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THE INHIBITORY EFFECT OF AUREOMYCIN (CHLORTETRACYCLINE) PRETREATMENT ON SOME RAT LIVER MICROSOMAL ENZYME ACTIVITIES

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Abstract—Rats were subjected to intraperitoneal injections of varying amounts of chlortetracycline to study the effects of this antibiotic on rat liver microsomal drug metabolism. The chlortetracycline-treated rats were then compared with sham-injected controls and phenobarbital-treated animals. Compared with the control animals, the chlortetracycline-treated animals gained weight more slowly, their livers were smaller, the microsomal protein per unit weight of liver was less, the hepatic microsomal cytochrome P-450 content and metabolism *in vitro* of aminopyrine and hexobarbital were decreased while the hexobarbital sleeping time was markedly increased. The phenobarbital-treated animals showed the usual increase in all of the hepatic microsomal components and a marked decrease in hexobarbital sleeping time. Our data would indicate a possible inhibition of microsomal protein synthesis by chlortetracycline treatment, supporting the suggested mechanism of action of the tetracycline antibiotics.

ICHIKAWA *et al.*[1] recently reported that livers from rats treated with Aureomycin (chlortetracycline), phenobarbital, chlordane and other drugs had markedly increased measurable liver microsomal cytochrome P-450. A recent review by Conney,[2] cites several investigators who have reported similar results with phenobarbital and chlordane pretreatment. In addition, after such pretreatment there is an apparent correlation between the increased hepatic microsomal cytochrome P-450 content and certain enhanced enzymic activities *in vivo* and *in vitro*. Electron microscopic studies have shown that treatment with either phenobarbital or chlordane also caused a proliferation of the smooth endoplasmic reticulum in rat liver associated with an increased liver microsomal protein content.[2, 3] These results provided evidence of enhanced protein synthesis.

A number of investigators have shown that chlortetracycline exerted its antibacterial action by suppressing protein synthesis.[4] Chlortetracycline also inhibited the incorporation of amino acids into rat liver ribosomal proteins. The increase in cytochrome P-450 content reported by Ichikawa *et al.*[1] could possibly be important to our concepts about the mechanism of action of chlortetracycline or microsomal enzyme induction or of both. Thus, a series of experiments was undertaken to determine the effect of chlortetracycline treatment on the cytochrome P-450 content and various other enzyme activities in liver microsomes.

MATERIALS AND METHODS

Male, Long–Evans rats weighing 100–150 g were obtained from Simonsen

Laboratories, Gilroy, Calif. 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 libitum*.

The rats were separated into groups of four animals each and subjected to various treatments. Three groups received intraperitoneal (i.p.) injections of chlortetracycline at doses of 25, 50 and 100 mg per kg per day for 5 days respectively. The chlortetracycline used was the commercially available preparation, Aureomycin (for i.v. use), obtained from Lederle Laboratories, Pearl River, N.Y. All doses were given as Aureomycin, not as the chlortetracycline base. A fourth group received phenobarbital (75 mg/kg/day for 3 days, i.p.) and served as a positive control. The untreated controls were subjected to daily sham injections. Twenty-four hr after the last injection, the animals were given 125 mg hexobarbital sodium per kg, i.p., and sleeping times were determined. One hr after waking the rats were sacrificed (between 7 and 8 a.m.) by cervical dislocation. The livers were immediately removed and placed in beakers immersed in ice. All subsequent procedures were carried out at 0–4°.

The livers were homogenized (1 g liver and 2 ml KCl) in ice-cold 1·15% potassium chloride containing 0·05 M Tris at a final pH of 7·4. The 9000 *g* supernatant fraction (microsomes and soluble fraction) was obtained by centrifuging the homogenate at 9000 *g* for 20 min. The microsomes were obtained by centrifuging the 9000 *g* supernatant fraction at 220,000 *g* for 30 min. The microsomal pellet was suspended and washed once in 1·15% KCl and resedimented as before. The washed microsomes were resuspended in a sufficient volume of 0·1 M phosphate buffer or 0·2 M HEPES buffer[5] at pH 7·4 to give a protein concentration of 10 mg microsomal protein per ml of suspension. Microsomal protein was determined by the method of Lowry *et al.*[6] with crystalline bovine serum albumin as the standard. The microsomal P-450 content was determined by measuring the difference spectrum essentially as described by Omura and Sato.[7] After this determination of the P-450, 2–3 mg of powdered bovine serum albumin was added to both the test and blank cuvettes and the difference spectrum was redetermined. This albumin was added to see if a reversibly bound material (chlortetracycline or a metabolite of chlortetracycline) was interfering with the assay of P-450.[8]

For the determination of drug-metabolizing enzyme activity *in vitro*, 1 ml of the microsomal suspension (10 mg of microsomal protein) was incubated in a Dubnoff shaking incubator for 30 min at 37° with oxygen as the gaseous phase. The final concentration of cofactors added was: nicotinamide adenine dinucleotide phosphate (NADP), 2·25 μmoles; glucose 6-phosphate, 25 μmoles; nicotinamide, 20 μmoles; 0·5 ml of soluble fraction (containing the NADPH generating system, glucose 6-phosphate dehydrogenase) and magnesium as indicated in the tables. The final volume of the incubation mixtures was brought to 5 ml with 0·1 M phosphate buffer or 0·2 M HEPES buffer, pH 7·4. HEPES buffer was used when the effects of various Mg^{++} concentrations were studied.

The enzyme pathways studied and substrate concentrations in micromoles per 5 ml of incubation mixture were: *N*-dealkylation of aminopyrine (10 μmoles) to 4-aminoantipyrine (4-AAP), measured by the method of LaDu *et al.*;[9] *N*-dealkylation of aminopyrine (20 μmoles) to formaldehyde, measured by the method of Cochin and Axelrod;[10] side chain oxidation of hexobarbital (12 μmoles), estimated by measuring the disappearance of substrate by a modification of the method of Axelrod *et al.*[11] as described by Roberts and Plaa.[12]

RESULTS

Chlortetracycline pretreatment of rats resulted in a decreased growth rate over a 5-day treatment period. In addition, the data presented in Table 1 show a decrease in liver weight and microsomal protein per unit weight of liver. Other alterations *in vivo* were seen as a decrease in P-450 content and an increased hexobarbital sleeping time. The metabolism *in vitro* of aminopyrine (measuring both formaldehyde and 4-aminoantipyrine formation) and hexobarbital by washed microsomal suspensions was decreased as well. However, pretreatment with phenobarbital caused a marked

TABLE 1. SOME ALTERATIONS IN RAT LIVER *IN VIVO* AND *IN VITRO* AFTER CHLORTETRACYCLINE PRETREATMENT*

Treatment	Liver wt. (g)	Microsomal protein (mg/g liver)	Sleeping time† (min)	P-450 (Δ O.D./mg protein/ml)	Microsomal metabolism of: Aminopyrine — Formaldehyde	Aminopyrine — 4-AAP	Hexobarbital
Control	7·3 ± 0·3	33·6 ± 1·9	37 ± 4	0·096 ± 0·011	210 ± 21	7·4 ± 0·4	175 ± 40
Phenobarbital (75 mg/kg)‡	9·5 ± 0·2§	37·4 ± 2·4	8 ± 1§	0·200 ± 0·004§	377 ± 9§	36·0 ± 2·3§	367 ± 13§
Chlortetracycline (25 mg/kg)	6·2 ± 0·3	21·9 ± 0·9§	44 ± 4	0·078 ± 0·003	165 ± 6	7·0 ± 0·5	128 ± 14
Chlortetracycline (50 mg/kg)	6·2 ± 0·4	21·4 ± 0·6§	61 ± 9	0·062 ± 0·002§	130 ± 7§	3·8 ± 0·1§	80 ± 7§
Chlortetracycline (100 mg/kg)	5·7 ± 0·5§	20·8 ± 2·2§	75 ± 13‡	0·071 ± 0·005	152 ± 12	5·7 ± 0·2§	68 ± 13§

* Values are the mean ± S.E. for four animals. Metabolism values *in vitro* are in terms of mμmoles of drug metabolized or product formed per mg microsomal protein per 30 min. Magnesium, 25 μmoles, was added to each of the incubation mixtures in the microsomal metabolism studies *in vitro*. All data within each treatment group were obtained from the same animals. Chlortetracycline treatment consisted of five daily injections and phenobarbital treatment consisted of three daily injections at the various doses listed. 4-AAP = 4-aminoantipyrine.

† Sleeping time was determined from the time of loss of righting reflex to the regaining of the righting reflex.

‡ Phenobarbital-treated animals were used as a positive control.

§ Significantly different from controls ($P < 0.05$).

increase in liver weight, microsomal protein per unit weight of liver, P-450 content, drug metabolism *in vitro* and a decrease in hexobarbital sleeping time. These alterations with phenobarbital pretreatment are indicative of microsomal enzyme induction.

Various possible explanations for the changes associated with chlortetracycline pretreatment were investigated and the results are presented in Tables 2–5. In Table 2 it can be seen that chlortetracycline pretreatment caused a marked decrease in metabolism *in vitro*, which was not corrected by the addition *in vitro* of excess magnesium. If the chlortetracycline were exerting its inhibitory effect by chelating the magnesium ion, thus effectively removing it from solution, the addition of excess magnesium might restore the activity. Such a possibility has been demonstrated with some enzyme systems (Brody *et al.*;[13] Weinberg[14]). The results from another experiment in which chlortetracycline was added *in vitro* to washed microsomes from non-chlortetracycline-pretreated animals are presented in Table 3. An inhibition of microsomal enzymes was observed which likewise was not reversed by adding varying concentrations of magnesium. It would appear that the inhibition is not caused by a

TABLE 2. INFLUENCE OF MAGNESIUM ION ON DRUG METABOLISM* BY HEPATIC MICROSOMES FROM CHLORTETRACYCLINE-PRETREATED RATS

Drug substrate and treatment	Magnesium concn/5 ml			
	0 μmoles	25 μmoles	100 μmoles†	200 μmoles†
Aminopyrine (4-AAP)‡				
Control	4·3 ± 0·3	8·5 ± 0·4	7·2 ± 0·6	8·1 ± 0·6
Chlortetracycline (50 mg/kg)	3·0 ± 0·3§	5·4 ± 0·3§	5·7 ± 0·0§	4·0 ± 0·1§
Hexobarbital‖				
Control	74 ± 6	150 ± 13	125 ± 14	93 ± 7
Chlortetracycline (50 mg/kg)	42 ± 6§	58 ± 7§	66 ± 8§	42 ± 6§

* Values are mμmoles drug metabolized or product formed per mg protein in 30 min; mean ± S.E. for four animals; in HEPES buffer.
† The final pH of the incubation mixture with these concentrations of Mg^{++} was 7·3 ± 0·1 Hepatic microsomes were isolated from normal, untreated animals (control) or from animals pretreated (i.p.) once a day for 5 days with chlortetracycline (50 mg/kg).
‡ Aminopyrine metabolism was measured by 4-aminoantipyrine production.
§ Significantly different from controls ($P < 0·05$).
‖ Hexobarbital metabolism was measured by hexobarbital disappearance.

TABLE 3. EFFECT OF ADDITION *IN VITRO* OF CHLORTETRACYCLINE AND MAGNESIUM ION ON DRUG METABOLISM* BY RAT LIVER MICROSOMES†

Hexobarbital	Magnesium added/5 ml			
	0 μmoles	25 μmoles	100 μmoles‡	200 μmoles‡
Control				
No chlortetracycline	130 ± 11	232 ± 1	234 ± 3	210 ± 1
Chlortetracycline $3·3 \times 10^{-3}$	95 ± 4§	85 ± 3§	62 ± 6§	15 ± 2§
Phenobarbital-treated				
No chlortetracycline	270 ± 7	414 ± 6	453 ± 8	488 ± 4
Chlortetracycline $3·3 \times 10^{-3}$	233 ± 5§	237 ± 2§	187 ± 24§	114 ± 14§

* Values are mμmoles drug metabolized per mg protein in 30 min; mean ± S.E. for three treatments. All treatments in the control group were on the same pool of microsomes. All treatments in the phenobarbital-treated group were on the same pool of microsomes. All assays were done in HEPES buffer.
† The microsomes were isolated from livers of untreated rats or rats treated for 3 days with phenobarbital (75 mg/kg) as indicated in the table.
‡ The final pH of the incubation mixture with these concentrations of Mg^{++} was 7·3 ± 0·1.
§ Significantly different from the respective incubation to which no chlortetracycline was added $P < 0·05$).

reversible magnesium-chelating effect of chlortetracycline, since excess magnesium does not prevent the inhibition caused by treating animals with chlortetracycline or by adding chlortetracycline to incubation mixtures *in vitro*. Table 4 shows the results of a series of experiments in which chemically reduced NADPH was used instead of the usual NADPH generating system. These results combined with the other data presented would suggest that the inhibitory effect seen with chlortetracycline pretreatment is associated with an alteration of the microsomal drug-metabolizing

enzyme systems themselves, rather than an inhibition of the NADPH generating system.

Dale *et al.*[15] have reported that tetracyclines are selectively localized in and firmly bound to the microsomal component of the cell. These findings offer another possible explanation for chlortetracycline inhibition. Castro *et al.*[8] have reported that pretreatment with SKF 525-A causes an apparent decrease in microsomal P-450 content which is partially reversed when serum albumin is added to the microsomes. These authors suggest that this reversal may be due to the partial removal of SKF 525-A and its metabolites which are reversibly bound to the liver microsomes. Table 5 gives the results of an experiment in which the P-450 content of liver microsomes

TABLE 4. CHLORTETRACYCLINE INHIBITION OF DRUG METABOLISM AND SOURCE OF NADPH: NADPH GENERATION VS. NADPH CHEMICALLY REDUCED*

Drug substrate and treatment	NADPH generation†	NADPH chemically reduced‡
Aminopyrine (formaldehyde)		
Control	186 ± 34	139 ± 4
Chlortetracycline (50 mg/kg)	83 ± 3§	78 ± 15§
Hexobarbital		
Control	91 ± 3	88 ± 2
Chlortetracycline (50 mg/kg)	38 ± 3§	51 ± 3§

* Values are mμmoles drug metabolized or product formed per mg protein in 30 min; mean ± S.E. for four animals. Hepatic microsomes were isolated from normal, untreated animals (control) or from animals pretreated (i.p.) once a day for 5 days with chlortetracycline (50 mg/kg).

† Generating system contained 2·25 μmoles NADP, 0·5 ml of soluble fraction from control animals, 25 μmoles magnesium, 20 μmoles nicotinamide, 25 μmoles G6-P, 10 mg microsomal protein, appropriate substrate and buffer to final volume of 5 ml.

‡ Incubation contained 10 mg NADPH, 25 μmoles magnesium, 20 μmoles nicotinamide, 10 mg microsomal protein, appropriate substrate and buffer to a final volume of 5 ml.

§ Significantly different from controls ($P < 0·05$).

TABLE 5. EFFECT OF ADDED ALBUMIN ON MEASURED CYTOCHROME P-450 CONTENT OF HEPATIC MICROSOMES FROM CONTROL VS. CHLORTETRACYCLINE-TREATED RATS*

Treatment	P-450 content	P-450 content with albumin†
Control	0·080 ± 0·005	0·080 ± 0·005
Chlortetracycline (50 mg/kg; exp. 1)	0·053 ± 0·002‡	0·053 ± 0·002‡
Chlortetracycline (50 mg/kg; exp. 2)§	0·052 ± 0·003‡	0·052 ± 0·003‡

* Values are Δ O.D. (450–500 mμ) per mg protein per ml of cuvette contents; mean ± S.E. for four animals. The chlortetracycline treatment consisted of 5 daily i.p. injections.

† Approximately 3 mg of powdered bovine serum albumin was added per 3 ml.

‡ Significantly different from control, $P < 0·05$.

§ This group of animals (but no other) was also treated with hexobarbital (125 mg/kg) to see whether sleeping time determinations altered the measurable P-450 in microsomal preparations from chlortetracycline-pretreated rats.

from chlortetracycline-pretreated animals was determined before and after the addition of approximately 3 mg of powdered serum albumin. The addition of serum albumin had no effect on the measured P-450 content (Table 5). Thus, chlortetracycline is bound more firmly, reacts with, or alters microsomal P-450 content in a way different from SKF 525-A, possibly by inhibiting microsomal protein synthesis, as suggested by the data presented in Table 1.

DISCUSSION

Ichikawa *et al.*[1] have reported that chlortetracycline pretreatment caused an increase in P-450 content in rat liver microsomal fractions. An increase in P-450 content is generally considered to be associated with enzyme induction and increased protein synthesis. Thus, the results of Ichikawa *et al.*,[1] might suggest that chlortetracycline is an enzyme inducer similar to phenobarbital, chlordane, etc. The present study was designed to determine the validity of this report and to determine more fully the effect of chlortetracycline pretreatment on microsomal drug metabolism. In considering the proposed mechanism of chlortetracycline action (suppression of protein synthesis[4]), it appeared that a decrease in P-450 content might be expected rather than an increase. One would also expect to see an inhibition of drug metabolism *in vivo* and *in vitro*.

The data presented in Table 1 contrast the results obtained from phenobarbital-pretreated and chlortetracycline-pretreated animals. The phenobarbital-treated rats showed evidence of microsomal enzyme induction, while chlortetracycline-treated rats provided evidence of microsomal enzyme inhibition.

Possible explanations for the inhibitory effect of chlortetracycline pretreatment on drug metabolism were investigated. Tetracycline antibiotics are known to bind to proteins in general and are selectively localized in the microsomal protein fraction.[15] This information presents several possible alternative explanations: (1) Is the chlortetracycline bound (reversibly or nonreversibly) to the microsomes, preventing interaction with the drug substrate? (2) Does the chlortetracycline prevent the synthesis of new protein, causing a gradual decrease in microsomal protein? (3) Does the chlortetracycline chelate the metal ions which are needed for the drug-metabolizing enzyme reactions or the generation of NADPH?

Castro *et al.*[8] have shown that pretreatment with SKF 525-A causes an apparent decrease in P-450 content which is partially reversed by the addition of serum albumin. This reversal is apparently due to removing the SKF 525-A from the microsomes. A similar experiment with microsomes from chlortetracycline-pretreated animals resulted in no reversal of the decrease in P-450 content. From the work of Castro *et al.*[8] and of Dale *et al.*,[15] it appears that some of the inhibitory effects of SKF 525-A can be removed from the liver microsomes within a few hours, whereas the chlortetracycline remains in the liver microsomes for several days. This would tend to support the idea of nonreversible binding of chlortetracycline or a metabolite, and reversible binding of SKF 525-A or removal of SKF 525-A by metabolism.[16]

Administration *in vivo* or addition *in vitro* of chlortetracycline caused an inhibition of hepatic microsomal drug metabolism as assayed *in vitro*. Brody *et al.*[13] have shown that the tetracycline inhibition of some hepatic enzymes can be reversed by the addition of excess amounts of Mg^{++} ion and thus concluded that these inhibitions were caused by the chelation of the essential Mg^{++} ion by the chlortetracycline. Franklin[17] has shown that chlortetracycline inhibition of protein synthesis was not

reversed by excess Mg^{++} ion, indicating that chlortetracycline did not exert its inhibitory action on protein synthesis merely by complexing with the essential Mg^{++} ion. Our attempts to reverse the inhibitory effect of chlortetracycline on microsomal drug metabolism by the addition of varying concentrations of Mg^{++} (Tables 2, 3) suggested that the inhibition seen was not a result of Mg^{++} chelation.

Using chemically reduced NADPH instead of an NADPH generating system did not overcome the inhibition seen with chlortetracycline pretreatment (Table 5). This suggested that the effect of chlortetracycline was not caused by inhibiting NADPH generation, but was instead an effect on the microsomal enzyme systems.

The data would suggest that chlortetracycline exhibits its inhibitory effect by very firmly combining with the hepatic microsomal proteins and/or interfering in some way with the synthesis of new microsomal protein. This latter would be consistent with our data of Table 1 which show a decrease in the hepatic cytochrome P-450 levels. We believe this evidence further substantiates the currently held concept of the mechanism of action of the tetracycline antibiotics, i.e. inhibition of protein synthesis.

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