Biochemical Pharmacology, Vol. 19, pp. 533–544. Pergamon Press. 1970. Printed in Great Britain

THE INFLUENCE OF MAGNESIUM AND SOME OTHER DIVALENT CATIONS ON HEPATIC MICROSOMAL DRUG METABOLISM *IN VITRO**

MARVIN A. PETERS and JAMES R. FOUTS

Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, Iowa 52240, U.S.A.

(*Received* 18 *March* 1969; *accepted* 20 *June* 1969)

Abstract—The addition of Mg^{2+} caused marked increases in rat liver microsomal metabolism *in vitro* of several drug substrates. The substrates studied have been grouped according to the responses seen with increasing concentrations of Mg^{2+}: (1) no effect or a decrease in enzyme activity; (2) increased enzyme activity with increased Mg^{2+} concentrations; and (3) peak enzyme activity with 6 mM Mg^{2+} or less, followed by a decreased activity with higher concentrations of Mg^{2+}. Enhanced drug metabolism appeared to be caused by the Mg^{2+} ion rather than by ionic strength or associated anion. Although Mg^{2+} may affect the NADPH-generating system, it also enhanced the microsomal drug-metabolizing enzyme systems directly (Mg^{2+} enhanced activity in the absence of an NADPH-generating system). Other divalent cations (Mn^{2+}, Ca^{2+} and Sr^{2+}) caused a marked enhancement of hepatic microsomal metabolism similar to that caused by Mg^{2+} with all substrates studied. Divalent cobalt in low concentration caused enhanced microsomal metabolism of hexobarbital, but was inhibitory with other substrates. Ferrous ion, Cu^{2+} and Zn^{2+} did not enhance any of the microsomal drug metabolisms studied. The monovalent cations, K^+ and Na^+, had little if any effect on drug metabolism. The effects of the various cations studied were qualitatively the same with hepatic microsomal preparations from both control and phenobarbital-treated rats.

DURING a study of the effects of chlortetracycline pretreatment on hepatic microsomal drug metabolism, it was observed that Mg^{2+} markedly stimulated the rate of metabolism *in vitro* of hexobarbital and aminopyrine by microsomes from control, phenobarbital-treated or chlortetracycline-treated rats.[1]

Some divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) have been shown to increase the activity of a nonmicrosomal enzyme, the NADP-linked aldehyde dehydrogenase from yeast.[2] Kornberg[3] found that Mg^{2+} (and Mn^{2+} to a lesser extent) enhanced the rate of reduction of NADP by glucose 6-phosphate dehydrogenase.

Murdoch and Heaton[4] have found relatively large amounts of Ca^{2+} and Mg^{2+} bound to hepatic microsomal membranes. Carvalho *et al.*[5] indicated that the lipoprotein fraction of hepatic microsomes was responsible for 45 per cent of the total binding of Ca^{2+} and Mg^{2+} by the whole microsomal fraction. These latter authors contended that cellular membrane structures (lipoprotein) can bind considerable quantities of the divalent cations. These cations may influence the enzyme systems associated with these membranous structures. Bogdanska *et al.*[6] have reported that Ca^{2+} and

*This work was supported by grants from the National Institutes of Health, National Institute of General Medical Sciences (GM 12675 and 5 TO 1-GM 141).

Mg^{2+} markedly stimulated the rate of NADPH oxidation by hepatic microsomes. Trivus and Spirtes[7] have reported a Mg^{2+} requirement for hexobarbital oxidation and chlorpromazine sulfoxidation by rabbit liver microsomal enzymes. These authors found that Ca^{2+}, Mn^{2+}, Cu^{2+}, Cu^{+},Mo^{6+}, Fe^{3+} and Fe^{2+} could not replace Mg^{2+} in their system. It appeared (from the results of Trivus and Spirtes[7]) that Mg^{2+} was quite specific for the rabbit liver microsomal enzyme systems metabolizing hexobarbital and chlorpromazine.

We have attempted to answer a number of questions about the effect of Mg^{2+} and other divalent cations on rat liver microsomal enzyme systems: (1) Does Mg^{2+} ion stimulate the metabolism of all types of drugs which are metabolized by the NADPH-requiring hepatic microsmal enzyme system, or is there a difference depending on the type of cytochrome P450-substrate interaction (type I vs. type II)[8] or enzymatic transformation taking place? (2) Is only the Mg^{2+} cation essential or is there an effect by the associated anion or overall ionic strength? (3) Does the Mg^{2+} ion influence the metabolism differently when the NADPH is generated than when chemically reduced NADPH is used? (4) What concentration of Mg^{2+} gives maximum effects on drug metabolism? (5) What is the response to other divalent cations?

MATERIALS AND METHODS

The experimental animals were obtained from Simonsen Laboratories and were male, Long–Evans rats weighing approximately 200 g. The animals were housed in an air-conditioned room (68–75° F), with 8–10 hr of light per day, and were maintained on a commercial Wayne Lab-Blox diet and water *ad lib.*

The rats were divided into two groups. One group received no treatment and the other group was given an intraperitoneal (i.p.) injection of 75 mg phenobarbital per kg, daily for 3 days. At 24–48 hr after the last injection, the animals were sacrificed. Both untreated and treated groups were killed between 6:00 and 8:00 a.m. by cervical dislocation. Livers were immediately removed and placed in an ice-cold solution having a pH of 7·35 and usually containing 1·15% KCl and 0·05 M Tris buffer. In some experiments livers were excised and placed in ice-cold Hepes (*N*-2-hydroxyethyl-piperazine-*N'*-2 ethanesulfonic acid)[9] buffer. All subsequent procedures were carried out at 0–4°.

The livers were homogenized (1 g of liver and 2 ml either of KCl with Tris buffer or of Hepes buffer) in a Potter homogenizer having a plastic pestle. The same homogenizer was used to prepare homogenates of liver from control and phenobarbital-pretreated rats. The 9000 *g* supernatant fraction (microsomes and soluble fraction) was obtained by centrifuging the homogenate in a Sorvall refrigerated centrifuge at 9000 *g* for 20 min. The microsomes were obtained by centrifuging the 9000 *g* supernatant fraction at 105,000 *g* for 60 min in a Beckman L2-65 refrigerated centrifuge. The microsomal pellet was suspended in either 1·15% KCl with 0·05 M Tris or in Hepes buffer and resedimented as before. The washed microsomes were resuspended in a sufficient volume of 0·1 M Hepes buffer at pH 7·35 to give a protein concentration of 10 mg microsomal protein per ml of suspension. The protein content was determined in duplicate essentially as described by Lowry *et al.*[10] Crystalline bovine serum albumin was used as the reference standard. The color interference of the Hepes buffer[11] was corrected for by running a buffer blank simultaneously with the unknown and standards.

The hepatic microsomes from one to four animals were pooled and aliquots were taken and subjected to the various treatments with metal ions, etc. Each treatment was repeated two or three times and the mean enzyme activity (± S.E.) was determined. Experiments were repeated at least once on a second pool of microsomes from different animals and qualitatively similar results were always obtained.

In determining the effect of ionic strength and monovalent cations (K^+ and Na^+) on microsomal drug metabolism, 0·1 M Hepes buffer was used throughout the entire procedure (including homogenizing and washing the microsomes) instead of the 1·15% KCl with Tris buffer solution. The soluble fraction (105,000 *g* supernatant fraction) and microsomal fraction were then dialyzed for 3 hr at 0–4° against 4 gallons of distilled water, on a rocking dialyzer. One-ml samples of the tissue fractions were taken before and after dialysis and digested with 1 ml of concentrated nitric acid. The digested samples were diluted to 10 ml with water and subsequent dilutions were made as needed. The Na^+ and K^+ content of the diluted samples were determined by using a model 21 Coleman flame photometer as outlined in the operators' manual. The dialyzing procedure removed approximately 95 per cent of the Na^+ and K^+ present in the original soluble fraction and washed microsomes.

For the determination *in vitro* of drug metabolism, 0·5 ml of the microsomal suspension (containing 5 mg of microsomal protein) was incubated in a Dubnoff shaking incubator for 30 min at 37° with oxygen as the gaseous phase when studying oxidative reactions. Nitrogen was used as the gaseous phase when the various reductive systems were studied. The gas flow rates were greater than 500 ml/min. The shaking rate was 100 rpm and a marble was added to each incubation mixture to insure vigorous agitation. The final amounts of cofactors added were: nicotinamide adenine dinucleotide phosphate (NADP), 1·12 μmoles; glucose 6-phosphate, 12·5 μmoles; nicotinamide, 10 μmoles; 0·25 ml of soluble fraction from control animals (containing the NADPH-generating system, glucose 6-phosphate dehydrogenase); and the various cations in the amounts listed in the tables. Hepes buffer, 0·2 M, pH 7·35 was added to give a final buffer strength of 0·1 M and distilled water was added to make a final volume of 2·5 ml of incubation mixture.

The enzyme pathways studied and the amount of substrate in μmoles per 2·5 ml of incubation mixture were: ring hydroxylation of benzpyrene (0·3 μmole);[12] ring hydroxylation of zoxazolamine (3 μmoles);[13] *O*-demethylation of *p*-nitroanisole (5 μmoles);[14] cleavage of the azo linkage of neoprontosil (3·25 μmoles);[15] reduction of *p*-nitrobenzoic acid (6 μmoles);[16] *N*-dealkylation of aminopyrine, (10 μmoles) to formaldehyde;[17] *N*-dealkylation of benzphetamine (2·4 μmoles) to formaldehyde;[17] *N*-dealkylation of aminopyrine (5 μmoles) to 4-aminoantipyrine;[18] *N*-dealkylation of *p*-chloromethylaniline (3·75 μmoles);[19] ring hydroxylation of aniline (10 μmoles);[20] side chain oxidation of hexobarbital (6 μmoles).[21]

The choice of a buffer was of importance in studying the effect of divalent cations on hepatic microsomal drug-metabolizing enzymes. Phosphate buffer is commonly used in drug metabolism studies such as these. However, as Good *et al.*[9] pointed out, phosphate buffer complexes or precipitates most polyvalent cations and can be an inhibitor in many enzyme systems. A number of the hydrogen ion buffers used in the study by Good *et al.*[9] have been used in microsomal drug metabolism studies in our laboratory. Studies with Hepes buffer showed marked increases in some drug metabolisms compared with phosphate or other buffers studied. In addition, the buffer

capacity of Hepes was as good as (or better than) most buffers at the pH we usually use (pH 7·35). Hepes buffer was therefore used in all these investigations.

RESULTS

The Mg^{2+} ion stimulated some microsomal enzyme pathways much more than others. One of the first concerns in our studies was whether Mg^{2+} exerted its effect on drug metabolism only because it enhanced the generation of NADPH by enzymes in the soluble fraction. The data in Table 1 shows the effect of various concentrations of Mg^{2+} on the rate of metabolism of hexobarbital, neoprontosil and *p*-chloromethylaniline with either the usual NADPH-generating system or chemically reduced NADPH. There appeared to be some differences in the concentration at which Mg^{2+} produced peak effects, but these data suggested that Mg^{2+} did not affect these drug metabolisms by hepatic microsomes only because it affected the NADPH-generating system.

TABLE 1. EFFECT OF MAGNESIUM ON HEPATIC MICROSOMAL METABOLISM OF HEXOBARBITAL, NEOPRONTOSIL AND *p*-CHLOROMETHYLANILINE WITH AN NADPH-GENERATING SYSTEM* VS. CHEMICALLY-REDUCED NADPH†

Substrate	Magnesium conc.‡ (μmoles)			
	0	1·5	15	150
Neoprontosil				
NADPH-generating system	111 ± 3	132 ± 4	198 ± 1§	225 ± 1§
NADPH-chemically reduced	165 ± 3	190 ± 4	219 ± 1§	186 ± 1§
-Chloromethylaniline				
NADPH-generating system	31 ± 3	34 ± 2	58 ± 2§	51 ± 2§
NADPH-chemically reduced	35 ± 1	41 ± 3	69 ± 9§	36 ± 2
Hexobarbital				
NADPH-generating system	76 ± 11	144 ± 13	212 ± 2§	131 ± 9§
NADPH-chemically reduced	31 ± 6	91 ± 4§	188 ± 3§	100 ± 6§

*The NADPH-generating system consisted of 0·25 ml of soluble fraction, 1·12 μmoles NADP and 12·5 μmoles glucose 6-phosphate in a total volume of 2·5 ml.
†Chemically reduced NADPH (5·5 μmoles) was added to a total volume of 2·5 ml.
‡Amount of $MgCl_2$ added to a total volume of 2·5 ml. Values are mμmoles of drug metabolized/mg of microsomal protein/30 min; mean ± S.E. of 3 different experiments. All values with any one substrate were obtained using the same pool of hepatic microsomes from control rats.
§Significantly different from 0 μmoles Mg^{2+}; $P < 0·05$.

Table 2 shows the effects of $MgCl_2$ vs. $MgSO_4$ on the metabolism of three substrates by hepatic microsomes from control and phenobarbital-pretreated rats. These data suggested that the anion had little effect on hepatic microsomal drug metabolism. The three substrates showed a difference in response to increasing concentrations of Mg^{2+} (i.e. increase to peak then decrease; only increase; no effect or decrease). However, the response to the $MgCl_2$ vs. $MgSO_4$ was the same in all cases. These three types of response were representative of the three groups into which the response to Mg^{2+} of a number of substrates currently used in our laboratory can be divided.

TABLE 2. INFLUENCE OF MAGNESIUM AS THE CHLORIDE VS. SULFATE ON MICROSOMAL METABOLISM *IN VITRO**

Substrate	Treatment	Magnesium conc.† (μmoles)						
		0	1·5		15		150	
			Cl^-	$SO_4^=$	Cl^-	$SO_4^=$	Cl^-	$SO_4^=$
Benzphetamine	Control	246 ± 3	279 ± 2‡	293 ± 18	497 ± 4‡	478 ± 8‡	352 ± 2‡	400 ± 8‡
	Phenobarbital§	628 ± 4	618 ± 4	633 ± 8	859 ± 4‡	868 ± 12‡	773 ± 0‡	833 ± 2‡
Neoprontosil	Control	92 ± 3	114 ± 1‡	117 ± 1‡	160 ± 2‡	168 ± 1‡	200 ± 11‡	202 ‖
	Phenobarbital§	126 ± 3	142 ± 3	149 ± 3	194 ± 2‡	190 ± 1‡	232 ± 4‡	233 ± 2‡
p-Nitroanisole	Control	50 ± 1	48 ± 2	51 ± 3	44 ± 1	48 ± 2	24 ± 2‡	23 ± 2‡
	Phenobarbital§	101 ± 2	96 ± 1	100 ± 2	103 ‖	103 ± 0	71 ± 6	90 ± 2

*Values are mμmoles of product formed/mg of microsomal protein/30 min; mean ± S.E. of two different experiments. All values for a given substrate and treatment were obtained with the same pool of microsomes.

†Amount of Mg^{2+} as chloride or sulfate added to a total volume of 2·5 ml.

‡Significantly different from activity without added Mg^{2+} (0 μmoles); $P < 0{\cdot}05$.

§Rats were pretreated with phenobarbital sodium at a dose of 75 mg/kg daily for 3 days. Enzyme assays were run 24–48 hr after the last dose of phenobarbital.

‖N = 1 in those cases where no S.E. is given.

Data with the various substrates studied are presented in Table 3 and are grouped according to the response seen with increasing concentrations of Mg^{2+}. No effect or a decrease in activity was seen with the substrates *p*-nitroanisole and benzpyrene. The substrates neoprontosil, *p*-nitrobenzoic acid and aminopyrine (measuring formaldehyde formation) showed only increased activity with increasing Mg^{2+} concentrations. The other substrates used showed a peak metabolism rate with 6 mM Mg^{2+} or less. Aniline, *p*-chloromethylaniline, benzphetamine, hexobarbital, aminopyrine (measured by 4-aminoantipyrine formation) and zoxazolamine are substrates falling into the latter category.

TABLE 3. CLASSIFICATION OF SUBSTRATES BASED ON ENZYME ACTIVITY RESPONSE TO Mg^{2+} IN HEPATIC MICROSOMAL METABOLISM *IN VITRO**

Classification and substrate	Magnesium conc.† (μmoles)			
	0	1·5	15	150
1. No enhanced activity				
a. Benzpyrene	8·5 ± 0·4	9·4 ± 0·3	8·9 ± 0·2	3·4 ± 0·1
b. *p*-Nitroanisole	47 ± 2	47 ± 2	44 ± 2	22 ± 0‡
2. Enhanced activity only				
a. Aminopyrine (formaldehyde)	118 ± 4	145 ± 5	203 ± 14‡	234 ± 21‡
b. Neoprontosil	87 ± 5	107 ± 4	155 ± 4‡	190 ± 2‡
c. *p*-Nitrobenzoic acid	8 ± 1	9 ± 1	13 ± 0‡	14 ± 1‡
3. Enhanced activity, peak activity at 15 μmoles or less				
a. Aminopyrine (4AAP)	12 ± 1	13 ± 2	18 ± 1‡	14 ± 2
b. Aniline	44 ± 1	45 ± 1	63 ± 1‡	53 ± 5
c. Benzphetamine	247 ± 2	284 ± 7‡	469 ± 7‡	367 ± 22‡
d. *p*-Chloromethylaniline	18 ± 3	30 ± 1	53 ± 5‡	50 ± 1‡
e. Hexobarbital	208 ± 18	258 ± 2	468 ± 18‡	228 ± 25
f. Zoxazolamine	80 ± 8	123 ± 7	116 ± 5	81 ± 18

*All values are mμmoles of drug metabolized or product formed/mg of microsomal protein/30 min; mean ± S.E. of two different experiments. All values for a given substrate were obtained with the same pool of microsomes from control rats. All concentrations of magnesium were incubated with any given substrate under the same conditions (same incubator, etc.).

†The amount of Mg^{2+} added to the total incubation volume of 2·5 ml.

‡Significantly different from no Mg^{2+}; $P < 0·05$.

Increased drug metabolism with increased Mg^{2+} concentration might be caused by increased ionic strength rather than by the Mg^{2+} ion. Table 4 compares the influence of Mg^{2+}, K^+ and Na^+ on the metabolism of aniline and hexobarbital when the chloride salts of these various cations were used at equal ionic strength. At all of the ionic strengths tested, Mg^{2+} caused a marked enhancement of aniline and hexobarbital metabolism, while the Na^+ and K^+ had relatively little effect. These data were obtained with microsomes from control animals. However, phenobarbital-treated animals responded in qualitatively the same manner.

The effects of EDTA on the enzyme activity responses seen with increased Mg^{2+} concentration are presented in Table 5. EDTA added to the incubation mixture caused an increase in the optimal concentration of Mg^{2+} needed for the metabolism of aniline, benzphetamine or hexobarbital by hepatic microsomes from control or phenobarbital-treated rats. The degree of stimulation of aniline hydroxylation, benzphetamine, dealkylation or hexobarbital oxidation, by whatever Mg^{2+} concentration was best

TABLE 4. INFLUENCE OF IONIC STRENGTH ON RAT LIVER MICROSOMAL DRUG METABOLISM *IN VITRO*

Substrate	Cation	Ionic strength of final solution*			
		0	0·0018	0·018	0·18
Aniline	Mg^{2+}	44 ± 1	56 ± 1† (0·6 mM)	81 ± 1† (6 mM)	64 ± 0† (60 mM)
	K^+	44 ± 1	49 ± 1 (1·8 mM)	52 ± 1 (18 mM)	43 ± 0 (180 mM)
	Na^+	44 ± 1	49 ± 1 (1·8 mM)	53 ± 0 (18 mM)	49 ± 1 (180 mM)
Hexobarbital	Mg^{2+}	99 ± 13	115 ± 5 (0·6 mM)	225 ± 3† (6 mM)	158 ± 15 (60 mM)
	K^+	99 ± 13	88 ± 1 (1·8 mM)	127 ± 12 (18 mM)	121 ± 0 (180 mM)
	Na^+	99 ± 13	103 ± 20 (1·8 mM)	141 ± 20 (18 mM)	91 ± 3 (180 mM)

*Ionic strength of final solution in terms of "added" ions (does not include ions donated by substrate, buffer and cofactors, which are the same in all samples). The tissue fractions used were dialyzed as described in Methods. The chloride salt of the various cations was used.

†Significantly different from 0 ionic strength; $P < 0·05$. Values are mμmoles of substrate metabolized/mg of protein/30 min; mean ± S.E. of two different experiments. All values were obtained with the same pool of microsomes from control rats.

TABLE 5. EFFECT OF EDTA ON THE Mg^{2+} ACTIVATION OF DRUG METABOLISM BY HEPATIC MICROSOMES*

	Magnesium conc.† (μmoles)			
	0	1·5	15	150
Aniline hydroxylation by:				
Control rats				
No EDTA	25 ± 1	32 ± 1	45 ± 1	35 ± 2
With EDTA‡	26 ± 1	25 ± 1§	39 ± 1§	44 ± 1§
Phenobarbital-treated rats‖				
No EDTA	35 ± 4	43 ± 1	68 ± 1	59 ± 1
With EDTA‡	34 ± 0	33 ± 1§	47 ± 1§	65 ± 1§
Benzphetamine dealkylation by:				
Control rats				
No EDTA	166 ± 6	152 ± 1	471 ± 1	380 ± 2
With EDTA‡	129 ± 4	124 ± 3§	273 ± 8§	351 ± 4§
Phenobarbital-treated rats‖				
No EDTA	466	485 ± 3	907 ± 35	948 ± 3
With EDTA‡	493 ± 10	472 ± 11	583 ± 8§	853 ± 11§
Hexobarbital oxidation by:				
Control rats				
No EDTA	76 ± 11	110 ± 14	208 ± 22	103 ± 6
With EDTA‡	56 ± 10	32 ± 4§	29 ± 7§	163 ± 9§
Phenobarbital-treated rats‖				
No EDTA	241 ± 9	280 ± 17	399 ± 27	366 ± 1
With EDTA‡	194 ± 3§	178 ± 6	157 ± 3§	337 ± 11

*Values are mμmoles of drug metabolized/mg of microsomal protein/30 min; mean ± S.E. of two different experiments. All values for a given substrate and treatment (control vs. phenobarbital-treated) were obtained with the same pool of microsomes. Where no S.E. is given, N = 1.

†The amount of Mg^{2+} as $MgCl_2$ added to a total incubation volume of 2·5 ml.

‡Final concentration of 0·01 M EDTA.

§Significantly different from "No EDTA"; $P < 0·05$.

‖Rats were pretreated with phenobarbital sodium at a dose of 75 mg/kg daily for 3 days. Enzyme assays were run 24–48 hr after the last dose of phenobarbital.

appeared to be similar whether EDTA was present or not (i.e. peak activity was the same with or without EDTA).

Table 6 compares the effects of several concentrations of five cations on the metabolism of three substrates by hepatic microsomes, in order to determine whether the enhanced metabolism was specific for Mg^{2+}, as suggested by the report of Trivus and Spirtes.[7] With neoprontosil as the substrate, there was a qualitatively similar response (increased metabolism) with Mg^{2+}, Mn^{2+}, Sr^{2+} and Ca^{2+}. However, with Co^{2+} there was only an inhibition of azo reductase at all Co^{++} concentrations. With *p*-nitroanisole as substrate, little except inhibition at higher metal ion concentrations was seen with all ions; Mn^{2+} and Co^{2+} caused a greater inhibition than the other ions tested. With hexobarbital as the substrate, all metal ions caused enhanced metabolism and the peak enzyme activity was seen with 15 μmoles or less of the various cations added to the 2·5 ml incubation mixture.

TABLE 6. EFFECT OF VARIOUS CATIONS ON RAT LIVER MICROSOMAL DRUG METABOLISM *IN VITRO**

Substrate	Cation	Cation conc.† (μmoles)			
		0	1·5	15	150
Neoprontosil					
	Mg^{++}	104 ± 4	94 ± 2	141 ± 1‡	189 ± 1‡
	Mn^{++}	100 ± 2	121 ± 1	177 ± 1‡	231 ± 1‡
	Ca^{++}	83 ± 1	94 ± 7	139 ± 2‡	153 ± 4‡
	Co^{++}	106 ± 1	105 ± 3	26 ± 1‡	2 ± 1‡
	Sr^{++}	96 ± 4	106 ± 3	153 ± 2‡	207 ± 2‡
p-Nitroanisole					
	Mg^{++}	39 ± 1	47 ± 1	42 ± 1	20 ± 0‡
	Mn^{++}	41 ± 3	47 ± 1	36 ± 1	6 ± 1‡
	Ca^{++}	42 ± 2	39 §	47 ± 2	19 ± 1‡
	Co^{++}	41 ± 2	46 ± 1	35 ± 8	1 ± 0‡
	Sr^{++}	48 ± 1	55 ± 1	48 ± 5	24 ± 2‡
Hexobarbital					
	Mg^{++}	129 ± 5	158 §	238 ± 10‡	111 ± 13
	Mn^{++}	112 ± 6	232 ± 17‡	365 ± 8‡	88 ± 3
	Ca^{++}	149 ± 8	140 ± 23	318 ± 8‡	194 ± 16
	Co^{++}	146 ± 21	283 ± 8‡	203 ± 11	60 ± 33
	Sr^{++}	195 ± 18	289 ± 27	312 ± 17	201 ± 26

*Values are mμmoles of drug metabolized/mg of microsomal protein in 30 min; mean ± S.E. of 2 different experiments. All values for a given substrate were obtained with the same pool of microsomes, but different pools were used with different substrates. All concentrations of a given metal and substrate were incubated under the same conditions (i.e. same gas flow in the same incubator on the same day). Microsomes came from control rats.

† Amount of metal iron added (as the cloride) to the total incubation volume of 2·5 ml.

‡ Significantly different from no added cation; $P < 0{\cdot}05$.

§ N = 1 in cases where no S.E. is reported.

In spite of the fact that these various cations and drug substrates vary in their acidity, the capacity of the Hepes buffer used was sufficient to maintain the pH of the incubation mixtures within a range of 0·2 to 0·3 of a pH unit with all metal ion concentrations used (minimum pH was 7·1). Thus, we can assume that the changes in enzyme activity seen are most likely caused by the metal ion rather than by any alteration in pH, since this range of pH causes little alteration in the microsomal metabolisms studied.

Table 7 shows the effect of some other cations studied. Some of these are known to inhibit certain enzymic reactions. A recent report by Dixon *et al.*[22] about the role of trace metals in chemical carcinogenesis reported that Fe^{2+}, Cu^{2+} and Zn^{2+} in trace amounts stimulated the metabolism of benzpyrene in the microsomal fraction of homogenates from rat lung. As can be seen from Table 7, these divalent cations did not stimulate the metabolism *in vitro* of benzphetamine or aniline by liver microsomal preparations at any concentration studied. It appears that Cu^{2+} and Zn^{2+} are much more inhibitory than Fe^{2+}, particularly at the higher concentrations studied. The data presented in Tables 6 and 7 were obtained by using microsomes from control

TABLE 7. EFFECT OF VARIOUS CATIONS ON RAT LIVER MICROSOMAL DRUG METABOLISM *IN VITRO**

Substrate	Cation	Cation conc.† (μmoles)				
		0	0·25	2·5	25	250
Benzphetamine						
	Fe^{2+}	189 ± 6	186 ± 3	173 ± 2	156 ± 3	108 ± 3‡
	Cu^{2+}	189 ± 6	185 ± 3	185 ± 3	149 ± 3‡	0 ± 0‡
	Zn^{2+}	189 ± 6	179 ± 3	161 ± 3‡	165 ± 6‡	0 ± 0‡
Aniline						
	Fe^{2+}	20 ± 1	21 ± 1	21 ± 1	19 ± 1	18 ± 1
	Cu^{2+}	19 ± 1	19 ± 1	19 ± 1	13 ± 1‡	2 ± 1‡
	Zn^{2+}	22 ± 1	21 ± 0	20 ± 1	17 ± 1	4 ± 1‡

*Values are the mμmoles of product formed/mg of microsomal protein/30 min; mean ± S.E. of two different experiments. All values for a given substrate were obtained using the same pool of microsomes. All concentrations of a given metal were incubated with any given substrate under identical conditions. Microsomes came from control rats.
†Amount of metal ion added (as the chloride) to the total incubation volume of 2·5 ml.
‡Significantly different from 0 μmoles of metal; $P < 0{\cdot}05$.

rats. However, microsomes from livers of phenobarbital-treated rats responded in a qualitatively similar manner in all cases. Again, the final pH of the incubation mixtures (in Table 7) having the highest metal ion concentrations did not differ from the least concentration by more than 0·3 pH unit.

DISCUSSION

Some of the questions we have attempted to answer in this paper are discussed below.

Does the Mg^{2+} ion influence the metabolism differently when NADPH is generated than when chemically reduced NADPH is used?

Kornberg[3] has shown that Mg^{2+} enhanced the activity of glucose 6-phosphate dehydrogenase at 10^{-2} to 10^{-3} M. Cooper and Brodie[23] concluded from their studies on the hepatic microsomal metabolism of hexobarbital that Mg^{2+} enhanced barbiturate metabolism because it stimulated the NADPH-generating system rather than by having a direct effect on the microsomal enzyme system metabolising the hexobarbital. We have shown (Table 1) that the rate of hexobarbital, *p*-chloromethylaniline and neoprontosil metabolism by hepatic microsomes was increased by increasing concentrations of Mg^{2+} with either chemically reduced NADPH or an NADPH-generating system. With the higher concentrations of Mg^{2+}, the stimulating effect in the system

having chemically reduced NADPH equalled or exceeded that with the NADPH-generating system. Some of the possible explanations for the lack of stimulation in the experiments of Cooper and Brodie[23] are: (a) These investigators used a 0·01 M concentration of Mg^{2+}, a concentration which could be less effective in enhancing hexobarbital metabolism than the lower concentrations we studied. (b) In the studies where chemically reduced NADPH was used, Cooper and Brodie[23] used considerably less NADPH than we did (0·8 μmole added to a 4-ml incubation volume every 10 min for 60 min). In our experiments (Table 1), 5·5 μmoles NADPH was initially added to the 2·5 ml incubation volume. When only 1·5 μmoles NADPH (per 2·5 ml volume) was added, we found little if any enhanced activity with added Mg^{2+} (unpublished observations). We also found that excess Mg^{2+} inhibited hexobarbital metabolism by microsomes (Tables 1, 3–6).

Does the Mg^{2+} ion stimulate the metabolism of all types of drugs which are metabolized by the NADPH-requiring microsomal enzyme system, or is there a difference depending on the cytochrome P450-substrate reaction (type I vs. type II)[8] or enzymatic pathway?

The enzyme-substrate responses to Mg^{+2} can be classified into three groups: (1) enzyme activity with these substrates either did not change or decreased with increased concentrations of Mg^{2+}; (2) enzyme activity with these substrates only increased with increasing Mg^{2+} concentrations; and (3) enzyme activity with these substrates showed a peak at 6 mM Mg^{2+} or less, followed by a decrease as the Mg^{2+} concentration increased.

At present we are unable to propose any correlation between the enzymatic processes studied (oxidation, reduction) and the response to Mg^{2+}. Likewise, there appears to be no correlation with the type of substrate–cytochrome P450 complex (type I vs. type II)[8] and the response to Mg^{2+}.

Is the Mg^{2+} cation responsible or is there an effect caused by the associated anion or by overall ionic strength?

An evaluation of the data suggested to us that the anion was relatively unimportant in enhancing hepatic microsomal metabolism, or that monovalent Cl^- exerts an effect comparable to that of divalent $SO_4^=$, since $MgCl_2$ and $MgSO_4$ caused similar increases in drug metabolism (Table 2).

The data presented in Table 4 compare the influence of the chloride salts of Mg^{2+} K^+ and Na^+, at equal ionic strength, on the microsomal metabolism of aniline and hexobarbital. This study suggested that the Mg^{2+} ion was the activator rather than the Cl^- or the ionic strength. It also indicated that these concentrations of K^+ and Na^+ had little if any influence on the microsomal drug metabolisms studied.

The addition of EDTA to incubation mixtures in the absence of added Mg^{2+} caused little reduction in metabolism. However, in the presence of added Mg^{2+}, EDTA shifted the peak enzyme activity to a higher concentration of Mg^{2+} (Table 5). These results indicated that if microsomal drug metabolism were absolutely dependent on the Mg^{2+} ion, the endogenous Mg^{2+} (i.e. 0 μmole of added Mg in Table 5) must be present in a nonchelatable form or the chelated Mg^{2+} must be active as far as the effect on drug metabolisms was concerned.

Trivus and Spirtes[7] reported that Mg^{2+} was essential for the metabolism of hexobarbital and chlorpromazine and that the addition of EDTA to metabolism studies

in vitro completely inhibited these metabolisms. The data shown in Table 5 would appear to disagree with those of Trivus and Spirtes.[7] A possible explanation of this disagreement may be that Trivus and Spirtes[7] used considerably lower amounts of cofactors in their incubation, and this could possibly mask some of the observations we have seen. Trivus and Spirtes[7] also used rabbit liver microsomes, whereas we have worked exclusively with rat liver enzymes.

What is the response to other divalent cations?

Our studies have shown that divalent cations other than Mg^{2+}, such as Sr^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+}, caused an enhanced metabolism of some drugs by hepatic microsomal enzymes *in vitro*. The response seen with these cations, with the exception of Co^{2+}, was qualitatively similar to that with Mg^{2+} in the metabolsim of neoprontosil, *p*-nitroanisole and hexobarbital. Co^{2+} in low concentrations produced an enhanced rate of metabolism of hexobarbital; however, at higher concentrations (6 mM or greater), Co^{2+} inhibited the drug metabolisms studied. The inhibition seen with Co^{2+} may be related to its tendency to precipitate the microsomes. Such precipitation occurred with Co^{2+} even though the pH of the incubation mixture was maintained relatively constant and above 7·1. Fe^{2+}, Cu^{2+} and Zn^{2+} were inhibitory to the metabolism of aniline and benzphetamine. Magnesium, on the other hand, only stimulated these metabolisms at all concentrations studied (compare Tables 5 and 7).

Dixon *et al.*,[22] in a recent study on the role of trace metals in chemical carcinogenesis, found that Cu^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} enhanced the metabolism of benzpyrene by lung microsomes, while Mn^{2+}, Ni^{2+}, Fe^{3+}, Cr^{6+}, Co^{2+} and Be^{2+} were inhibitory. Nebert and Gelboin[24] have recently shown that Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+} and Co^{2+} caused a marked increase in the aryl hydroxylase activity in the microsomal fraction isolated from hamster fetus cell cultures, while Cu^{2+}, Fe^{2+} and Zn^{2+} were inhibitory in that system.

The data presented by these two groups of investigators studying the effects of metals on benzpyrene hydroxylase activity from different tissue sources were therefore different. Their results in turn differ from ours. In our study with rat hepatic microsomes, Mg^{2+} did not enhance benzpyrene metabolism at any of the Mg^{2+} concentrations studied (Table 3). In the studies of Dixon *et al.*[22] and of Nebert and Gelboin,[24] Mg^{2+} did cause an enhanced metabolism of benzpyrene, but the systems differed markedly in response to some of the other cations studied. Thus it appears that the effect of various cations on microsomal drug metabolism may depend on the tissue from which the microsomes are obtained. Metal ions appear to affect microsomal drug metabolism in lung, in fetal hamster cells and in adult rat liver differently.

Acknowledgements—The authors wish to thank Mrs. Roberta Pohl and Mrs. Gladys Rios for technical assistance in this research.

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