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INTERACTION OF FLAVINS AND CHLORAMPHENICOL WITH MICROSOMAL ENZYME SYSTEMS*

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Abstract—Possible inter-relationships between flavins and chloramphenicol with drug-metabolizing enzyme systems of liver were studied. Chloramphenicol competitively inhibited *N*-demethylation of aminopyrine, *S*-demethylation of 6-methylmercaptopurine, hydroxylation of acetanilid, *O*-demethylation of *o*-nitroanisole and side chain oxidation of hexobarbital. Riboflavin-5′-monophosphate, FAD and riboflavin were inhibitory to all of the enzyme systems, but reversed the inhibitory effects of chloramphenicol on the *O*-demethylation of *o*-nitroanisole and side chain oxidation of hexobarbital reactions. FMN was a competitive inhibitor of hydroxylation of acetanilid and side chain oxidation of hexobarbital, but a non-competitive inhibitor of the *N*-demethylation of aminopyrine and *S*-demethylation of 6-methylmercaptopurine reactions. The mechanism of inhibition of flavins and chloramphenicol of these enzyme systems is undetermined.

SEVERAL investigators have reported that flavins influence the activity of some of the drug-metabolizing enzymes. Nitroreductase of rabbit liver microsomes[1] and mouse placental homogenates[2] is stimulated anaerobically by flavins, but the reduction is blocked in aerobic conditions.[3] Riboflavin (B_2) also stimulates the nitroreductase enzymes but inhibits the demethylase activity of rat liver.§ Shortly after being placed on a riboflavin-deficient diet, weanling mice showed a decrease in hexobarbital sleeping time, but the sleeping time increased above controls when the diet was continued for several weeks.[4] It has also been reported that riboflavin will stimulate the microsomal azoreductase activity of rabbit liver while inhibiting the neotetrazolium diaphorase activity.[5] All of these reports demonstrate the complexity of flavin interaction with drug-metabolizing enzyme systems of the liver and other tissues.

Recent findings in this laboratory have demonstrated that flavins may interact with chloramphenicol (CAM) to modify the normally toxic effect of this drug. It was shown that injections of riboflavin-5′-monophosphate (FMN) protect guinea pigs from the lethal and bone marrow suppressive actions of CAM[6] and that FMN will reverse the inhibitory effects of CAM on *O*-demethylation of *o*-nitroanisole in the rat.[7] The present investigation was designed to study further this flavin–CAM interaction and to

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§ F. Rosen, M. Hillman and C. A. Nichol, personal communication.

determine whether flavins could also modify the action of CAM[8] on other liver drug-metabolizing enzyme systems.

MATERIALS AND METHODS

Sources of materials. Chemicals were purchased from the sources indicated: glucose-6-phosphate, nicotinamide, NADP, riboflavin-5′-monophosphate and L-phenylalanine from CalBiochem; *o*-nitroanisole and acetanilid from Eastman Organic Chemicals; riboflavin and FAD from Sigma Chemical Co.; aminopyrine from Matheson, Coleman & Bell; 6-methylmercaptopurine from Nutritional Biochemical Corp.; sodium hexobarbital from K & K Laboratories; and semicarbazide hydrochloride from J. T. Baker Chemical Co. Chloramphenicol was supplied gratis by Parke, Davis & Co.

Preparation of animals. Male 25–35 g Swiss albino mice were used for all experiments unless otherwise stated. They were maintained on Purina laboratory chow and water *ad lib.*

Preparation of liver homogenates. All mice were killed by decapitation; livers were quickly removed, and placed immediately in a beaker containing ice-cold 1·15% KCl, pH 7·5. All enzyme studies *in vivo* were conducted using either a 9000 *g* supernatant or a microsomal preparation. To obtain a 9000 *g* supernatant, livers were weighed and homogenized in 2 vol. (w/v) of cold 1·15% KCl, pH 7·5, and centrifuged for 30 min at 9000 *g* at 0°. The pellet was discarded and the supernatant fluid was utilized for the subsequent experiments. To obtain a microsomal preparation the livers were weighed and homogenized in 9 vol. (w/v) of cold 0·25 M sucrose. Nuclei, mitochondria, and other contaminating materials were removed by centrifuging twice for 10 min at 30,000 *g* at 0°. The microsomal pellet was obtained by centrifuging the 30,000 *g* supernatant for 1 hr at 105,000 *g* in the cold. The final pellet was suspended in 0·25 M sucrose, 2 ml for each gram original wet wt of liver. In all experiments the final preparation was used immediately unless otherwise stated.

Reaction mixtures. All experiments utilizing the 9000 *g* supernatant as the enzyme source contained the following components in the incubation system:[9] 9000 *g* supernatant, 1 ml; glucose 6-phosphate, 25 μmoles; $MgSO_4$, 25 μmoles; nicotinamide, 100 μmoles; NADP 0·6 μmole; various concentrations of substrate; and 0·01 M semicarbazide when formaldehyde was measured. Chloramphenicol was used in concentrations ranging from $5{\cdot}0 \times 10^{-5}$ to 10^{-3} M. Concentrations of flavins ranged from 10^{-5} to 10^{-3} M. Incubation mixtures were brought to a final volume of 5 ml with 0·1 M phosphate buffer, pH 7·35, and incubated 1 hr at 37°. These reactions were found to be linear for 1 hr when studied repeatedly at 10-min intervals. Linearity of drug-metabolizing enzyme activity for 60 min and longer has been reported by Gram and Fouts[9] for mouse microsomes.

For the microsomal system, the conditions were the same except for the following: 0·5 ml microsomal suspension, 10^{-3} M CAM, 200 units (according to Bucher) of glucose 6-phosphate dehydrogenase (Nutritional Biochemicals Corp.), and a final volume of 2 ml was used.

Assay procedures. For quantitative determination of the rate of *N*-demethylation of aminopyrine, the rates of formation of 4-aminoantipyrine and of formaldehyde were measured. Formation of formaldehyde was determined using a combination of methods described by Mueller and Miller[10] and Nash[11] as modified by Cochin and Axelrod.[12]

The reaction was terminated by adding 2 ml of 20% trichloroacetic acid. The samples were allowed to stand 10 min in the ice and then were centrifuged 15 min at 2000 *g* in a PR2 International centrifuge at 0°. Four ml of this protein-free supernatant were pipetted into a 50-ml Erlenmeyer flask which contained 10 ml of distilled water. Exactly 10 ml was then distilled off. Two ml of Nash reagent (150 g ammonium acetate and 2 ml of 2,4-pentadione/500 ml) were added to 5 ml of distillate and the solution was heated 30 min in a 60° water bath. The samples were removed and the optical density was measured immediately at 415 nm. The method described by Brodie and Axelrod[13] was utilized to measure formation of 4-aminoantipyrine. The rate of *S*-demethylation of 6-methylmercaptopurine was determined using the method described above for measuring the formaldehyde formed from aminopyrine.

Hydroxylation experiments, utilizing acetanilid as the substrate, were carried out utilizing either formation of *N*-acetyl-*p*-aminophenol or disappearance of substrate as an index of hydroxylation. The method used was that described by Brodie and Axelrod.[14] Hexobarbital metabolism was determined by measuring the amount of hexobarbital remaining after incubation.[15,16]

O-demethylation experiments utilizing *o*-nitroanisole as the substrate were carried out on male albino Holtzman rats (Cheek–Jones Co., Houston, Texas) weighing 75–150 g. In all experiments which utilized *o*-nitroanisole as substrate, a microsomal suspension, prepared according to Netter,[17] was used as the enzyme source. Once prepared, the microsomes were stored at −15° in 1- to 2-ml aliquots and used within 4 days after preparation. During storage, the microsomal preparation did not lose enzyme activity.[2,17] The *O*-demethylation reaction was measured according to Netter.[17] The final reaction mixture was 1·5 ml and contained 1·5 mg of microsomal protein/ml.Incubation was carried out at 37° for 20 min during which time the reaction rate was linear. When experiments *in vivo* were conducted, the rats were injected intramuscularly (i.m.) with CAM (7·5 mg/kg) 1 hr prior to decapitation. Rats were injected with FMN (100 mg/kg) intraperitoneally (i.p.) either 30 min before and 30 min after CAM injection or with 200 mg/kg as a single injection 30 min after CAM injection.

For protein estimation of liver 9000 *g* supernatant or microsomal preparation, the method described by Lowry *et al.*[18] was utilized.

Hexobarbital sleeping time. Male 40–50 g albino Holtzman rats (Cheek–Jones Co., Houston, Texas) were separated at random into ten groups of four animals each. CAM was dissolved in distilled water. FMN was dissolved in 0·85% saline and the solution adjusted to pH 7·3. CAM was injected i.m. and FMN was administered i.p. The concentrations of the above solutions were adjusted so that they could be administered on the basis of 0·01 ml/g of body wt. A 0·75% solution of hexobarbital was prepared. A 2·5% solution of L-phenylalanine in distilled water was used for intraperitoneal injection. Sleeping time was defined as the interval between initial loss and spontaneous return of the righting reflex.

The significance of the difference between means was determined by Student's *t*-test.

RESULTS

Effects of flavins and chloramphenicol on mouse liver N-*demethylation enzyme system.* Figure 1 presents the effects of CAM on *N*-demethylation of aminopyrine as measured by formation of formaldehyde. The apparent K_m of the control reaction is $5{\cdot}4 \times 10^{-4}$

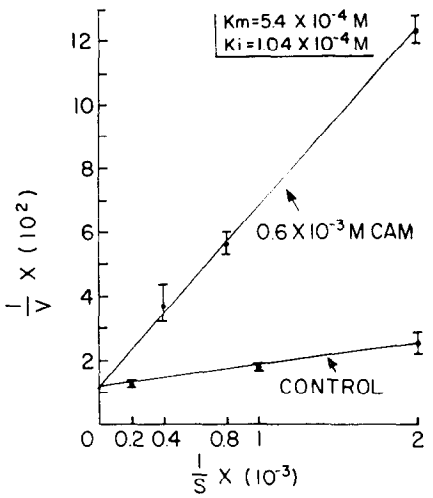

FIG. 1. Inhibition by chloramphenicol of *N*-demethylation of aminopyrine based on formation of formaldehyde. V = micromoles of formaldehyde formed per g of 9000 *g* protein/hr. S = molarity of aminopyrine.

M. CAM is a competitive inhibitor of aminopyrine and the K_i of this inhibited reaction is $1{\cdot}04 \times 10^{-4}$ M. Inhibition of *N*-demethylation of aminopyrine by chloramphenicol is not reversed by flavins, but flavins themselves are inhibitors of the *N*-demethylation reaction. As shown in Table 1, FMN, FAD and riboflavin are all inhibitors

TABLE 1. EFFECTS ON THE *N*-DEMETHYLATION OF AMINOPYRINE BY FMN, FAD AND RIBOFLAVIN (B_2)*

Treatment	Units†	Inhibition (%)
Control	77·8 ± 2·2	0
FMN (10^{-5} M)	81·3 ± 5·3	0
FMN ($2{\cdot}5 \times 10^{-5}$ M)	40·8 ± 2·4	47·6‡
FMN (10^{-4} M)	25·2 ± 3·4	67·6‡
FMN (10^{-3} M)	5·2 ± 1·5	93·3‡
FAD ($2{\cdot}5 \times 10^{-5}$ M)	77·3 ± 3·2	0·7
FAD (10^{-4} M)	46·3 ± 8·7	40·5‡
FAD (10^{-3} M)	29·1 ± 2·2	62·6‡
B_2 (10^{-5} M)	78·3 ± 0·9	0
B_2 ($2{\cdot}5 \times 10^{-5}$ M)	70·1 ± 3·9	9·9
B_2 (10^{-4} M)	30·1 ± 13·4	61·3‡

* The values are the mean ± S. E. from three to nine experiments.
† Micromoles of CH_2O formed per hr/g of 9000 *g* protein.
‡ Significant inhibition compared to control reaction ($P < 0{\cdot}05$).

of the *N*-demethylation reaction, with FMN and riboflavin being most effective. However, at $2{\cdot}5 \times 10^{-5}$ M flavin, FMN was more inhibitory than was riboflavin, and it was, therefore, selected for the subsequent studies.

Both end products of aminopyrine metabolism were measured. Figure 2 shows the effects of FMN on *N*-demethylation of aminopyrine measured by formation of formaldehyde. FMN is a non-competitive inhibitor of aminopyrine and the reaction has a K_i of $2{\cdot}7 \times 10^{-5}$ M. The same results were obtained when 4-aminoantipyrine production was used to measure aminopyrine metabolism (Fig. 3). These data also demonstrate that FMN is a non-competitive inhibitor of aminopyrine metabolism.

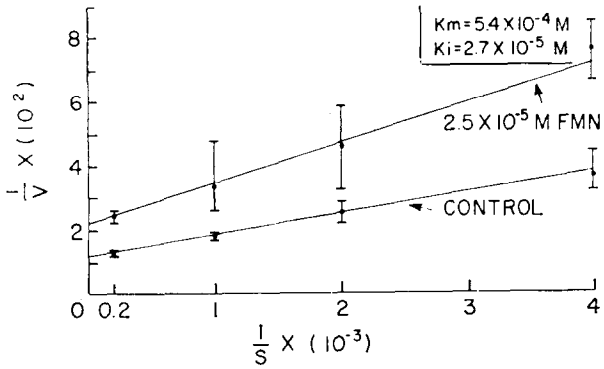

FIG. 2. Inhibition by FMN of *N*-demethylation of aminopyrine based on formation of formaldehyde. V = micromoles of formaldehyde formed per g of 9000 *g* protein per hr. S = molarity of aminopyrine.

The K_i using 4-aminoantipyrine production as an index of aminopyrine metabolism is $2{\cdot}45 \times 10^{-5}$ M. The apparent K_m of the control reaction is $1{\cdot}2 \times 10^{-4}$ M. The maximum velocity for formation of formaldehyde was obtained at approx. 5×10^{-3} M aminopyrine and that for 4-aminoantipyrine production at $2{\cdot}5 \times 10^{-4}$ M. The theoretical 2 : 1 ratio of formaldehyde to 4-aminoantipyrine production could not be demonstrated except at very low concentrations of aminopyrine (10^{-4} M); at aminopyrine concentrations above this molarity the ratio increased considerably. At $2{\cdot}5 \times 10^{-4}$ M aminopyrine the ratio was 4·5; at 5×10^{-4} M, 8·5; at 10^{-3} M, 12·0; and at 5×10^{-3} M it was 26·5.

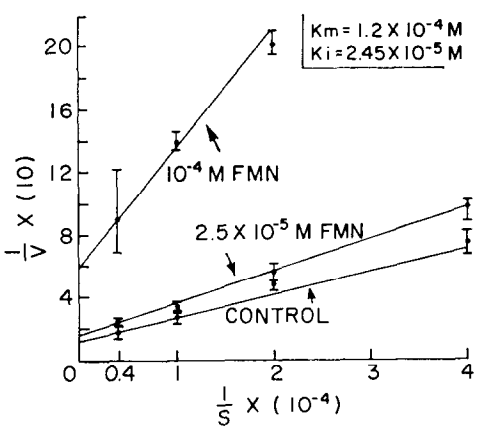

FIG. 3. Inhibition of FMN of *N*-demethylation of aminopyrine based on formation of 4-aminoantipyrine. V = micromoles of 4-aminoantipyrine formed per g of 9000 *g* protein per hr. S = molarity of aminopyrine.

Studies were conducted in an attempt to eluciate the mechanism by which FMN inhibited the aminopyrine-metabolizing enzyme system. Inhibition is not by means of a "nonspecific" effect of nucleotides, since the reaction is not inhibited by 5'-AMP, nor is it through an inability to convert NADP to NADPH since NADP was converted to NADPH within 3 min in the presence and absence of FMN. Other characteristics of FMN inhibition were also studied. The inhibition resulted regardless of the sequence of addition of the components to the reaction mixture. Inhibition was the same in reactions conducted in light or darkness. When chloramphenicol was used in the reaction mixture, no FMN–CAM complex could be demonstrated by spectrophotometric methods.

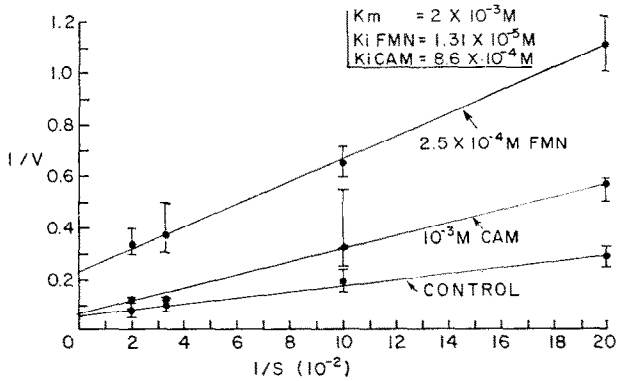

FIG. 4. Inhibition by chloramphenicol and FMN on *S*-demethylation of 6-methylmercaptopurine estimated by formation of formaldehyde. V = micromoles of formaldehyde formed per g of 9000 *g* protein per hr. S = molarity of 6-methylmercaptopurine.

Effects of flavins and chloramphenicol on mouse liver S-*demethylation enzyme system.* The results from *S*-demethylation of 6-methylmercaptopurine are very similar to those for *N*-demethylation of aminopyrine. Chloramphenicol is a competitive inhibitor of the reaction and the K_i of the reaction is $8{\cdot}6 \times 10^{-4}$ M (Fig. 4). FMN was also inhibitory, but the inhibition was non-competitive. The K_i of the reaction is $1{\cdot}31 \times 10^{-5}$ M. The apparent K_m of the control reaction is 2×10^{-3} M. Of the flavins studied, riboflavin was the best inhibitor and produced 70 per cent inhibition at 10^{-4} M (Table 2). FMN and FAD were also inhibitory, but at 10^{-3} M these two flavins demonstrated only 35 and 19 per cent inhibition, respectively. Only riboflavin was inhibitory at 10^{-5} M. When used in combination with CAM, the inhibition produced was less than additive.

Effects of flavins and chloramphenicol on mouse liver hydroxylation enzyme system. Chloramphenicol inhibits hydroxylation of acetanilid (Fig. 5). Approximately 50 per cent inhibition was obtained with 10^{-3} M CAM. Inhibition was competitive in nature, as was shown when *N*-acetyl-*p*-aminophenol was measured as a metabolic product (Fig. 5) or when the rate of disappearance of acetanilid was determined (Fig. 6). The apparent K_m for hydroxylation of acetanilid determined by measuring *N*-acetyl-*p*-aminophenol as a metabolic product is $2{\cdot}4 \times 10^{-3}$ M. Based on the disappearance of substrate, it is $8{\cdot}5 \times 10^{-4}$ M. Flavins were inhibitory (Table 3) and did not reverse chloramphenicol inhibition.

TABLE 2. EFFECTS ON THE *S*-DEMETHYLATION OF 6-METHYLMERCAPTOPURINE, 0·003 M, BY FLAVINS AND CHLORAMPHENICOL ALONE AND IN COMBINATION*

Treatment	−CAM		+CAM†	
	Units‡	Inhibition (%)	Units‡	Inhibition (%)
Control	11·1 ± 0·7	0	7·9 ± 0·2	28·8§
FMN (10^{-5} M)	12·3 ± 1·5	0	7·1 ± 0·9	34·0§
FMN (10^{-4} M)	7·2 ± 0·1	35·1§	4·2 ± 0·3	62·2§
FMN ($2·5 \times 10^{-4}$ M)	2·7 ± 0·7	75·7§		
FMN (5×10^{-4} M)	1·9 ± 0·1	82·9§	1·9 ± 0·1	82·9§
FMN (10^{-3} M)	1·8 ± 0·1	83·8§		
FAD (10^{-3} M)	14·4 ± 0·1	0	9·1 ± 0·3	18·0§
FAD (10^{-5} M)	8·9 ± 0·9	19·2	7·4 ± 1·1	33·3§
FAD (10^{-4} M)	2·8 ± 1·0	74·8§	3·2 ± 0·4	71·2§
B_2 (10^{-5} M)	8·7 ± 1·2	21·6	5·2 ± 1·0	53·2§
B_2 (10^{-4} M)	3·3 ± 0·9	70·3§	2·2 ± 0·2	80·2§

* The values are the mean ±S. E. from two to nine experiments.
† Chloramphenicol (10^{-3} M) was used in all experiments.
‡ Micromoles of CH_2O per hr/g 9000 *g* protein.
§ Significant inhibition compared to control reaction ($P < 0·05$).

FMN is a competitive inhibitor of the hydroxylation reaction (Figs. 5 and 6). The K_i with *N*-acetyl-*p*-aminophenol production as an index for acetanilid metabolism is $3·2 \times 10^{-4}$ M. When disappearance of acetanilid was used as an index, the K_i is $9·5 \times 10^{-3}$ M. Of the flavins tested for their inhibitory activity, riboflavin was the most effective, giving 46 per cent inhibition at 10^{-4} M (Table 3). FMN and FAD were also inhibitory, but 10^{-3} M concentrations were required for 50 per cent inhibition. Inhibition by flavins or chloramphenicol was not prevented by preincubation nor did the sequence of addition of the components to the reaction mixture influence the inhibitory activity of flavins or chloramphenicol.

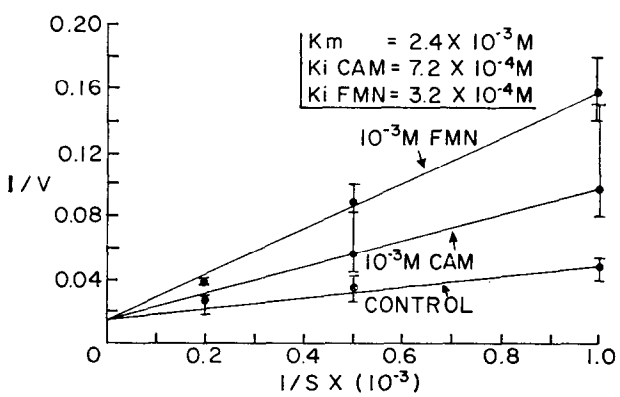

FIG. 5. Inhibition of hydroxylation of acetanilid by FMN and chloramphenicol using formation of *N*-acetyl-*p*-aminophenol as an index for acetanilid metabolism. V = micromoles of *N*-acetyl-*p*-aminophenol formed per g of 9000 *g* protein per hr. S = molarity of acetanilid.

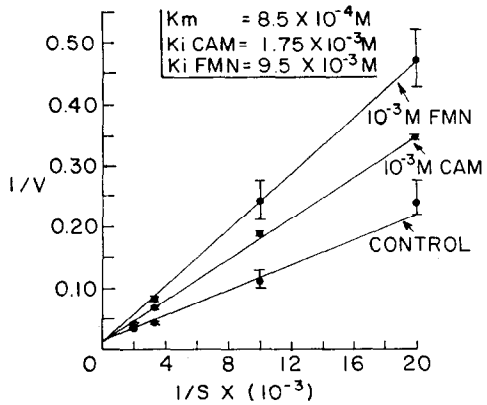

FIG. 6. Inhibition of hydroxylation of acetanilid by FMN and chloramphenicol using disappearance of acetanilid as an index of metabolism of acetanilid. V = micromoles of acetanilid metabolized per g of 9000 *g* protein per hr. S = molarity of acetanilid.

Effects of flavins and chloramphenicol on rat liver O-*demethylation enzyme system.* Figure 7 presents Lineweaver–Burk plots of the *O*-demethylation of *o*-nitroanisole and of the effects of 10^{-3} M CAM alone and in combination with 10^{-6} M FMN. The apparent K_m of the control reaction is $1{\cdot}9 \times 10^{-3}$ M. Chloramphenicol is a competitive inhibitor and the K_i of the reaction is $0{\cdot}5 \times 10^{-3}$ M. This inhibitory effect of 10^{-3} M CAM is nullified by 10^{-6} M FMN. FMN, FAD or riboflavin at this concentration (10^{-6} M) do not influence the rate of the control reaction. The results of the experiments in which rats were treated with 7·5 mg/kg of body wt of CAM alone

TABLE 3. EFFECTS ON THE HYDROXYLATION OF ACETANILID BY FMN, FAD AND B_2*

Treatment	Units†	Inhibition (%)
Control	48·4 ± 1·9	0
FMN (10^{-5} M)	50·3 ± 0·8	0
FMN (10^{-4} M)	31·1 ± 1·5	35·7‡
FMN (10^{-3} M)	27·1 ± 1·0	44·0‡
FAD (10^{-5} M)	50·6 ± 6·8	0
FAD (10^{-4} M)	42·8 ± 1·0	11·6‡
FAD (10^{-3} M)	26·5 ± 2·2	45·2‡
B_2 (10^{-5} M)	46·3 ± 6·3	4·3
B_2 (10^{-4} M)	26·1 ⊥ 2·0	46·1‡

* All data are based on the degree of inhibition noted at 0·005 M acetanilid, measuring *N*-acetyl-*p*-aminophenol formation. The values are the mean ±S. E. from two to nine experiments.

† Micromoles of product per hr/g of 9000 *g* protein.

‡ Significant inhibition compared to control reaction ($P < 0{\cdot}05$).

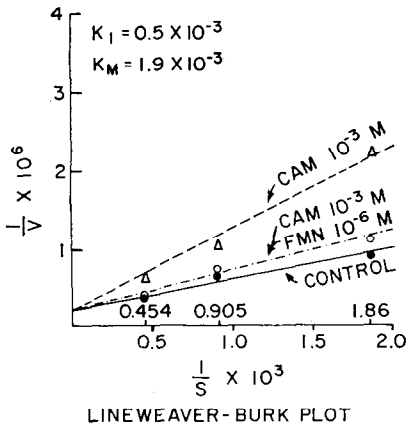

FIG. 7. Effect of chloramphenicol alone and in combination with FMN on the *O*-demethylation of *o*-nitroanisole by rat liver microsomes. V = micromoles of *o*-nitrophenol formed per milliliter. min. S = molarity of *o*-nitroanisole.

and with both CAM and FMN are shown in Table 4. Liver microsomes from CAM-treated animals show a markedly depressed demethylation activity. Injections of FMN, 100 mg/kg of body wt 30 min before and 30 min after CAM restored microsomal enzyme activity as did 200 mg/kg of body wt of FMN injected 30 min after CAM.

Effects of flavins and chloramphenicol on rat and mouse liver hexobarbital-metabolizing enzyme systems. Chloramphenicol and FMN are each competitive inhibitors of the mouse liver enzyme system that metabolizes hexobarbital (Figs. 8 and 9). The K_I is $3{\cdot}8 \times 10^{-3}$ M with CAM and $8{\cdot}9 \times 10^{-4}$ M with FMN. However, when

TABLE 4. EFFECTS OF INJECTION OF RATS WITH CHLORAMPHENICOL ALONE AND WITH FMN ON *o*-NITROANISOLE DEMETHYLATION BY LIVER MICROSOMES

Treatment	Units*
	1·92 ± 0·08
CAM†	6·0 ± 0·08‡
FMN§, CAM, FMN§	2·46 ± 0·32
CAM, FMN‖	1·86 ± 0·16

* Values are expressed as nanomoles of *o*-nitrophenol formed per milligram of microsomal protein per min, mean ±S. E.; n = 4.

† Body wt (7·5 mg/kg) injected intramuscularly 1 hr before removal of liver.

‡ Significant inhibition compared to control reaction ($P < 0{\cdot}05$).

§ Body wt (100 mg/kg) injected intraperitoneally 30 min before and after CAM injection.

‖ Body wt (200 mg/kg) injected intraperitoneally 30 min after CAM injection.

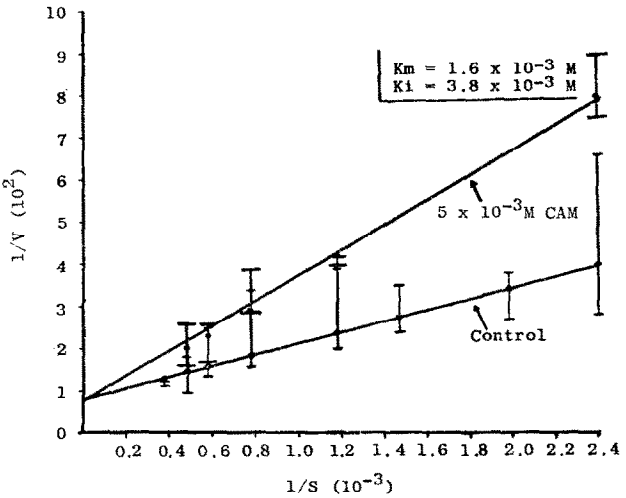

FIG. 8. Inhibition by chloramphenicol of hexobarbital metabolism by 9000 *g* supernatant of mouse liver. The lines are regression lines calculated by method of least squares. V = micromoles of hexobarbital metabolized per g of 9000 *g* protein per hr. S = molarity of hexobarbital.

both CAM and FMN are added to the reaction mixture, no inhibition results (Table 5). Flavins other than FMN also inhibit the hexobarbital-metabolizing enzyme system and reverse inhibition by chloramphenicol as well (Table 5). FAD is a stronger inhibitor of the enzyme system than riboflavin, but less effective in reversing chloramphenicol inhibition. Of the three flavins studied, FMN was the most potent inhibitor as well as the most effective in reversing chloramphenicol inhibition.

Reversal of chloramphenicol inhibition by flavins was also observed if a microsomal enzyme system was used. Table 6 shows a typical experiment in which a microsomal

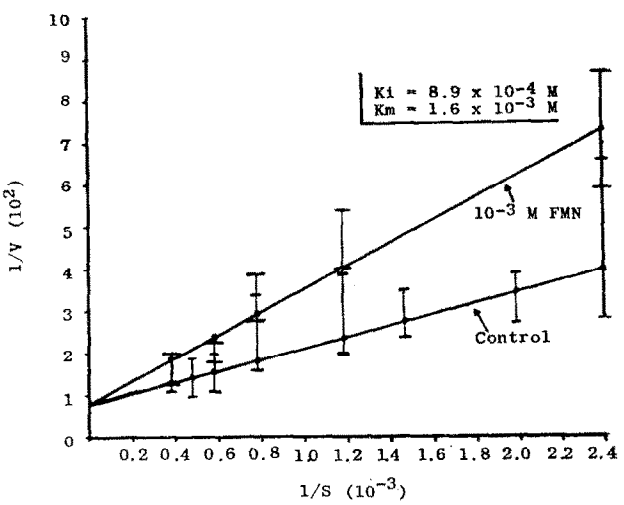

FIG. 9. Inhibition of FMN of hexobarbital metabolism by 9000 *g* supernatant of mouse liver. The lines are regression lines calculated by method of least squares. V = micromoles of hexobarbital metabolized per g of 9000 *g* protein per hr. S = molarity of hexobarbital.

TABLE 5. EFFECTS ON THE 9000 *g* HEXOBARBITAL-METABOLIZING ENZYMES BY FLAVINS, CHLORAMPHENICOL, AND COMBINATIONS OF FLAVINS WITH CHLORAMPHENICOL AT 500 μg HEXOBARBITAL/ml OF REACTION MIXTURE*

Treatment	−CAM		+CAM†	
	Units‡	Inhibition (%)	Units‡	Inhibition (%)
Control	75·7 ± 5·7	0	52·5 ± 3·5	30·6§
FMN (10^{-5} M)	62·0 ± 1·5	18·1§	72·4 ± 5·4	4·4
FMN (10^{-4} M)	48·4 ± 3·1	36·1§	84·8 ± 16·2	0
FMN (10^{-3} M)	56·3 ± 2·8	25·6§	103·3 ± 1·2	0
FAD (10^{-5} M)	77·9 ± 0·2	0	59·5 ± 9·0	21·4
FAD (10^{-4} M)	62·5 ± 6·5	17·4	61·0 ± 7·2	19·4
FAD (10^{-3} M)	48·9 ± 5·9	35·4§	66·0 ± 0·5	12·8
B_2 (10^{-5} M)	78·9 ± 8·7	0	65·6 ± 7·1	13·3
B_2 (10^{-4} M)	79·8 ± 4·8	0	73·0 ± 12·2	3·6

* The values given are the mean ±S. E. from two to eighteen experiments.
† Chloramphenicol (5×10^{-3} M) was used in all experiments.
‡ Micromoles of hexobarbital metabolized per hr/g of 9000 *g* protein.
§ Significant inhibition compared to control reaction ($P < 0.05$).

preparation was used as the enzyme source. The best results were obtained when the 105,000 *g* supernatant was added back to the incubation media. In none of the experiments were the results influenced by preincubation or by the sequence of addition of CAM, flavins or the enzyme source.

Hexobarbital sleeping time experiments were also utilized to demonstrate reversal by flavins of CAM inhibition. Table 7 shows that injection of 100 mg/kg of body wt of FMN 30 min prior and 30 min after injection of CAM prevented the prolongation of

TABLE 6. TYPICAL EXPERIMENT DEMONSTRATING THE EFFECTS OF FMN, CHLORAMPHENICOL, AND COMBINATIONS OF FMN AND CHLORAMPHENICOL ON THE MICROSOMAL HEXOBARBITAL-METABOLIZING ENZYME SYSTEM: 750 μg HEXOBARBITAL/ml REACTION MIXTURE

Conditions	Micrograms of hexobarbital metabolized per milligram of microsomal protein per hr	Control (%)
Microsomes + enzyme*		
Complete	33·4	100
Complete + FMN†	31·2	94
Complete + CAM‡	15·7	47
Complete + FMN + CAM	29·4	88
Microsomes + supernatant§		
Complete	55·0	100
Complete + FMN	41·8	76
Complete + CAM	36·4	66
Complete + FMN + CAM	55·5	101

* Two hundred units of glucose 6-phosphate dehydrogenase.
† FMN (10^{-3} M).
‡ CAM (10^{-3} M).
§ One-half of 105,000 *g* supernatant was added back to the system.

TABLE 7. EFFECTS OF CHLORAMPHENICOL AND FMN ON SLEEPING TIME OF RATS GIVEN HEXOBARBITAL, 75 mg/kg OF BODY WT

CAM (mg/kg)	−FMN (min ± S. E.)	+FMN* (min ± S. E.)
	38 ± 2	44 ± 6
0·1	56 ± 7†	53 ± 3†
1·0	60 ± 9†	39 ± 2
5·0	> 105†	42 ± 1
10·0	> 120†	120†

* FMN, 100 mg/kg body wt injected i.p. 30 min before and 30 min after i.m. injection of CAM.

† Significant prolongation of sleeping time compared to controls ($P < 0·05$).

sleeping time effect of 1 and 5 mg/kg of body wt of CAM, but did not overcome the action of 10 mg/kg of CAM. In other studies, the injection of two doses of 50 mg/kg of FMN did not appreciably influence the effect of CAM. A single dose of FMN of 200 mg/kg injected before CAM was effective, but when injected 30 min after CAM it did not prevent sleeping time prolongation. L-Phenylalanine injected intraperitoneally, 200 mg/kg of body wt 30 min before CAM injection did not influence hexobarbital sleeping time and did not prevent prolongation of sleeping time by CAM.

DISCUSSION

All of the drug-metabolizing enzyme systems studied were oxygen-requiring systems. The reactions were linear with time and at the substrate concentrations used in these experiments. Chloramphenicol competitively inhibited all of the drug-metabolizing systems studied; FMN competitively inhibited only the acetanilid hydroxylation and hexobarbital side chain oxidation reactions. FMN reversed CAM inhibition of *O*-demethylation of *o*-nitroanisole and side chain oxidation of hexobarbital.

All flavins studied are effective inhibitors of the *N*-demethylation of aminopyrine. FMN and riboflavin are the best inhibitors exhibiting about twice the inhibitory activity of FAD at 10^{-4} M. FMN produced 50 per cent inhibition at approx. $2·5 \times 10^{-4}$ M and is a more efficient inhibitor at this concentration. The mechanism of inhibition is unknown, but it may be due to some allosteric effect. It has been pointed out by Gillette[19] that FMN may inhibit drug metabolism by limiting NADPH required for the reaction. However, studies conducted in this laboratory to measure NADPH availability were unsuccessful in demonstrating a NADPH lack in the presence of flavins. NADP was converted to NADPH equally well in a 30,000 *g* supernatant in the presence or absence of FMN. The inhibition of *N*-demethylation of aminopyrine by FMN was not reversed by a 5-fold addition of NADP. Also, after 5- and 20-min incubation periods oxidized glutathione was added to the system and the change in o.d. at 340 nm was found to be the same in the presence or absence of FMN.

Theoretically, if *N*-demethylation goes to completion, 2 moles of formaldehyde are produced for each mole of 4-aminoantipyrine. We were unable to demonstrate a 2 : 1 ratio except at low concentrations of aminopyrine. Similar results have been

reported by other workers for rat and rabbit enzymes.[20] They postulated that one methyl group at a time is removed from aminopyrine, with the initial formation of monomethyl 4-aminoantipyrine as an intermediate. The rate of demethylation of monomethyl 4-aminoantipyrine appeared to be slower than the preceding reaction and the rate of 4-aminoantipyrine production decreased as the aminopyrine concentration increased. The same results were found in our experiments using mouse liver preparations. Dingell and D'Encarnacao[21] reported that only about 20 per cent of the aminopyrine metabolized in the isolated perfused rat liver is converted to 4-aminoantipyrine. They suggested that the tertiary amine competitively inhibits the further demethylation of its secondary amine metabolite.

The method utilized to measure 4-aminoantipyrine content cannot be utilized when CAM is incubated with FMN. A color forms during the final isoamyl-alcohol extraction step that interferes with normal color development. The color formed absorbs maximally at 473 nm, and is noted when CAM and FMN are incubated separately. The same problem was confronted when measuring *N*-acetanilid. The color formed in this reaction absorbed maximally at 510 nm, and was produced in the absence of substrate.

Hydroxylation of acetanilid and side chain oxidation of hexobarbital is competitively inhibited by CAM and FMN. This is in contrast to a report by Dixon and Fouts[8] for rat liver microsomes. In their report they found that CAM inhibition was noncompetitive and irreversible in both systems. However, species difference in the type of response to such inhibitors as SKF 525A[22] and SKF 2675A[23], has been noted. The same principal may also apply to CAM inhibition. Riboflavin is the most effective inhibitor of acetanilid hydroxylation giving 50 per cent inhibition at 10^{-4} M.

It would appear that the site of action of FMN and CAM are different in acetanilid hydroxylation and *S*-demethylation of 6-methylmercaptopurine as they obviously are in the *N*-demethylation of aminopyrine. However, in *O*-demethylation of *o*-nitroanisole and side chain oxidation of hexobarbital, the inhibitory sites may be similar since flavins will reverse the inhibitory effects of CAM on these drug-metabolizing enzyme systems. It is possible that these inhibitors compete for allosteric sites on the enzyme systems. Other actions of flavins on drug-metabolizing enzymes have been reported. Rabbit and rat liver microsomal nitroreductase activity is stimulated by flavins under anaerobic conditions;[1,2] flavins stimulate rat liver demethylase activity; they also stimulate rabbit liver azoreductase and inhibit neotetrazolium diaphorase activity.[5] It is evident that the interactions of flavins with drug-metabolizing enzyme systems are complex and that the mechanisms require elucidation.

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