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EFFECT OF RIBOFLAVIN DEFICIENCY ON PHENOBARBITAL AND 3-METHYLCHOLANTHRENE INDUCTION OF MICROSOMAL DRUG-METABOLIZING ENZYMES OF THE RAT

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Abstract—The effect of riboflavin deficiency on the induction of hepatic microsomal enzymes by phenobarbital and 3-methylcholanthrene has been investigated. A decrease in microsomal flavin levels of 56 per cent was associated with a decrease in NADPH cytochrome *c* reductase (52 per cent), azoreductase (71 per cent) and benzpyrene hydroxylase (74 per cent). Microsomal cytochrome P-450 content and aminopyrine demethylase were not significantly affected by flavin deficiency. Phenobarbital or 3-methylcholanthrene pretreatment did not affect hepatic microsomal flavin levels in normal or deficient animals. In flavin-deficient animals, phenobarbital pretreatment significantly increased cytochrome *c* reductase, cytochrome P-450 content, aminopyrine demethylase and azoreductase. Thus the carbon monoxide-sensitive pathway (cytochrome P-450 mediated) of azoreductase was essentially unaffected by flavin deficiency. In deficient animals, the carbon monoxide-insensitive microsomal azoreductase pathway (non-cytochrome P-450 mediated) normally induced by 3-methylcholanthrene was unaffected. Thus, induction of azoreductase by 3-methylcholanthrene was found to be flavin dependent. However, 3-methylcholanthrene did increase cytochrome P-450 content and benzpyrene hydroxylase in flavin-deficient animals. The induction of benzpyrene hydroxylase by 3-methylcholanthrene increased with increasing microsomal flavin content. Part of the mechanism of azoreductase induction by 3-methylcholanthrene was due to an induced change in the structure or composition of microsomal flavoprotein. This interpretation is supported by the findings that: (1) induction by 3-methylcholanthrene in riboflavin-deficient rats required a minimal flavin level, (2) increased enzyme activity was not compensated by an increase in microsomal flavin and (3) induction by 3-methylcholanthrene augmented FMN-stimulation of microsomal azoreductase *in vitro*.

THE ACTIVITY of hepatic microsomal drug-metabolizing enzymes can be altered by a variety of factors including age, sex, environment, nutritional status and drug pretreatment. The administration of the inducing agents phenobarbital (PB) and 3-methylcholanthrene (3-MC) to animals increases the activity of these microsomal enzymes.[1] However, there are differences in the pattern of enzymes which are induced by these agents.[1,2]

The reduction of the azo compound, neoprontosil, utilizes the same microsomal enzymes involved in oxidative drug metabolism. Studies by Hernandez *et al.*[3]

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demonstrated that neoprontosil was reduced by at least three microsomal enzyme systems which used reduced NADPH as the electron donor. These include: (1) cytochrome P-450, (2) NADPH cytochrome *c* reductase and (3) a third NADPH specific pathway which can be demonstrated indirectly by differential induction utilizing 3-methylcholanthrene and phenobarbital.

Pretreatment with the inducing agent, phenobarbital, will increase the activity of the NADPH pathway involving cytochrome P-450. Increased azoreductase activity after phenobarbital induction corresponded directly to an increase in cytochrome P-450 content. In addition, carbon monoxide, which blocks cytochrome P-450, inhibited the phenobarbital-induced microsomal azoreductase activity. The NADPH specific pathway which was induced by phenobarbital and inhibited by carbon monoxide has been termed the carbon monoxide-sensitive pathway of hepatic microsomal azoreductase. On the other hand, 3-methylcholanthrene induced a NADPH specific pathway in hepatic microsomes which was independent of an increase in cytochrome P-450, since carbon monoxide failed to inhibit completely the 3-methylcholanthrene-induced azoreductase activity. Moreover, 3-methylcholanthrene did not stimulate microsomal NADPH cytochrome *c* reductase activity. Thus, 3-methylcholanthrene induced a NADPH specific pathway which was carbon monoxide insensitive.[3,4]

The importance of flavins in the reductive pathways has been demonstrated by Kensler[5] who found a direct correlation between hepatic flavin levels and the ability of rat liver slices to reduce the carcinogenic azo dye, dimethylamino azobenzene. Furthermore, the addition of various flavins, *in vitro*, stimulated the reduction of nitro compounds[6] and azo compounds.[7,8] Moreover, inducing agents such as phenobarbital and 3-methylcholanthrene which increased microsomal azoreductase activity failed to increase microsomal flavin content.[9,10]

This report investigates the effect of flavin deficiency on microsomal oxidative and reductive pathways with particular emphasis on microsomal azoreductase.

MATERIALS AND METHODS

Chemicals

Most reagents and solvents were purchased from J. T. Baker Chemical Co., including Dowex 50W-X8 and were used without further purification. Sodium phenobarbital was purchased from Merck & Co., Inc., and 3-methylcholanthrene from Mann Research Laboratories, Inc. NADP, NADPH, glucose 6-phosphate, nicotinamide, riboflavin, FMN, FAD, cytochrome *c* (horse heart, type III), NED [N-(1-naphthyl)-ethylenediamine diHCl] were purchased from Sigma Chemical Co. and ultra high purity nitrogen from Southern Oxygen, Division of Air Products; carbon monoxide from Matheson Co.; crystalline bovine serum albumin from Armour Pharmaceuticals Co. Aminopyrine was purchased from Matheson, Coleman & Bell. Riboflavin-deficient diet was supplied by Nutritional Biochemicals Corp. Neoprontosil was generously supplied by Sterling Winthrop Research Institute, Rensselaer, N.Y.

Animals

Riboflavin-deficient rats. Weanling 40–50 g male Sprague–Dawley rats were maintained either on a riboflavin-deficient diet (riboflavin-deficient rats) or on the

same diet supplemented with 30 mg of riboflavin (RF)/kg of diet (normal diet rats). The rats were allowed to eat and drink freely.

After 18 days, those rats which showed typical signs of riboflavin deficiency, including absence of weight gain, alopecia and scaly skin,[11] were divided into four groups: (1) control groups which received either corn oil or saline, (2) drug-treated groups which received either 3 MC or PB, (3) a riboflavin-treated group and (4) a group which received either 3-MC + RF or PB + RF. 3-Methylcholanthrene was dissolved in corn oil and injected i.p., 40 mg/kg daily for 4 days. Phenobarbital was injected i.p., 90 mg/kg in normal saline daily for 3 days. Riboflavin was injected i.p., 10 mg/kg in normal saline daily for 3 days.

The normal diet rats (RF supplemental) were divided into two groups: (1) a control group which received either corn oil or saline and (2) a drug-treated group which received either 3-MC or PB.

After pretreatment, the animals were sacrificed, approx. 21 or 22 days from the start of diet. Microsomes were prepared as previously described by Hernandez *et al.*[3]

Enzyme assays

Microsomal azoreductase was assayed by the method of Hernandez *et al.*[12] All values were corrected for non-enzymatic reduction due to the NADPH generating system. The non-enzymatic generating system consisted of 0·5 μmole of $NADP^+$, 50 μmoles of glucose 6-phosphate, 100 μmoles of nicotinamide, soluble fraction (105,000 g liver supernatant equivalent to 250 mg of liver obtained from normal diet rats), 10 μmoles of neoprontosil, 5 μmoles of EDTA, 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6, in a final incubation volume of 5·0 ml. The reduction of neoprontosil by the NADPH generating system was considered non-enzymatic. The millimicromoles of sulfanilamide formed after incubation with the NADPH generating system (non-enzymatic activity) was subtracted from the millimicromoles of sulfanilamide obtained after incubation of the complete incubation system (total activity) which included the liver microsomal fraction. Non-enzymatic reduction of neoprontosil was generally less than 10 per cent of the total reduction obtained by the complete incubation system. Nicotinamide was in the incubation mixture to prevent the inactivation of NADPH by microsomal enzymes. NADPH cytochrome *c* reductase was measured according to the procedure of Philips and Langdon.[13] *N*-demethylation was measured by determining the amount of formaldehyde formed.[14] Benzpyrene hydroxylase was kindly performed by Dr. H. V. Gelboin (National Cancer Institute, National Institute of Health, Bethesda, Md.) according to the procedure of Nebert and Gelboin.[15]

Cytochrome P-450 was determined according to the procedure of Omura and Sato[16] using a Cary model 15 spectrophotometer and the extinction coefficient of 91 mM^{-1} cm^{-1}. Protein was measured by the method of Lowry *et al.*[17] as modified by Miller[18] using crystalline bovine serum albumin as the standard. The determination of flavins was performed by a modification of the procedure of Burch[19] as described by Udenfriend[20] which differentiates between FAD-flavin and total tissue flavin.

Kinetics of the enzymatic reduction of neoprontosil have been studied by Hernandez *et al.*[12] in hepatic microsomal enzymes from normal diet rats. This study demonstrated that the reductase enzyme(s) could not be saturated with neoprontosil. Although complete kinetics were not performed on hepatic microsomal enzymes from

flavin-deficient rats, cofactors were added in the same concentrations as the previous study.[12] These cofactor concentrations did not appear to be rate limiting.

RESULTS

Effect of phenobarbital and 3-methylcholanthrene pretreatment on hepatic microsomal flavin levels. The total microsomal flavin levels of the control riboflavin-deficient rats were less than half of the value for the normal diet rats (Table 1). Administration of riboflavin to deficient rats resulted in microsomal and total flavin levels which were between those of the completely deficient and normal rats. Pretreatment of rats with phenobarbital or 3-methylcholanthrene did not significantly affect the flavin content in the total liver or microsomal fraction.

TABLE 1. EFFECT OF RIBOFLAVIN DEFICIENCY AND 3-METHYLCHOLANTHRENE AND PHENOBARBITAL PRETREATMENT ON HEPATIC MICROSOMAL FLAVIN LEVELS*

Treatment	Total flavin	FAD
Riboflavin deficient diet		
Saline-control (4)	0·17 ± 0·01	0·14 ± 0·01
Phenobarbital (3)	0·15 ± 0·01	0·15 ± 0·01
Corn-oil-control (4)	0·21 ± 0·02	0·14 ± 0·02
3-Methylcholanthrene (4)	0·15 ± 0·01	0·11 ± 0·01
Normal diet		
Saline-control (3)	0·39 ± 0·04	0·30 ± 0·04
Phenobarbital (3)	0·39 ± 0·04	0·30 ± 0·04
Corn oil-control (4)	0·43 ± 0·03	0·24 ± 0·03
3-Methylcholanthrene (4)	0·49 ± 0·03	0·25 ± 0·02

* Phenobarbital (90 mg/kg) in saline or 3-methylcholanthrene (40 mg/kg) in corn oil was administered for 3 days prior to sacrifice. The results are expressed as the mean ± S. E. of the values obtained from three or four separate groups of two pooled livers per group. Flavin concentration is expressed as millimicromoles per milligram of protein and was measured by the fluorimetric procedure as described in Materials and Methods.

Effect of riboflavin deficiency and pretreatment on microsomal cytochrome c reductase and cytochrome P-450. The effect of riboflavin deficiency, phenobarbital and 3-methylcholanthrene pretreatment on microsomal NADPH cytochrome *c* reductase and cytochrome P-450 is shown in Table 2.

Riboflavin deficiency caused a marked decrease (52 per cent) in the enzymatic reduction of cytochrome *c* by hepatic microsomes. Cytochrome P-450 content was slightly decreased in riboflavin-deficient animals. Interestingly, phenobarbital pretreatment significantly stimulated NADPH cytochrome *c* reductase (46 per cent) and cytochrome P-450 formation (182 per cent) in the deficient animals. 3-Methylcholanthrene pretreatment increased cytochrome P-450 in deficient animals by 108 per cent but did not affect NADPH cytochrome *c* reductase activity. 3-Methylcholanthrene has been shown not to affect NADPH cytochrome *c* reductase.[3] Thus, measurement of these two microsomal parameters indicated induction even though microsomal flavin levels were reduced by 56 per cent.

TABLE 2. EFFECT OF RIBOFLAVIN DEFICIENCY, PHENOBARBITAL AND 3-METHYLCHOLANTHRENE PRETREATMENT ON NADPH CYTOCHROME *c* REDUCTASE AND CYTOCHROME P-450*

Treatment	NADPH–cytochrome *c* reductase†	Difference from controls (%)	Cytochrome P-450‡	Difference from controls (%)
Riboflavin-deficient diet				
Control (4)	12·8 ± 1·0		0·56 ± 0·06	
Phenobarbital (3)	18·7 ± 4·1	+ 46	1·58 ± 0·11	+ 182
3-Methylcholanthrene (4)			1·17 ± 0·02	+ 108
Normal diet				
Control (4)	26·3 ± 7·1		0·67 ± 0·05	
Phenobarbital (3)	41·0 ± 5·6	+ 60	1·27 ± 0·06	+ 90
3-Methylcholanthrene (4)	27·6 ± 1·3		1·10 ± 0·02	+ 64

* Phenobarbital (90 mg/kg) in saline or 3-methylcholanthrene (40 mg/kg) in corn oil was administered for 3 days prior to sacrifice. The results are expressed as the mean ± S. E. of the values obtained from 3 or 4 separate groups of two pooled livers per group.
† Millimicromoles of cytochrome *c* reduced per min per milligram of protein.
‡ Millimicromoles per milligram of protein.

Effect of riboflavin deficiency and pretreatment on microsomal aminopyrine demethylase and benzpyrene hydroxylase. Riboflavin deficiency did not significantly decrease aminopyrine demethylase activity (Table 3). Furthermore, phenobarbital pretreatment increased the *N*-demethylation of aminopyrine by 94 per cent in the deficient animals. Aminopyrine demethylase is not normally induced by 3-MC[1] and therefore was not investigated in this experiment.

In contrast, benzpyrene hydroxylase was markedly decreased in riboflavin-deficient animals. However, 3-MC pretreatment dramatically increased benzpyrene hydroxylase in deficient and control animals. The enzymatic activity also increased in riboflavin-deficient animals when they were administered riboflavin in (10 mg/kg) for 3 days.[21]

TABLE 3. EFFECT OF RIBOFLAVIN DEFICIENCY, PHENOBARBITAL AND 3-METHYLCHOLANTHRENE PRE TREATMENT ON AMINOPYRINE DEMETHYLASE AND BENZPYRENE HYDROXYLASE*

Treatment	Aminopyrine demethylase†	Difference from controls %	Benzpyrene hydroxylase‡	Difference from controls %
Riboflavin-deficient diet				
Control (4)	289 ± 28	+ 94	279 ± 15	
Phenobarbital (3)	561 ± 36			+ 1006
3-Methylcholanthrene (4)			3087 ± 333	
Normal diet				
Control (3)	319 ± 27	+ 139	1075 ± 89	
Phenobarbital (4)	764 ± 39			+ 708
3-Methylcholanthrene (4)			8688 ± 1012	

* Phenobarbital (90 mg/kg) in saline or 3-methylcholanthrene (40 mg/kg) in corn oil was administered i.p. for 3 days prior to sacrifice. The results are expressed as the mean ± S. E. of the values obtained from 3 or 4 separate groups of two pooled livers per group.
† Millimicromoles of HCHO formed per milligram of protein per 30 min.
‡ Millimicromoles of hydroxylated product per milligram of protein per 30 min.

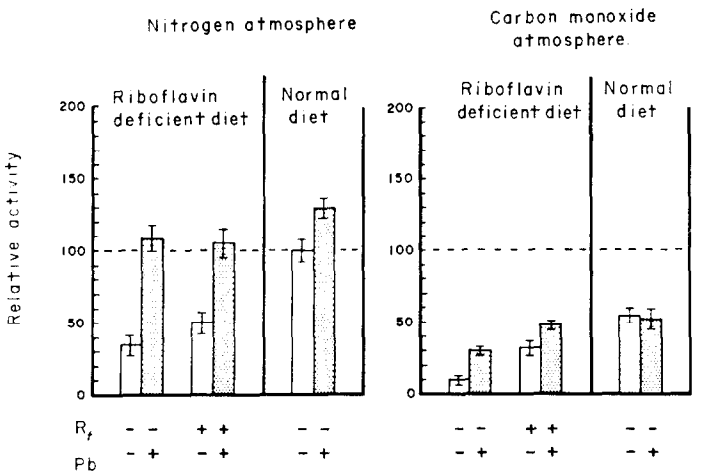

FIG. 1. Effect of riboflavin deficiency and phenobarbital pretreatment on microsomal azoreductase. Phenobarbital (90 mg/kg) and/or riboflavin (10 mg/kg) were administered i.p. for 3 days prior to sacrifice. Azoreductase relative activity = millimicromoles of sulfanilamide formed per 30 min per milligram of protein. The results are expressed as the mean ± S. E. of the values obtained from three or four separate groups of two pooled livers per group.

Effect of riboflavin deficiency and pretreatment on microsomal azoreductase. In riboflavin-deficient animals, with microsomal flavin levels decreased by 56 per cent, azoreductase activity was decreased by 71 per cent (Fig. 1). Phenobarbital pretreatment increased microsomal azoreductase in deficient animals by 212 per cent. Administration of riboflavin to deficient animals also increased enzymatic activity.

In an atmosphere of carbon monoxide, with cytochrome P-450 blocked, azoreductase from normal diet animals was decreased by 45 per cent, whereas in the riboflavin-

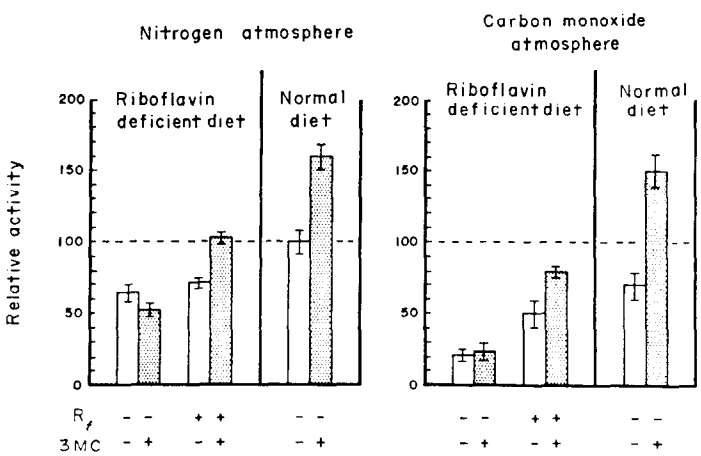

FIG. 2. Effect of riboflavin deficiency and 3-methylcholanthrene pretreatment on microsomal azoreductase. 3-Methylcholanthrene (40 mg/kg) in corn oil and/or riboflavin (10 mg/kg) were administered i.p. for 3 days prior to sacrifice. Azoreductase relative activity = millimicromoles of sulfanilamide formed per 30 min per milligram of protein. The results are expressed as the mean ± S. E. of the values obtained from four separate groups of two pooled livers per group.

deficient rats the decrease was 67 per cent. However, the sensitivity of azoreductase to carbon monoxide was increased in all groups after phenobarbital pretreatment. Thus, it appeared that the CO-sensitive (cytochrome P-450) pathway was still intact in the riboflavin-deficient animals.

In contrast to PB, 3-MC did not induce azoreductase in riboflavin-deficient rats. 3-MC pretreatment induced microsomal azoreductase in normal diet and in riboflavin-pretreated rats (Fig. 2).

The experiments with carbon monoxide demonstrated that 3-methylcholanthrene induced the carbon monoxide-insensitive pathway of microsomal azoreductase only in the riboflavin-pretreated group and normal diet animals.

Riboflavin pretreatment (10 mg/kg i.p., for 7 days) to rats on a nutritionally adequate diet produced no significant alterations in microsomal enzyme activities.[21] The lack of an effect due to high RF intake may be due to the observations by Bessey *et al.*[22] and Christensen[23] that much of the RF injected intraperitoneally in rats is destroyed or rapidly eliminated.

DISCUSSION

The utilization of riboflavin-deficient rats has permitted further characterization of the hepatic microsomal drug-metabolizing enzymes.

The data indicate that microsomal cytochrome P-450 content and aminopyrine demethylase activity were independent of liver flavin levels. The depression of NADPH cytochrome *c* reductase in riboflavin-deficient animals, without a concomitant effect on cytochrome P-450 and aminopyrine demethylase, further supports the contention that NADPH cytochrome *c* reductase is not rate limiting in the drug-metabolizing oxidative pathways.[21]

On the other hand, the ability of hepatic microsomes to hydroxylate 3,4-benzpyrene was depressed in RF deficiency. With increasing microsomal flavin content, benzpyrene hydroxylase activity increased. This finding indicated that the oxidative microsomal pathway which hydroxylates 3,4-benzpyrene was dependent upon a flavoprotein enzyme which was rate limiting. Benzpyrene hydroxylase activity was induced in RF-deficient rats by 3-MC pretreatment. Moreover, the ability to induce benzpyrene hydroxylase by 3-MC pretreatment increased with an increased microsomal flavin.

In agreement with our studies, Catz *et al.*[24] found that *p*-nitrobenzoic acid reductase and 3,4-benzpyrene hydroxylase were reduced in rats maintained on a riboflavin-deficient diet, whereas aminopyrine demethylase was unaffected. Moreover, these investigators found an increase in microsomal cytochrome P-450 content as well. Thus, our studies and those of Catz *et al.*[24] demonstrate that not all mixed-function oxidases are affected by flavin deficiency.

Hepatic microsomes from RF-deficient rats had less azoreductase activity than microsomes from normal diet rats. In addition, azoreductase activity of microsomes from RF-deficient animals was more sensitive to CO inhibition than microsomes from normal diet rats.

The CO-sensitive pathway of microsomal azoreductase which is induced by PB was demonstrated by Hernandez *et al.*[3] to be related to microsomal cytochrome P-450 content. On the other hand, 3-MC induced a CO-insensitive microsomal azoreductase activity which was unrelated to microsomal cytochrome P-450 content. Hepatic

microsomes from RF-deficient rats had the same cytochrome P-450 levels as did normal diet animals. These findings imply that the CO-insensitive pathway of microsomal azoreductase is depressed during RF deficiency. Thus, PB-induced azoreductase activity, whereas 3-MC did not induce azoreductase in RF-deficient animals.

PB and 3-MC pretreatment did not significantly alter the microsomal flavin content in the livers of each group of rats. This has been previously observed.[9,10] Induction by 3-MC required a minimum of flavin, whereas PB induction did not have a minimum flavin requirement. These findings imply that PB and 3-MC pretreatment may cause a shift in the flavin compartment of the microsomes.

Assuming that the flavins, FMN and FAD, are the active moieties of the microsomal drug-metabolizing enzymes, then it is possible that induction by 3-MC and PB may be due to a favorable shift in the configuration of the flavin-prosthetic groups of the enzyme to expose more active centers. The possibility that induction might represent a change in the orientation of the enzymes within the microsomes rather

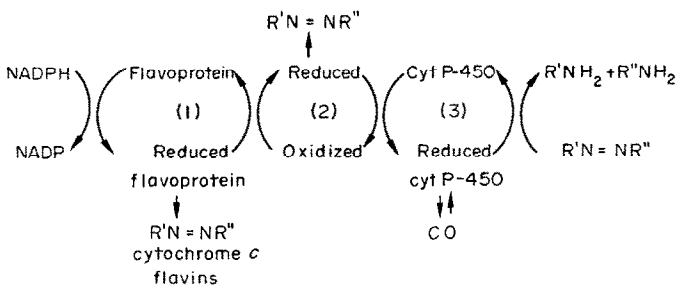

FIG. 3. Effect of flavin deficiency and induction on pathways for the reduction of azo dyes by hepatic microsomal enzymes.

than a change in enzyme concentration has also been suggested by Kamin.[25] While studying the mechanism of NADPH cytochrome *c* reductase, Masters *et al.*[26] were unable to get consistent flavin to protein ratios in order to determine whether this enzyme has one or two molecules of FAD per molecule of enzyme. Thus flavin/protein ratios might be important as part of the mechanism of induction. Moreover, Alvares *et al.*[27] have shown that 3-MC induction altered the apparent K_m for the microsomal hydroxylation of 3,4-benzpyrene, whereas PB induction did not. This could be interpreted as an alteration of microsomal enzyme configuration having occurred due to 3-MC induction.

Differential induction and carbon monoxide sensitivity as reported here for azoreductase have also been observed with the oxidative enzyme systems for microsomal hydroxylations. For example, hydroxylation of testosterone at the 7α position was three times less sensitive to CO inhibition than is hydroxylation at the 16α position.[28] Furthermore, 7α-hydroxylation of testosterone was induced by 3-MC, whereas 16α-hydroxylation of testosterone was induced by PB. Thus, Kuntzman *et al.* [28] found two steroid hydroxylase pathways in liver microsomes which differed in the CO to O_2 ratio for inhibition after induction by PB and 3MC.

The effect of flavin deficiency and induction on the previously described pathways for the reduction of azo-dyes by hepatic microsomal enzymes is shown in Fig. 3.

Pathway (1) (Fig. 3), which represents microsomal azoreductase contributed by NADPH-cytochrome *c* reductase (3), is decreased by flavin deficiency. Pathway (2) is the CO-insensitive 3MC-inducible pathway and is decreased by flavin deficiency. Pathway (3) represents the CO-sensitive pathway which is induced by phenobarbital and little affected by flavin deficiency.

The fact that pathway (2) (Fig. 3) was stimulated by flavins *in vitro*,[29] decreased by RF deficiency, and induced by 3MC identifies (2) as a flavoprotein enzyme.

REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
2. N. E. Sladek and G. Mannering, *Molec. Pharmac.* **5,** 174 (1969).
3. P. H. Hernandez, P. Mazel and J. R. Gillette, *Biochem. Pharmac.* **16,** 1877 (1967).
4. J. R. Gillette and T. E. Gran, in *Microsomes and Drug Oxidative* (Eds. J. R. Gillette *et al.*) p. 133. Academic Press, New York (1969).
5. C. J. Kensler, *Cancer, N.Y.* **1,** 483 (1948).
6. J. R. Fouts and B. B. Brodie, *J. Pharmac. exp. Ther.* **115,** 68 (1955).
7. J. R. Fouts, J. J. Kamm and B. B. Brodie, *J. Pharmac. exp. Ther.* **120,** 291 (1957).
8. L. Shargel, *Fedn Proc.* **26,** 461 (1967).
9. Y. Ichikawa, B. Hagihara and T. Yamano, *Archs Biochem. Biophys.* **120,** 204 (1967).
10. R. W. Estabrook and B. Cohen, in *Microsomes and Drug Oxidations* (Eds. J. R. Gillette *et al.*) p. 95. Academic Press, New York (1969).
11. C. L. Bliss and P. Gyorgy, in *Vitamin Methods* (Ed. P. Gyorgy) Vol. II, p. 201. Academic Press, New York (1951).
12. P. H. Hernandez, J. R. Gillette and P. Mazel, *Biochem. Pharmac.* **16,** 1859 (1967).
13. A. V. Philips and R. G. Langdon, *J. biol. Chem.* **237,** 2652 (1962).
14. P. Mazel, J. F. Henderson and J. J. Axelrod, *J. Pharmac. exp. Ther.* **143,** 1 (1964).
15. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243,** 6242 (1968).
16. T. Omura and R. Sato, *Biochim. biophys. Acta* **71,** 224 (1963).
17. O. H. Lowry, H. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951)
18. G. L. Miller, *Analyt. Chem.* **31,** 964 (1959).
19. H. B. Burch, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan) Vol. III, p. 960. Academic Press, New York (1957).
20. S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, p. 236. Academic Press, New York (1962).
21. J. R. Gillette, D. C. Davis and H. A. Sasame, *A. Rev. Pharmac.* **12,** 57 (1972).
22. O. A. Bessey, O. H. Lowry, E. B. Davis and J. L. Dorn, *J. Nutr.* **64,** 185 (1958).
23. S. Christensen, *Acta pharmac. tox.* **27,** 27 (1969).
24. C. S. Catz, M. R. Juchau and S. J. Yaffee, *J. Pharmac. exp. Ther.* **174,** 197 (1970).
25. H. Kamin, in *Microsomes and Drug Oxidations* (Eds. J. R. Gillette *et al.*), pp. 105–106. Academic Press, New York (1969).
26. B. S. S. Masters, M. H. Bilimoria, H. Kamin and Q. H. Gibson, *J. biol. Chem.* **240,** 4081 (1965).
27. A. P. Alvares, G. R. Schilling and R. Kuntzman, *Biochem. biophys. Res. Commun.* **30,** 588 (1968).
28. R. Kuntzman, W. Levin, M. Jacobson and A. H. Carney, *Life Sci.* **7,** 215 (1968).
29. L. Shargel and P. Mazel, *Toxic. appl. Pharmac.* **12,** 317 (1968).