contained in 500 mg of liver, were incubated at 37° for 1 hr with 50 μg of [^{3}H]RME (specific activity 4×10^5 cpm/mg) and cofactors as described in the Materials and Methods section. EDTA (10^{-3} M) and $MgCl_2$ (10^{-3} M) were also added to each flask to obtain maximal activity.

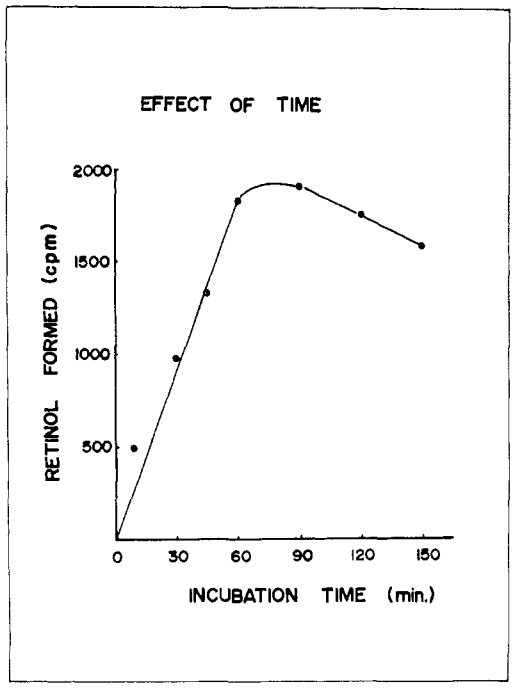

FIGURE 1: Enzymatic cleavage of RME with respect to time. Microsomal fractions each equivalent to 500 mg of rat liver were incubated at 37° with 50 μg of [^{3}H]RME (specific activity 4 $\times$ 10^5 cpm/mg) under standard conditions as described in the Materials and Methods section for varying intervals of time. EDTA (10^{-3} M) and Mg^{2+} (10^{-3} M) were added to each flask to obtain maximal activity.

The Effect of Metal Ions on RME Conversion in Liver Homogenates. A liver homogenate, or a 9000*g* supernatant fraction which contained microsomes, was able to convert RME into retinol. Magnesium and manganese ions as well as EDTA stimulated retinol formation significantly, whereas cupric ion was inhibitory (Table II). Ferric and ferrous ions had little or no effect.

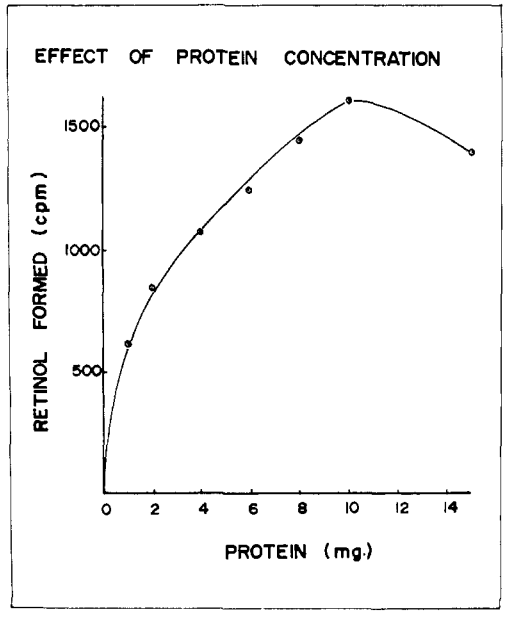

FIGURE 2: Effect of enzyme concentration. [^{3}H]RME (50 μg; specific activity 4 $\times$ 10^5 cpm/mg) was incubated at 37° for 1 hr under standard conditions as described in the Materials and Methods section, with varying amounts of the microsomal fraction. EDTA (10^{-3} M) and Mg^{2+} (10^{-3} M) were added to each flask to obtain maximal activity.

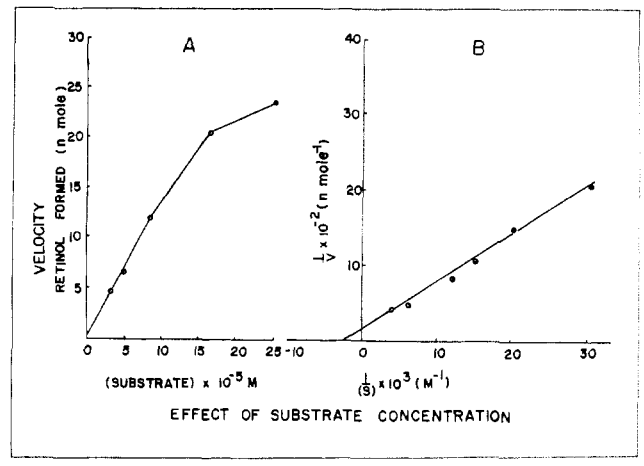

FIGURE 3: (A) Effect of substrate concentration. The microsomal protein fraction (10 mg) was incubated at 37° for 1 hr under standard conditions as described in the Materials and Methods section, with the indicated final concentrations of RME in a volume of 2 ml. (B) Lineweaver–Burk plots for the rat liver microsomal enzyme, drawn from data given in Figure 3A.

Subcellular Localization of the Enzyme System. The crude microsomal fraction was about twice as active as either the mitochondrial or supernatant fractions, and its activity was not enhanced by the addition of other fractions (Table III). Boiled microsomes were inactive.

The Effect of Incubation Time, Enzyme Concentration, and Substrate Concentration on RME Cleavage by the Microsomal Fraction. The rate of RME cleavage was linear over the first 60 min and thereafter gradually declined (Figure 1). When increasing amounts of microsomal protein were present, the rate of retinol formation increased to a maximum, at about 10 mg of protein, and then declined (Figure 2). The effect of substrate concentration on the rate of retinol formation is shown in Figure 3A, and the Lineweaver–Burk plot of the same data is presented in Figure 3B. The K_m for RME cleavage was about 4 $\times$ 10^{-4} M and the V_{max} was about 6.6 nmole/hr per mg of protein at 37°.

Cofactor Requirements for RME Cleavage. Unlike intact microsomes, the enzyme in acetone powder preparations was unaffected by the addition of Mg^{2+} and EDTA (Table IV). Apparently heavy metal inhibitors present in the fresh microsomal preparation were removed by acetone treatment.

In keeping with the cofactor requirements of some other monooxygenases, the cleavage of RME seemed to require tetrahydropteridine as a cofactor. When either tetrahydropteridine or NADPH was omitted, for example, about one-third of the activity was lost. The addition of the tetrahydropteridine inhibitor, 2,4-diamino-5,6,7,8-tetrahydroquinazoline, to reaction flasks lacking either tetrahydropteridine or NADPH further reduced the activity by 10–30% (Table IV). When tetrahydroquinazoline was added in the absence of *both* tetrahydropteridine and NADPH, the activity was sharply reduced further to a basal level (*ca.* 300 cpm of retinol).

A more striking activation of the enzyme by tetrahydropteridine could be shown by preincubating the acetone powder enzyme, dissolved in 0.2 M phosphate buffer, with 10^{-3} M tetrahydroquinazoline at 0° for 1 hr, followed by dialysis against 0.02 M phosphate buffer for 4 hr with one change of

TABLE IV: Cofactor Requirements for the Conversion of RME to Retinol by the Acetone Powder Preparation.

Treatment	Retinol Formed (cpm)
Complete system	1496
All cofactors omitted	266
Mg^{2+} omitted	1498
EDTA omitted	1478
Mg^{2+} and EDTA omitted	1480
Tetrahydropteridine omitted	1065
Enzyme preincubated with 10^{-3} M tetrahydroquinazoline, tetrahydropteridine omitted	622
Tetrahydropteridine omitted, tetrahydroquinazoline added	632
NADPH omitted	1052
NADPH-generating system omitted	1208
Enzyme preincubated with 10^{-3} M tetrahydroquinazoline, NADPH omitted	874
NADPH generating system and tetrahydropteridine omitted, tetrahydroquinazoline added	320
Enzyme preincubated with 10^{-3} M tetrahydroquinazoline, tetrahydropteridine and NADPH omitted	293

buffer. Although some residual activity still existed, a marked stimulation of RME cleavage was observed in the presence of increasing concentrations of tetrahydropteridine (Table V). The K_m for tetrahydropteridine, determined from a Lineweaver–Burk plot (Figure 4A) was 3×10^{-4} M. Tetrahydroquinazoline competitively inhibited tetrahydropteridine, as indicated by the same Lineweaver–Burk plot (Figure 4A). A Dixon plot of the same data (Figure 4B) gave a K_i of 4.25×10^{-4} M for tetrahydroquinazoline. The inhibitory action of any concentration of tetrahydroquinazoline could be completely reversed by the addition of tetrahydropteridine.

Requirement for Molecular Oxygen. The replacement of air or oxygen by a helium–butane mixture reduced the activity of the complete enzyme system to about one-third of normal (Table VI). Since the K_m for oxygen of most oxygenases is low and the removal of the last traces of oxygen from biological

TABLE V: Requirement of Tetrahydropteridine for Optimal Activity of RME-Cleavage Enzyme.

Concn of Tetrahydropteridine Added (M)	Retinol Formed (cpm)
No tetrahydropteridine added	346
6.25×10^{-5}	977
1.25×10^{-4}	1523
2.5×10^{-4}	1692
5×10^{-4}	1650
1×10^{-3}	1895
2×10^{-3}	1911
Nonpreincubated enzyme + 1.25×10^{-4}	1339

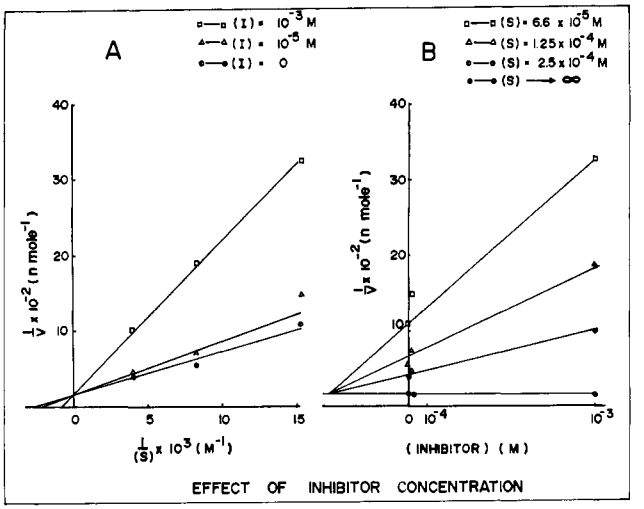

FIGURE 4: Competitive inhibition of RME cleavage by tetrahydroquinazoline. The apoRME enzyme was prepared as described in the text, and the assay was carried out with the indicated final concentrations of the inhibitor under standard conditions as described under the Materials and Methods section. Lineweaver–Burk plots of the reaction are given at various inhibitor concentrations in part A, and Dixon plots are depicted in part B.

preparations is both tedious and difficult, the residual activity is not unexpected.

Discussion

The observations made in this paper on the conversion of RME to retinol *in vitro* mesh nicely with earlier studies on RME metabolism *in vivo* (Narindrasorasak *et al.*, 1971). The serosal fluid in experiments with everted intestinal sacs, for example, contained appreciable amounts of both RME and retinol, an observation in keeping with the fact that a significant portion of a large oral dose of RME is transported across the intestine and is initially stored as RME in the liver. The subsequent decrease in the relative amount of RME and increase in retinyl ester in the liver implies that the liver also contains the cleavage enzyme, a suggestion fully confirmed by present studies with slices and subcellular fractions of liver. Although other tissues might also contain the cleavage enzyme, the intestine and the liver are probably the most active tissues (Pfleger *et al.*, 1967) in ether cleavage, and certainly would be most involved when RME is administered orally.

The cleavage enzyme is mainly localized in the microsomal fraction, as is the monooxygenase which cleaves glyceryl ethers

TABLE VI: Requirement of Molecular O_2 for the Maximal Activity of the RME-Cleavage Enzyme.[a]

Gas Phase	Retinol Formed (cpm)
Normal air (control)	1611
Oxygen	1478
95% helium–5% butane gas	587

[a] The acetone powder preparation with all cofactors added was assayed in the indicated gas phase by the use of Thunberg tubes.

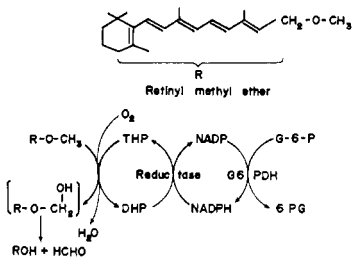

FIGURE 5: Postulated reaction scheme for the oxidation of RME.

(Tietz *et al.*, 1964 Pfleger *et al.*, 1967). The cleavage activity found in mitochondrial and 100,000*g* supernatant fractions is probably due to contamination with the microsomal fraction, inasmuch as combination experiments between various active and boiled fractions gave no indication of a separate enzyme in other fractions. These experiments also showed that the microsomal enzyme was neither stimulated nor inhibited by factors present in other subcellular fractions.

The 9000*g* supernatant fraction, which contained microsomes + cytosol, but not the microsomal acetone powder preparation, was activated by EDTA. Presumably some heavy metal inhibitor initially present in the fresh microsomal preparation was removed during acetone precipitation of the proteins. Similarly Mg^{2+} ions enhanced the cleavage rate in fresh microsomes but not in the acetone powder preparation. Although Mg^{2+} also stimulates the activity of a brain enzyme which hydrolyzes the vinyl ether linkage of ethanolamine plasmalogen (Ansell and Spanner, 1965), it has little effect on the activity of a liver enzyme cleaving aromatic ethers (Axelrod, 1956), and is not required for the oxidation of glyceryl ethers (Tietz *et al.*, 1964, Pfleger *et al.*, 1967). Although monooxygenases often possess tightly bound heavy metal ions at catalytic sites, added Mg^{2+} is rarely if ever required (Hayaishi, 1969). In all likelihood, therefore, neither Mg^{2+} nor EDTA play any basic role in RME cleavage.

The enzyme cleaving RME is clearly a pteridine containing monooxygenase, along with an increasing number of other such enzymes (Hayaishi, 1969). Tetrahydropteridine seems to be tightly bound to the enzyme, and could only be removed by preincubation with tetrahydroquinazoline followed by dialysis. From the Lineweaver–Burk and Dixon plots of the kinetics of inhibition, tetrahydroquinazoline was shown to inhibit the enzyme competitively. The K_i for the analog (4.25×10^{-4} M) is only slightly higher than the K_m for tetrahydropteridine (4×10^{-4} M). The fact that 0.625 mM tetrahydropteridine can completely reverse the inhibition caused by 1 mM tetrahydroquinazoline implies that the affinity of the enzyme for the normal substrate is significantly higher than for its analogue.

The stimulation of RME cleavage by NADPH is also a common property of several pteridine requiring oxygenases (Hayaishi, 1969). Presumably the microsomal fraction contains a dihydropteridine reductase which employs NADPH to reduce dihydropteridine to the tetrahydro derivative, which in turn reduces molecular oxygen during ether cleavage. These enzymatic relationships are summarized in Figure 5.

It seems unlikely that an enzyme specific for RME cleavage exists. Firstly, RME is a synthetic substrate which does not occur naturally, and secondly, many relatively nonspecific monooxygenases are localized in the microsomes (Hayaishi, 1969). The glyceryl ether cleavage enzyme of the microsomes, for example, has a K_m for glyceryl ethers of 2.5×10^{-4} M (Kapoulas *et al.*, 1969) almost identical with the K_m for RME (4×10^{-4} M), requires tetrahydropteridine and molecular oxygen, and utilizes NADPH as the ultimate reductant. Whether or not the RME cleavage enzyme is inducible, as are microsomal monooxygenases which inactivate drugs, has yet to be determined.

Acknowledgment

The authors express their sincere appreciation to Dr. J. A. Olson for his constructive criticism during the course of this work, and to Miss Patchari Karnasuta for secretarial assistance.

References

Ansell, G. B., and Spanner, S. (1965), *Biochem. J. 94*, 252.

Axelrod, J. (1956), *Biochem. J. 63*, 634.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Glover, J., Goodwin, T. W., and Morton, R. A. (1948), *Biochem. J. 43*, 109.

Hanze, A. R., Conger, T. W., Wise, E. C., and Weisblat, D. I. (1948), *J. Amer. Chem. Soc. 70*, 1253.

Hayaishi, O. (1969), *Ann. Rev. Biochem. 38*, 21.

Isler, O., Ronco, A., Guex, W., Hindley, N. C., Huber, W., Dialer, K., and Kofler, M. R. (1949), *Helv. Chim. Acta 32*, 489.

Kapoulas, V. M., Thompson, G. A., and Hannahan, D. J. (1969), *Biochim. Biophys. Acta 176*, 250.

Narindrasorasak, S., Pimpa, P., and Lakshmanan, M. R. (1971), *Biochem. J. 122*, 427.

Pfleger, R. C., Piantadosi, C., and Snyder, F. (1967), *Biochim. Biophys. Acta 144*, 633.

Thompson, J. N., and Pitt, G. A. J. (1963), *Biochim. Biophys. Acta 78*, 753.

Tietz, A., Lindberg, M., and Kennedy, E. P. (1964), *J. Biol. Chem. 239*, 4081.