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THE ROLE OF MICROBIAL FLORA IN THE HEPATOTOXICITY OF CHLORTETRACYCLINE *IN VIVO*: A STUDY WITH GERMFREE MICE*

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Abstract—Studies with germfree mice have revealed that chlortetracycline (CTC) hepatotoxicity does not depend on secondary effects related to modification of the indigenous microflora but that it is the consequence of a direct drug action. Germfree (GF) and conventionalized (CONV) mice, injected intraperitoneally with large doses of CTC, both developed signs of hepatotoxicity: liver weight, total lipids, and lipid fractions (glycerides, cholesterol, phospholipids) increased, while liver water and protein decreased; activity of the liver enzymes (glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, glutamic dehydrogenase, urea cleavage enzyme, and arginase) all decreased, even below that of liver protein concentration. In general, the effect of CTC on the GF mouse was more severe than on the CONV: lipids rose higher, while protein fell lower. In each type of mouse, the concentration of liver glycerides increased most. Fasting exacerbated all these effects especially in the CONV mouse. There were no significant differences between the livers of GF and CONV *untreated* mice in any of the parameters measured, except for total lipids and glycerides, which were lower in the GF liver. Thus, the presence of an indigenous flora appears not only to attenuate the lipogenic response of the liver to CTC but also to influence the liver lipid composition of the untreated mouse.

Our results are consistent with the hypothesis that CTC interferes with protein metabolism of the liver in at least two broad ways: by exerting a catabolic or antianabolic affect, in that liver protein, in general, is decreased after CTC; and by interfering with metalloenzyme action by chelation with metal cofactors, leaving those enzymes relatively unaffected which require no metal cofactors.

IT WAS discovered in 1951 that large parenteral doses of the tetracycline antibiotics caused fatty infiltration of the liver of man[1–3] and that this could be reproduced in experimental animals.[4] The effects of the tetracyclines have been intensively investigated *in vitro* and *in vivo* since then, and a number of human deaths due to tetracycline toxicity have been reported;[5–8] chlortetracycline has been shown to lose its hepatotoxicity for rats when its antibacterial effects are abolished by heat before administration.[9] In addition, Faloon *et al.* showed that chlortetracycline produces antianabolism or catabolism of protein in man.[10]

The interrelationships of antibiotics, microflora, and the host remain largely to be elucidated, and the germfree animal is the tool *par excellence* for this work. It has

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been shown, for example, that small doses of penicillin are lethal for conventional guinea pigs, but not harmful to germfree guinea pigs.[11] It is inferred by the authors that the lethal effect arises secondarily to modifications of the microflora, and is not a direct effect on the host. Germfree animals, in this way, provide a unique opportunity to study the direct drug effects of an antibiotic, uncomplicated by any indirect effects that may arise in ordinary bacteria-laden animals from a modification of their indigenous microflora.

We set out to determine in these experiments: (a) whether the germfree mouse would develop a fatty liver after parenteral treatment with large doses of fresh chlortetracycline and, if so, (b) the nature of the hepatic lipid components accumulated, and (c) the effect on liver protein and some enzymes. Each germfree (GF) group was compared with a like group of mice which had been germfree, but were at the time of the study "conventionalized" (CONV), that is, they had been purposely contaminated with cecal material from other conventionally reared mice.

MATERIALS AND METHODS

Experiment 1. In this experiment, emphasis was put on the characterization of the liver lipids, both with and without chlortetracycline (CTC) treatment, as we had no certain knowledge that the presence or absence of a microbial flora per se influences the liver lipids of the mouse. Moreover, since the inception of our study, we have been able to find only one fragmentary report[7] dealing with the nature of the lipids of tetracycline-induced fatty liver. Three-month-old ICR-strain male and female mice were used. All mice were originally germfree (GF); half of these were conventionalized (CONV) 5 weeks earlier according to procedures established in this laboratory.[12] They were maintained in isolators throughout the experiment, whether CONV or GF, and all were handled and monitored microbiologically by established procedures.[12] Steam-sterilized, semi-synthetic L-356 diet (General Biochemicals, Inc., Chagrin Falls, Ohio) and water were provided *ad libitum* pre- and post-treatment. The mice were housed in plastic cages with wood-chip litter in groups of 5–6 per cage, and were maintained on an alternating 12-hr schedule of illumination and darkness. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Fresh chlortetracycline (Aureomycin, Lederle) (CTC) solution was prepared daily by adding 50 ml 0·9% saline to 500 mg CTC; these components had been previously introduced into the isolators (whether GF or CONV) by the usual cold sterilization procedure of spraying the exterior of the sealed vials containing the sterile preparations with peracetic acid. CTC was injected i.p. at a dose of 100 mg/kg body weight, with controls receiving an equivalent volume of saline only (0·01 ml/g body weight). The same dose and treatment were repeated once daily for 4 consecutive days; animals were sacrificed 24 hr after their last injection.

Since we had no data regarding the liver lipid composition of the untreated animals of either sex, extra control animals were included in the first experiment in order to accumulate enough data to enable meaningful statistical evaluation for each sex.

At sacrifice, accomplished by CO_2 inhalation, blood was withdrawn from the still-beating heart of the dying mouse, after which the liver was promptly excised, patted dry with a gauze pad, weighed, and divided into three portions, each of which was immediately weighed. One portion was set aside for subsequent histological study

(to be reported later), one for determination of water content, and one for lipid analysis. Liver water was determined by weight difference after drying a pre-weighed sample of fresh liver to constant weight at 105°. Lipid analyses were done within one week on samples of liver that had been sealed fresh in plastic bags, quick frozen to, and stored at, −70°.

Total lipids were extracted from a portion of the liver according to the procedure of Folch *et al.*[13] The lipid fractions were evaporated at room temperature under a stream of nitrogen and then taken up with 6 ml hexane. Here we noticed that a small portion of the GF or CONV sample remained insoluble. One ml of the hexane solution was chromatographed on minus 325 mesh silicic acid (Bio-Rad Labs , Richmond, Calif.), according to the techniques of Freeman *et al.*[14] and Hirsch and Ahrens,[15] with minor modifications. These techniques separate the total lipids into three fractions: cholesterol esters, glycerides plus fatty acids plus free cholesterol, and phospholipids. The cholesterol ester fraction was discarded, since the total cholesterol was assayed later in the entire lipid sample.[16] The second fraction was assayed in infrared analysis for total glycerides; tri-, di-, and monoglycerides were not differentiated. Free cholesterol did not interfere, and the free fatty acids were negligible. The phospholipid fraction was assayed by infrared in the third fraction.[14]

A Perkin–Elmer model 221 infrared spectrophotometer was used in these assays with 1-mm NaCl cells Each fraction emerging from the column was evaporated as before with nitrogen gas and taken up with 2 ml carbon disulfide. Glycerides were measured at a wavelength of 8·70μ, and phospholipids at 9·35 μ. Glyceryl palmitate and lecithin were used for standards (Applied Science Labs., Inc., State College, Pa.).

Blood urea nitrogen (BUN) was determined with diacetyl monoxide according to Friedman.[17]

Experiment 2. In this experiment we examined some of the effects of CTC on the liver proteins and certain enzymes, because the accumulation of liver fat is probably secondary to an induced defect in protein metabolism, irrespective of the microbial status of the mouse. Four-month-old ICR-strain female mice were used. All procedures were as before. Body weights, liver weights, liver water, liver total lipids, and BUN were determined as before. Liver glycogen was quantitated by the procedure of Carroll *et al.*,[18] and liver protein was determined gravimetrically after trichloroacetic acid precipitation and removal of this acid by ether extraction.

Liver glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) were assayed according to Tohazy *et al.*[19] and glutamic dehydrogenase (GDH) by the method of Lowry *et al.*[20] with a fluorescent determination for NAD according to Kaplan.[21] The "cleavage enzyme" of the urea cycle, and arginase were assayed by the methods of Brown and Cohen,[22] except that the tissues were ground in water as recommended by Schimke.[23]

Liver glycogen was determined promptly on fresh liver. Liver lipids, protein, and enzymes were determined within one week on liver specimens that had been sealed fresh in plastic bags and quick frozen to −70°; preliminary observations indicated that these substances were stable under these conditions of storage.

Experiment 3. This experiment was performed simultaneously with Experiment 2 and was in all respects identical with it except that the GF and CONV female mice were deprived of food and water (fasted) for 4 days, starting with the saline or CTC treatment until they were sacrificed. We did this experiment because we, as well as

others,[4] have observed that the drug-treated mice tended to eat less than normally, and we wished to determine whether, or how much of, the effect of the CTC might have been due to lessened dietary intake.

The statistical significance of the difference between mean values was assessed by the *t*-test, and was set at a probability level of 5% or less.

RESULTS

Experiment 1. No obvious signs of distress were noted among the mice, except that some of the CTC-treated animals, both CONV and GF, showed evidence of diarrhea up to 4 days after their first injection; none of the saline controls was affected in this way.

Table 1 shows the data gathered from control mice only, both male and female; there were no significant differences between the CONV and GF mice with respect to

TABLE 1. NORMAL VALUES FOR GERMFREE AND CONVENTIONALIZED MICE

Variable	Males		Females	
	CONV	GF	CONV	GF
Initial body wt. (g)	33·6 ± 1·1	31·6 ± 0·6	29·4 ± 0·9	28·7 ± 0·6
BUN (mg/100 ml)	25 ± 4	26 ± 3	21 ± 5	25 ± 4
Liver wt. (g/100 g b.w.)	5·20 ± 0·23	4·98 ± 0·18	4·65 ± 0·13	4·12 ± 0·10
Liver (% water)	68·1 ± 0·5	69·5 ± 1·1	69·5 ± 1·1	69·3 ± 0·8
Liver lipids (mg/g liver)				
Total lipids	53·3 ± 3·1[a]	42·4 ± 2·4[a]	56·1 ± 2·1[b]	45·4 ± 2·1[b]
Glycerides	14·5 ± 1·0[c]	10·5 ± 0·7[c]	14·6 ± 1·0	12·0 ± 0·6
Cholesterol	4·0 ± 0·4	3·2 ± 0·2	5·3 ± 0·5	4·2 ± 0·4
Phospholipids	28·4 ± 1·2	28·4 ± 1·4	30·0 ± 1·4	30·0 ± 1·4

Each group consisted of 13–15 mice. Mean ± S.E. of the mean in all tables.

Differences between values with the same superscript (a, b, or c) are statistically significant ($P < 0·05$ by *t*-test) for all tables.

body weight, BUN, liver weight per unit body weight, or liver water. The liver lipids, including glycerides and cholesterol, however, were clearly higher in the CONV livers; phospholipids were the same in both groups.

Table 2 presents the results of four injections of chlortetracycline or saline; the results for the males and females are combined (there was an equal number of each), since there were no obvious differences in response, and since this procedure made statistical evaluation possible.

The only significant differences in response to CTC occurred in the liver lipid fractions and in the BUN. All liver lipid components increased in concentration in both GF and CONV mice after CTC, but the glycerides showed the greatest increase. CTC increased the CONV liver glycerides about 2·6 times above the control level, but raised the GF liver glycerides 3·8 times above the control level. For the CONV and GF mice, respectively, the corresponding ratios (CTC-treated:saline-treated) for total liver lipids were 2·3 and 3·1; for cholesterol 1·4 and 2·2; and for phospholipids 2·1 and 2·5. Thus the GF mice, in general, showed greater increases in all liver components

TABLE 2. EFFECTS OF CHLORTETRACYCLINE ON FED MICE; EXPERIMENT 1

Variable	Conventionalized			Germfree		
	Saline	CTC	CTC-Sal.	Saline	CTC	CTC-Sal.
Initial body wt. (g)	30·6 ± 0·5	31·1 ± 0·8	+ 0·5	30·0 ± 0·6	30·2 ± 0·6	+ 0·2
Final body wt. (g)	29·5 ± 0·4	27·9 ± 1·1	− 1·6	29·8 ± 0·5	27·3 ± 0·8	−2·5
BUN (mg/100 ml)	20 ± 3	46 ± 14	+26[a]	29 ± 7	17 ± 1	−12[a]
Liver wt. (g/100 g body wt.)	5·39 ± 0·24	5·41 ± 0·22	+ 0·02	4·42 ± 0·18	5·09 ± 0·20	+ 0·67
Liver (% water)	69·1 ± 0·9	64·9 ± 1·1	− 4·2	69·4 ± 0·8	64·3 ± 0·9	− 5·1
Liver lipids (mg/g liver)						
Total lipids	51·9 ± 4·7	118·9 ± 13·8	+67·0[b]	42· 4 ± 3·6	134·0 ± 6·8	+91·6[b]
Glycerides	14·3 ± 2·2	37·5 ± 3·8	+23·2[c]	12·5 ± 1·2	48·1 ± 3·3	+35·6[c]
Cholesterol	4·4 ± 0·6	6·1 ± 0·7	+1·7[d]	3·4 ± 0·7	7·6 ± 0·8	+4·2[d]
Phospholipids	28·3 ± 1·9	58·4 ± 5·0	+30·1	28·3 ± 2·1	69·6 ± 4·7	+41·3

Each group consisted of 9–10 mice.

TABLE 3. EFFECTS OF CHLORTETRACYCLINE ON FED MICE; EXPERIMENT 2

Variable	Conventionalized			Germfree		
	Saline	CTC	CTC-Sal.	Saline	CTC	CTC-Sal.
Initial body wt. (g)	29·7 ± 1·7	27·7 ± 1·5	−2.0	35·4 ± 1·4	32·9 ± 1·9	−2·5
Final body wt. (g)	29·5 ± 1·3	26·9 ± 1·4	−2·6	33·2 ± 1·3	30·9 ± 1·6	−2·3
BUN (mg/100 ml)	19·7 ± 1·2	16·4 ± 1·4	−3·3	16·5 ± 3·1	15·3 ± 5·1	−1·2
Liver						
Wt. (g/100 g body wt.)	4·21 ± 0·30	5·34 ± 0·22	+1·13	3·74 ± 0·08	4·67 ± 0·13	+0·93
Protein (g/100 g body wt.)	1·09 ± 0·07	0·96 ± 0·09	−0·13[a]	1·03 ± 0·09	0·73 ± 0·06	−0·30[a]
Protein (mg/g liver)	289 ± 22	211 ± 30	−78	283 ± 21	176 ± 49	−107
Glycogen (mg/g liver)	34·3 ± 5·0	29·5 ± 3·8	−4·8[b]	30·4 ± 5·1	35·0 ± 4·2	+4·6[b]
Total lipids (mg/g liver)	61·1 ± 6·6	134·4 ± 8·6	+73·3[c]	48·6 ± 4·0	149·8 ± 12·2	+101·2[c]
Water (%)	66·0 ± 0·5	56·3 ± 1·0	−9·7	67·3 ± 1·1	56·8 ± 1·2	−10·5
GOT, GPT, GDH (μmoles α-ketoglutarate formed/min/g protein)	727 ± 109	615 ± 49	−112	686 ± 60	587 ± 78	−99
GPT (μmoles α-ketoglutarate formed/min/g protein)	265 ± 32	185 ± 30	−80	273 ± 38	207 ± 40	−66
GDH (μmoles α-ketoglutarate formed/min/g protein)	44·0 ± 6·1	40·6 ± 8·1	−3·4	43·7 ± 5·3	40·5 ± 12·2	−3·2
Cleavage Arginase (μmoles urea formed/min/g protein)	28·0 ± 3·0[d]	13·6 ± 2·1	−14·4	42·3 ± 6·0[d]	23·7 ± 5·8	−18·6
Cleavage Arginase (μmoles urea formed/min/g protein)	3811 ± 525	2478 ± 74	−1333	4850 ± 486	2915 ± 82	−1935

Each group consisted of 5–6 mice.
GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase;
GDH, glutamic dehydrogenase.

than their CONV counterparts after CTC, the differences being statistically significant for the total lipids, glycerides, and cholesterol. Moreover, those liver lipid components which were present in relatively lower concentration in the GF than in the CONV controls showed the greatest increases after CTC, especially in the GF; the liver phospholipid fraction, which was almost identical in both control groups, rose essentially to the same extent after CTC.

Two of the other parameters listed in Table 2 are worthy of note: both groups showed an increase in liver weight after CTC, but only CONV animals showed an increase in BUN.

Experiment 2. All mice in the second experiment were females. It must be noted that the course of the second experiment was not completely comparable to the first: the fatty liver infiltration was more severe in the CTC-injected mice than in the comparable groups of the first experiment, whether GF or CONV; the relationships between the GF and CONV groups, however, were the same—that is, the response of the GF mouse was more severe. In addition, the CONV CTC-treated mice experienced no rise in BUN; one GF mouse died.

Table 3 shows the results of the CTC treatment, as well as control data including liver enzyme activities. Of these control data, only the cleavage enzyme levels were significantly different in the two groups, with the GF showing the higher control activity. Liver lipids of the control CONV were again higher, as in Experiment 1, although this time the difference was not statistically significant. The effects of CTC treatment were as follows: liver protein per unit body weight declined significantly less in the CONV; liver glycogen declined in the CONV, while it rose slightly in the GF; and liver total lipids again increased significantly more in the GF. As for the liver enzymes, the CTC treatment had a universally depressive effect. The activities expressed on a per unit protein basis show that CTC exerted a more deleterious effect on enzyme activities than it did on protein levels in general. Further, the approximate percentage depression was about the same for both CONV and GF for all enzymes tested: GOT, −15%; GPT, −25%; GDH, −8%; cleavage, −50%; arginase, −40%.

Experiment 3. Four fasting mice died during the course of CTC administration in this experiment, of which three were GF.

From the data presented in the first and fourth columns of Table 4, it is evident that the GF mouse reacted to four days of food and water deprivation in a manner that was not statistically different from the reaction of the CONV mouse: relative to the corresponding *fed* control mice of Experiment 2 (Table 3, columns 1 and 4), there was a sharp loss of liver glycogen and a mild fatty liver; BUN, which was originally somewhat less in the GF mouse, dropped even lower; total liver lipids were also less in the GF. As regards the liver enzymes, fasting increased the activity of GDH and arginase somewhat in both groups, decreased the cleavage enzyme activity, and had little or no effect on GOT and GPT.

Columns 2, 3, 5, and 6 of Table 4 shows the effects of CTC on these fasted animals as compared with their fasted saline controls (columns 1 and 4). Blood urea nitrogen increased in the CONV mice particularly, liver weights increased markedly, although more in the CONV; liver glycogen declined even further and significantly more so in the CONV mice; liver total lipids increased again, but there was no difference between the GF and CONV mice, as was seen in Experiments 1 and 2; liver water content declined markedly in both groups.

TABLE 4. EFFECTS OF CHLORTETRACYCLINE ON FASTED MICE; EXPERIMENT 3

Variable	Conventionalized			Germfree		
	Saline	CTC	CTC-Sal.	Saline	CTC	CTC-Sal.
Initial body wt. (g)	30·1 ± 1·8	31·2 ± 1·4	+1·1	34·0 ± 2·0	35·0 ± 1·8	+1·0
Final body wt. (g)	24·5 ± 1·4	25·6 ± 1·6	+1·1	25·0 ± 1·8	26·6 ± 1·4	+1·6
BUN (mg/100 ml)	20·2 ± 2·3	30·8 ± 8·1	+10·5	11·6 ± 3·5	13·3 ± 4·9	+1·7
Liver						
Wt. (g/100 g body wt.)	3·76 ± 0·39	6·49 ± 0·26	+2·73[a]	3·60 ± 0·20	4·92 ± 0·16	+1·32[a]
Protein (g/100 g body wt.)	1·04 ± 0·07	0·93 ± 0·06	−0·11[b]	1·07 ± 0·08	0·85 ± 0·04	−0·22[b]
Protein (mg/g liver)	267 ± 26	108 ± 12	−159	293 ± 11	186 ± 17	−107
Glycogen (mg/g liver)	17·4 ± 7·1	3·1 ± 0·5	−14·3[c]	10·2 ± 2·6	7·7 ± 5·0	−2·5[c]
Total lipids (mg/g liver)	69·0 ± 7·2	207·7 ± 12·3	+138·7	59 2 ± 2·6	192·3 ± 0·3	+133·1
Water (%)	64·2 ± 1·7	47·8 ± 1·8	−16·4	67·4 ± 2·6	47·4 ± 0·7	−20·0
GOT (μmoles α-ketoglutarate formed/min/g protein)	778 ± 108	904 ± 17	+126	545 ± 62	651 ± 75	+106
GPT (μmoles α-ketoglutarate formed/min/g protein)	260 ± 30	262 ± 28	+2	177 ± 33	131 ± 11	−46
GDH (μmoles α-ketoglutarate formed/min/g protein)	59·9 ± 6·4	61·2 ± 12·1	+1·3	55·9 ± 3·5	64·9 ± 14·1	+9·0
Cleavage (μmoles urea formed/min/g protein)	24·3 ± 3·6	15·2 ± 1·6	−9·1	32·7 ± 5·6	15·4 ± 20	−17·3
Arginase (μmoles urea formed/min/g protein)	4467 ± 804	2945 ± 180	−1522	7738 ± 909	2845 ± 92	−4893

Each group consisted of 5–6 mice.

The effect of starvation plus CTC on the liver enzymes was obviously complex. The urea cycle enzymes declined further in both groups after CTC, but GDH and the transaminases did not undergo remarkable further declines, and in most cases even rose.

DISCUSSION

Influence of CTC *on liver lipids.* The major findings of our experiment are that the liver of *normal* GF mouse has a lower lipid content than that of the CONV mouse, but that the GF liver undergoes a more severe fatty infiltration than the CONV consequent to CTC administration. The type of fatty infiltration is the same in both groups of animals—that is, mainly glycerides, with smaller elevations in cholesterol and phospholipids; in this respect, CTC acts on both GF and CONV livers like most other lipogenic agents. It is also of interest that the lower levels of total liver lipids in the normal GF mouse are due entirely to lower glycerides and cholesterol, the phospholipids being identical with those of the CONV. Thus, the microbial status of the mouse appears to influence the lipid composition of its liver and to influence profoundly (for the worse) the reaction of its liver to large doses of CTC.

Influence of CTC *on liver proteins and enzymes.* After CTC, the protein of the GF livers declined 29% on the average as compared with a decline of 12% for the CONV. This confirms our previous impression from the lipid data that the GF liver was more severely affected. Van Meter *et al.*[24, 25] showed that chlortetracycline impaired oxidative phosphorylation in rat liver mitochondria, and Rendi and Ochoa[26] suggested that the antibiotic exerts an antianabolic action through inhibition of amino acid incorporation into protein. It has also been shown that the major mechanism for lipid transport from the liver is the very low-density protein glyceride complex.[27] It may be that inhibition of biosynthesis of the protein moiety of this lipoprotein complex is involved here, and that the inhibition is of a more severe nature in the germfree mouse.

The enzyme activities declined to a greater extent than did the concentration of liver protein after CTC administration in both GF and CONV animals. It is well known that in addition to its protein synthesis-inhibiting property, CTC also has the faculty of chelating metals.[28] It is of interest that the depression of activities was about the same for GF and CONV, and ranged from 15%–25% for the transaminases to 40%–50% for the urea cycle enzymes. Glutamic dehydrogenase alone among these enzymes requires no metal cofactor, and the decline in activity of this enzyme was only of the order of 8% below the decline in protein for both GF and CONV livers. The hypothesis that metal chelation is a factor in enzyme inactivation by the tetracyclines was first put forward by Zimmerman and Humoller, working with choline, citrate, and succinate dehydrogenases.[29] Our results are consistent with this hypothesis.

Influence of CTC *on blood urea nitrogen.* The failure of the BUN of the germfree mice in the first experiment to rise in response to the antibiotic while the BUN of the conventionalized mouse did rise is, at present, inexplicable. This suggests that in addition to physical differences in liver response to tetracycline, there may also be differences in kidney sequelae. It is unlikely that the failure of BUN to rise in the CTC-treated GF mouse is due to a failure of urea synthesis, since our second experiment showed that arginase levels of the GF liver did not decline more sharply than they did in the CONV liver after CTC, nor did the "cleavage" enzyme, one of the limiting enzymes of the urea cycle, decline in activity more than in the CONV.

Role of microbial flora and fasting on hepatotoxicity of CTC. The interrelationships of microbial status, nutritional status, and CTC as they affected the weights and the compositions of the mouse livers, are shown in Fig. 1. The inside circles represent the control livers, the outside circles the CTC-treated livers, their radii being proportional to the weights of the livers they represent. It seems clear that the effects of CTC on

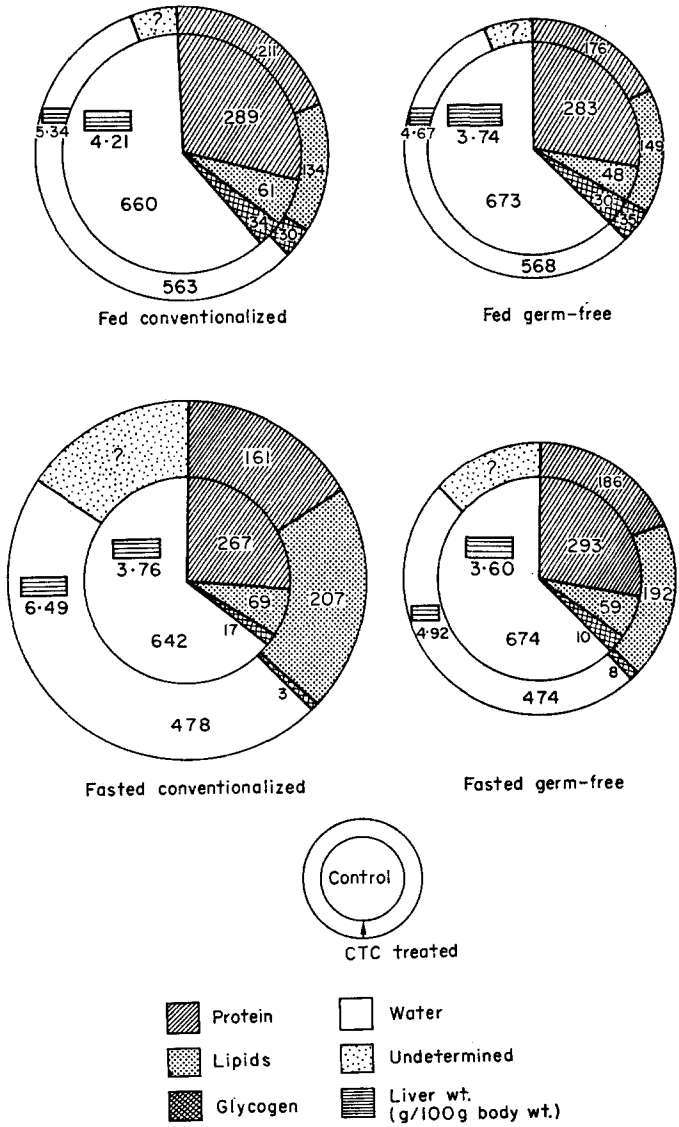

FIG. 1. Diagrammatic representations of livers. Each circle (inner or outer) represents the mean of 5–6 livers; the inner circles correspond to livers of control mice (fed or fasted), and the outer circle to those of CTC-treated mice. The size of each circle is exactly proportional to the liver weight; the sectors, representing the liver components are proportional to the amounts of these components in units of milligrams per gram liver. The figures under the boxes refer to liver weights; the other figures refer to the liver components in milligrams per gram liver.

the liver of a mouse which is eating and drinking *ad libitum* is surely not due primarily to reduced food intake, since the livers of the mice which were fasted and also given CTC were clearly different from those of mice which were ostensibly eating. Referring to the CONV mice, the *fed*, CTC-treated animals experienced a lesser increase in liver weights than the *fasted*, CTC-treated mice, and in addition, the *fasted* CTC-treated CONV mice had much less water, glycogen, and protein, and much more fat than their *fed* counterparts. Obviously, fasting markedly worsened the sequelae of CTC administration in all parameters of the CONV mouse livers which we measured. This exacerbating effect curiously was not so evident in the GF animals; fasting and CTC administration increased the liver lipids in these mice so little beyond that produced by CTC alone that the lipid differences noted between the GF and CONV fed animals had disappeared. In addition, the GF liver glycogen did not decline further with fasting and CTC nor did the protein. Thus, CTC had a more pronounced toxic effect on the liver of the fed GF mouse than on the fed CONV mouse but this hepatotoxicity was potentiated more by fasting in the CONV mouse than in the GF. We do not yet know the cause of the increased total weight of the liver after CTC, nor what constitutes the unknown portions illustrated in Fig. 1, which were apparent only after CTC treatment in both CONV and GF mice; the great increase in this unknown portion with fasting poses an interesting problem for further investigation.

We conclude that: (a) the observed effects on the liver of large doses of parenterally administered chlortetracycline are due to a direct drug action of this antibiotic, i.e. effects secondary to an alteration of the indigenous microflora via its antibacterial activity are *not* essential for the production of a fatty liver—in fact, the GF mice suffered a more severe fatty infiltration and reduction in liver proteins than their CONV counterparts; (b) while all major hepatic lipid components rose as a result of chlortetracycline treatment, the glyceride concentration rose most markedly in both CONV and GF mice; (c) the hepatotoxic effects of chlortetracycline in mice eating *ad libitum* are not ascribable to a lessened food intake—fasting exacerbated the hepatic sequelae of chlortetracycline treatment, and more so in the CONV than in the GF mouse; and (d) the livers of *normal* GF mice have less lipid, in general, than have livers of *normal* mice living in association with a microbial flora.

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