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TOLERANCE AND ACCEPTABLE DAILY INTAKE OF CHLORINATED FUMIGANTS IN THE RAT DIET*

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Abstract—Long-term trials were carried out with rats fed mash fumigated with [redacted]. The difficulty of maintaining a constant residual level of fumigant in the feed was overcome by adopting suitable storage and feeding procedures. The amounts of fumigant actually consumed accounted for 60–70% of the residue initially present in the mash. Triglyceride accumulation in the liver was the most sensitive sign of chronic poisoning with carbon tetrachloride and was apparent after only 6 wk of treatment with a daily dose of about 1% of the LD_{50}. Ethylene dichloride caused only a slight increase in liver fat at very high doses. Carbon tetrachloride and ethylene dichloride were each administered to rats for 2 yr in fumigated mash at levels below the obviously harmful ones. Growth, fertility, reproduction and biochemical tests were used as criteria for establishing the no-effect levels. On the basis of these data, acceptable daily intakes of 10 mg/kg body weight for carbon tetrachloride and 25 mg/kg for ethylene dichloride are proposed. The respective tolerances are 100 and 250 ppm. The proposed tolerances in human food are discussed.

INTRODUCTION

Fumigation of cereals with haloalkanes is a common practice to prevent storage losses due to insect infestation. The most frequently used fumigants in this group, methyl bromide, ethylene dichloride (dichloroethane; EDC), ethylene dibromide (dibromoethane; EDB) and carbon tetrachloride (CCl_4) are highly toxic to living organisms. Despite their volatility, all fumigants except methyl bromide leave unchanged residues, which persist for up to several months after fumigation (Bielorai & Alumot, 1966; Olomucki & Bondi, 1955; Scudamore & Heuser, 1973). Residues of EDB in cereal grain were found to affect egg weight and egg production in laying hens (Bondi, Olomucki & Calderon, 1955) and spermatogenesis in the bull (Amir & Volcani, 1965). Levels as low as 1–2% of the LD_{50} were effective, when fed daily in the fumigated feed for several weeks.

It was clear that the fumigant residues in grain could cause chronic poisoning, differing in effects from acute poisoning and specific for each species and compound. The chronic effects are of special importance in animal nutrition since, in contrast to man, the animals are fed unprocessed grain. Although baking or cooking reduce the residues in human food to low levels, the safety of the repeated consumption of such levels is still to be proved. Data indicating no-effect levels of residual fumigants for man and animals are lacking. The 1968 Joint FAO Working Party/WHO Expert Committee Meeting concluded that the available toxicological evidence was insufficient to enable calculation of an acceptable daily intake for several fumigants, and recommended that long-term feeding trials should be carried out in two mammalian species. One of the reasons for the lack of such research is the difficulty of maintaining constant residue levels in a fumigated food. Kenaga, McCollister, Spencer & Torkelson (1969) observed correctly that other methods of dosing were not representative of normal conditions of consumption. For the study now reported, special storage and feeding procedures, suitable for long-term trials, were established, and rats were fed over a 2-yr period with mash fumigated with EDC or CCl_4.

EXPERIMENTAL

Fumigation of the mash. In preliminary experiments designed to demonstrate the no-effect levels, 3-kg batches of commercial mash were fumigated in special 20-litre fumigation containers (Alumot & Calderon, 1965). The levels tested were 150–520 ppm for CCl_4 and 300–1600 ppm for EDC. For the 2-yr trials, commercial mash was fumigated for 48 hr in hermetically sealed 3-litre containers. The following residue levels were assayed: EDC, 250 ± 30 ppm (low level) and 500 ± 40 ppm (high level); CCl_4, 80 ± 5 ppm (low level) and 200 ± 20 ppm (high level).

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9197

03/22/2000 10:49 5056617468 P NEWELL PAGE 02

The fumigated mash was allowed to aerate for 5 min, to enable the 'free' fumigant to evaporate from the air spaces between the mash particles. It was then distributed either into polyvinyl bags, coated inside with polyamide to prevent loss of fumigant by sorption on to the polyvinyl, or into glass jars sealed hermetically with a plastics screw cover. The inside of the cover was inlaid with a polyamide sheet. The food was stored for 7–10 days, and checks of the residue concentration showed that about 5% was lost between the beginning and end of the storage period. The residues were determined by the cold-extraction method of Heuser & Scudamore (1969).

Feeding procedure. Following studies designed to determine the feeding procedure that involved minimal loss of fumigant, the animals were fed weighed portions twice daily from 11–12 noon and from 7–9 p.m. The evening portion comprised 80% of the daily intake, since rats are nocturnal animals. About 66% of the feed was consumed during the first hour after distribution, and practically no feed remained after the second hour. During the day the animals had access to feed for 1 hr only and became accustomed to consuming it quickly. Any remaining food was removed after each feed and weighed weekly. Fumigant residues in the mash were tested at the beginning of the distribution, and after 1 and 2 hr in the feed troughs. Since the amount eaten and the residue level were known, the amount of fumigant actually consumed was calculated with fair accuracy. It comprised 60–70% of the residue present initially in the feed.

Preliminary study. Groups of six weanling rats aged 4 wk were fed diet containing 150, 275 or 520 ppm CCl_4 for 6 wk (males) or 5 wk (females) at the end of which the animals were weighed and killed and the livers were analysed for total lipids, triglycerides and phospholipids (the latter in the females only). Total liver lipids were determined and liver weights were recorded in groups of six rats fed dietary levels of 300 or 600 ppm EDC for 5 wk or 1600 ppm for 7 wk and hepatic triglyceride levels were also measured in the rats fed 1600 ppm.

Long-term study

Growth experiments. The rats used were bred locally, 90 male and 90 female litter mates being divided into five equal groups, with 18 animals of each sex per group. Males and females were housed separately in stainless-steel cages each holding six animals. The experiment was started about 2 wk after weaning. Feed consumption and weight gain were recorded weekly up to wk 13 and in every second week thereafter.

Reproduction tests. After 6 wk on the experimental diets, the 3-month old females were mated with untreated males for a preliminary test of their reproductive capacity. Thereafter, at about 2-monthly intervals, 45 experimental males were used to breed all the females, in cages housing one male and two females for 10 days. In order to give the remaining 45 males an opportunity to mate, they were housed with hysterotomized (normally cycling but sterile) females. The control diet was fed during these 10-day periods. Males mated with treated females in one trial were switched to the hysterotomized females in the next one, and *vice versa*. This alteration allowed the reasonably accurate elimination of infertile males and females after four trials.

Following mating, the experimental animals were returned to their original cages. Females were weighed twice a week and when they had gained 60 g they were placed in individual cages until parturition. At parturition, litter size and weight were recorded. After 10 days with the dams, the pups were again counted, the litters were weighed, and the dams were returned to the communal cages.

Biochemical tests. Total liver fat was determined by the method of Folch, Lees & Sloane Stanley (1957), involving homogenization of 3-g liver samples with 20 vols chloroform-methanol (2:1, v/v), filtration and evaporation of the solvent in a rotary evaporator, after which the fat was dried at 60°C to constant weight. For triglyceride determinations, the extracted fat was dissolved in 25 ml chloroform and aliquots of this solution were treated by the method of Van Handel & Zilversmit (1957). Phospholipids were determined after wet digestion by the colorimetric method for phosphorus (Gomori, 1942).

For serum analyses, the animals were killed by decapitation, blood was collected and left to coagulate, and serum was obtained after centrifugation for 20 min at 5000 rev/min in a Servall centrifuge at 5°C. Most of the tests were performed on the same day, and some of them on the next day, if the procedure allowed storage in a refrigerator. Total protein was determined by the biuret reaction (Weichselbaum, 1946), albumin by the procedure using bromcresol green, described in Technical Bulletin No. 11 (British Association of Clinical Biochemists, Scientific and Technical Committee), glucose and urea according to the Technicon Autoanalyzer method (Technicon Instrument Corp., Method File N-16 b), cholesterol by the Lieberman-Burchardt reaction (Rappaport & Eichhorn, 1960), uric acid with Folin reagent according to the modification of Eichhorn, Zelmanowski, Lew, Rutenberg & Fanias (1961) and transaminases according to Reitman & Frankel (1957).

Statistical evaluation. All results were subjected to analysis of variance, and the multiple range test of Duncan (1955) was used to evaluate treatment means.

RESULTS

Preliminary studies

The growth rate of the male rats fed mash containing 150 or 275 ppm CCl_4 did not differ significantly from that of the controls. The average total weight gain of male rats after 6 wk on trial was 175·3, 176·0, 161·8 and 152·9 g for control and ascending CCl_4 levels, respectively. The highest CCl_4 level, 520 ppm, depressed weight gain significantly ($P < 0·05$) compared with the other groups. The corresponding values for females after 5 wk on trial were 92, 94, 94 and 92 g, the weight gain being clearly unaffected by the treatment.

The most characteristic sign of CCl_4 poisoning was evident in the liver, in which total lipids and triglyceride levels were significantly higher in the CCl_4-treated animals, except the group fed 150 ppm, than in the controls. The levels of phospholipids were unchanged (Table 1). The calculated amount of CCl_4 consumed

03/22/2000 10:49 5056617468 P NEWELL PAGE 03

Table 1. *Determination of fat in the livers of male and female rats fed for 6 and 5 wk, respectively, on diets containing 0–520 ppm CCl_4*

Dietary level (ppm)	Mean levels (mg/g liver, wet weight) of		
	Total fat	Triglycerides	Phospholipids
	Males		
0 (control)	61·0 ± 6·6	27·0 ± 3·4	
150	71·0 ± 6·0	25·0 ± 3·0	
275	136·0 ± 21·0**	53·4 ± 21·4**	
520	229·0 ± 49·0**	99·5 ± 16·8**	
	Females		
0	70·0 ± 8·2	7·0 ± 3·6	38·5 ± 4·0
150	62·0 ± 7·0	7·0 ± 3·0	35·0 ± 3·1
275	96·0 ± 7·0*	32·5 ± 7·7**	39·5 ± 0·8
520	174·0 ± 22·0**	73·5 ± 14·5**	39·3 ± 2·7

Values are means for groups of six rats and those marked with asterisks differ significantly from the control value: $*P < 0·05$; $**P < 0·01$.

ily by the rats given the high CCl_4 level approached % of the LD_{50} for this species (about 5 g/kg body ight).

The rats fed EDC in the mash did not show anges in liver fat with dietary levels of EDC below 00 ppm (Table 2). With 1600 ppm, a fat accumulation of about 15% was noted, an increase much aller than that induced by levels of CCl_4 several nes lower.

The reproductive activity of male and female rats as not affected by either fumigant.

ong-term study

On the basis of the above preliminary experiments, e fumigant-residue levels chosen for 2-yr trials were) and 200 ppm for CCl_4 and 250 and 500 ppm for DC. The rate of growth of the male and female rats recorded in Table 3. No significant differences were ound between the different groups either in growth r in feed consumption and feed efficiency.

The reproductive findings in the females are presented in Table 4. The difference between the igh-CCl_4 group and the other groups in the number f parturitions was due to a very low parturition percentage in the fourth pregnancy. In the fifth pregnancy, the high-CCl_4 group returned to normal and he number of parturitions was higher than that in he control group. The difference may be regarded, herefore, as incidental. The size and weight of litters and the mortality rate of the pups were unaffected by the treatments.

In yr 2, the fertility of the females dropped steadily and after five pregnancies only a few females from each group conceived. Seven matings were conducted during the whole period. In the seventh, the females had to be assigned to young control males, due to high mortality among the experimental males at the end of the 2-yr trial.

The distribution of sterile males after four matings was normal, with one in the control, high-CCl_4 and low-EDC groups and two in the high-EDC and low-CCl_4 groups. The treatments had no effect on male fertility and, in general seemed to have no effect on the reproductive activity of the rats of either sex.

From the age of 14 months, all the animals began to suffer from chronic respiratory disease, and the mortality rate increased (Table 5). The females were more resistant to such illness than the males, but no effects of treatment were observed.

After 2 yr, surviving animals were killed and the biochemical tests for general metabolic and liver functions were carried out. The results (Table 6) showed no significant differences between the control and the treatment groups except for the serum-protein values, which were significantly ($P < 0·05$) higher in the male rats of the control and low-CCl_4 groups than in those of the other groups. The most pronounced difference was found in the globulin fraction, indicating a more

Table 2. *Weight and fat content of the livers of rats fed EDC in the diet at levels of 300–600 ppm for 5 wk (Trial 1) or 1600 ppm for 7 wk (Trial 2)*

Dietary level (ppm)	Liver weight (g/100 g body weight)		Liver content (mg/g wet liver) of		
			Total fat		Triglycerides
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 2
0 (control)	3·89	3·41	66·0	67·0	16
300	3·93		63·0		
600	3·81		65·0		
1600		3·53		76·0*	28*

Values are means for groups of six rats and those marked with an asterisk differ significantly ($P < 0·05$) from the control value.

03/22/2000 10:49 5056617468 P NEWELL PAGE 04

Table 3. *Mean body weights of male and female rats fed mash fumigated with EDC or CCl_4*

Duration of treatment (wk)	Body weight (g) of rats fed diet containing: No fumigant (control)	CCl_4 (ppm) 80	CCl_4 (ppm) 200	EDC (ppm) 250	EDC (ppm) 500
		Males			
0	130	131	130	131	130
1	160	160	158	160	159
2	192	190	184	186	190
3	218	218	210	216	216
4	244	250	239	249	246
5	271	275	263	274	268
6	297	301	290	299	292
7	313	314	304	314	306
8	328	334	322	334	323
9	340	346	337	345	334
10	354	362	348	360	354
11	362	366	356	367	362
12	370	373	362	376	368
13	376	380	367	384	376
		Females			
0	110	110	110	110	110
1	133	133	130	133	132
2	153	151	152	155	152
3	170	169	169	170	170
4	189	186	186	188	187
5	202	201	197	202	199

advanced stage of chronic disease in these than in the other animals. This was confirmed by a post-mortem examination of the respiratory system.

The biochemical tests did not reveal any effect either on liver function, as indicated by transaminases and cholesterol values, or on kidney function, shown by the urea and uric acid levels. In additi to the values given in Table 6, serum levels of chlo ide, sodium and potassium were determined a found to be (in mequiv./litre): 100 and 103, 142 a

Table 4. *Reproductive performance of female rats fed mash fumigated with EDC or CCl_4*

Parameter	Values for groups fed diet containing: No fumigant (control)	CCl_4 (ppm) 80	CCl_4 (ppm) 200	EDC (ppm) 250	EDC (ppm) 500
	Pregnancy 1				
No. of females mated	18	18	18	18	18
No. pregnant (% of total)	83	89	72	83	100
No. with litters (% of total)	72	89	67	83	100
Mean litter size	10·3	12	11·8	11·3	11·7
Mortality of young (%):					
at birth	16·4	0	8·5	0	0·9
at weaning	6·0	11·4	9·2	8·9	9·9
Mean body weight (g) of young:					
at birth	5·4	5·1	5·0	5·6	5·4
at weaning	14·5	13·0	13·3	13·7	14·3
	Pregnancies 2–5				
No. of females mated	66	72	72	72	72
No. pregnant (% of total)	58	70	56	72	67
No. with litters (% of total)*	53	64	44	71	64
Mean litter size	9·4	9·2	9·8	8·8	9·8
Mortality of young (%) at 10 days	9·8	23	11·3	11·1	13·7
Mean body weight (g) of young:					
at birth	5·7	5·5	5·4	5·5	5·5
at weaning	14·2	15·9	14·3	15·9	15·5

* No. of females producing litters declined sharply in pregnancies 4 and 5 (i.e. in females more than 1 yr old).

03/22/2000 10:49 5056617468 P NEWELL PAGE 05

Table 5. *Survival of male and female rats fed mash fumigated with EDC or CCl_4 in a 2-yr feeding study*

Duration of study (months)	No. of survivors in groups fed diet containing									
	No fumigant (control)		CCl_4 (ppm)				EDC (ppm)			
			80		200		250		500	
	M	F	M	F	M	F	M	F	M	F
0	18	18	18	18	18	18	18	18	18	18
9	17	17	18	18	18	18	17	18	18	18
12	16	16	17	18	17	18	15	18	15	18
15	16	16	16	17	16	18	13	18	14	18
18	15	15	15	16	11	16	10	17	12	18
21	7	12	8	10	6	10	3	12	3	14
24	4	9	3	5	4	7	3	12	2	10

and 6·7 and 7·6 respectively, in females and males ll groups. No fatty livers were detected in the ted animals.

: seems that the levels of fumigants used did not ct the growth, health or reproductive activity of male or female rats.

DISCUSSION

he difficulty of dealing with volatile compounds he mash was overcome by finding storage condi- ns that caused minimal changes in fumigant levels by training the animals to eat the fumigated mash ing a relatively short period of time. Although re are some approximations in calculating the ounts of residue consumed, the methods seem suit- : for the purpose of establishing the no-effect levels of fumigant for the experimental animals. The calculation is as follows: the maximal level of CCl_4 fed to rats without harmful effect during the 2-yr trial was 200 ppm; young and adult rats consumed daily from 10 to 30 g feed, according to age, and since they weighed from about 100 g at the beginning to about 400 g at the end of the experiment, it can be calculated that the CCl_4 dose consumed with the high-CCl_4 diet ranged from 15 to 25 mg/kg body weight; as only about 70% of the dose was actually consumed because of desorption of the residues during the eating period, the rats ate from 10 to 18 mg CCl_4/kg body weight. According to criteria of growth, sexual development, reproductive activity and general clinical symptoms, these levels were completely safe. The acceptable daily intake and tolerance proposed, therefore, are 10 mg/kg body weight and 100 ppm in the diet, respectively.

Table 6. *Terminal serum analyses in rats fed mash fumigated with EDC or CCl_4 for 2 yr*

Serum component	Mean values* for groups fed diet containing				
	No fumigant (control)	CCl_4 (ppm)		EDC (ppm)	
		80	200	250	500
		Males			
cose (mg/100 ml)	128·0 ± 15·1	139·0 ± 23·0	102·8 ± 6·5	94·3 ± 12·6	109·0 ± 17·0
tein (g/100 ml)	7·6 ± 0·09	7·5 ± 0·5	7·1 ± 0·13	6·9 ± 0·35	7·0 ± 0·05
umin (g/100 ml)	3·5 ± 0·06	3·5 ± 0	3·3 ± 0·06	3·0 ± 0·2	3·2 ± 0
bulin (g/100 ml)	4·1 ± 0·12	4·0 ± 0·5	3·7 ± 0·11	3·85 ± 0·55	3·8 ± 0
a (mg/100 ml)	46·5 ± 4·0	48·5 ± 7·5	45·8 ± 3·6	50·0 ± 5·9	42·5 ± 5·5
c acid (mg/100 ml)	1·37 ± 0·06	1·34 ± 0·05	1·48 ± 0·06	1·31 ± 0·05	1·15 ± 0·05
olesterol (mg/100 ml)	103·3 ± 3·3	166·5 ± 20·5	115·7 ± 12·1	149·3 ± 12·5	131·8 ± 31·8
T (IU)	103·8 ± 12·8	100·5 ± 10·5	109·3 ± 13·4	89·0 ± 13·0	69·5 ± 8·5
T (IU)	20·0 ± 2·9	22·5 ± 2·5	19·0 ± 2·9	16·7 ± 1·7	22·5 ± 2·5
		Females			
cose (mg/100 ml)	97·5 ± 5·0	89·7 ± 2·3	94·6 ± 3·6	91·8 ± 7·9	100·8 ± 9·0
tein (g/100 ml)	6·9 ± 0·30	7·6 ± 0·34	7·0 ± 0·18	7·2 ± 0·40	7·0 ± 0·36
umin (g/100 ml)	3·8 ± 0·35	3·5 ± 0·35	3·6 ± 0·09	3·2 ± 0·23	3·5 ± 0·20
bulin (g/100 ml)	3·1 ± 0·33	4·0 ± 0·18	3·4 ± 0·17	4·0 ± 0·39	3·3 ± 0·40
a (mg/100 ml)	43·8 ± 5·3	50·6 ± 5·5	43·4 ± 1·5	47·0 ± 1·9	45·8 ± 5·4
c acid (mg/100 ml)	1·60 ± 0·11	1·87 ± 0·29	1·47 ± 0·22	1·45 ± 0·05	1·60 ± 0·09
olesterol (mg/100 ml)	87·3 ± 9·6	65·6 ± 4.7	86·7 ± 9·4	76·5 ± 16·0	103·0 ± 20·7
T (IU)	119·3 ± 26·0	145·0 ± 25·4	158·6 ± 25·4	123·8 ± 20·2	115·8 ± 18·3
T (IU)	15·9 ± 3·6	20·5 ± 0·5	25·8 ± 3·3	14·1 ± 2·4	15·0 ± 0.5

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase

* Mean ± SEM for groups of three or four males and four or five females.

03/22/2000 10:49 5056617468 P NEWELL PAGE 06

The best proof of the reliability of the method seems to be the appearance of fatty livers after 5–6 wk on diets containing 275 ppm CCl_4. Rats on this diet consumed about 40 mg/kg body weight daily, an amount close to 1% of the LD_{50}.

Osumi, Amano & Shimamoto (1967) administered CCl_4 to rats orally twice weekly at doses from 38 to 200 mg/kg body weight. The calculated daily dose given to these rats was in the range used in the present feeding experiments. After 8 wk, the rats developed dose-dependent fatty livers and, at high levels, histochemical changes were observed in the livers.

The proposed acceptable daily intake and tolerance for EDC, calculated on the same basis as those for CCl_4, are 25 mg/kg body weight and 250 ppm, respectively.

Kenaga *et al.* (1969) calculated the safe residues of fumigants in food from the threshold limit values, the atmospheric concentrations considered safe for industrial workers to breathe for many years. Using a safety factor of 10, they calculated that the safe level, based on a 60-kg man consuming 400 g cereal products/day, was 49 ppm for CCl_4 and 150 ppm for EDC. The respective acceptable daily intakes were 0·33 and 1 mg/kg body weight.

We propose a tolerance of 10 ppm for CCl_4 and EDC in human food. The daily intake, calculated on the above basis, will be 0·07 mg/kg body weight, which is about 100 times less than the acceptable daily intake for animals (see also Alumot, Meidler, Holstein & Herzberg, 1976). A safety factor of 100 is usually applied to laboratory-animal data. A level of 10 ppm of fumigant is not likely to occur in cereal foods for human consumption, since these are generally cooked or baked. The residue level in bread baked from fumigated wheat containing 10 ppm CCl_4 was less than 0·5 ppm (Bondi & Alumot, 1974). Similar results were reported by Dutch scientists (Wit, Besemer, Das, Goedkoop, Loosjes & Meppelink, 1969). The normal fumigation practice seems to be a harmless procedure for man.

Olson, Haberman, Weisburger, Ward & Weisburger (1973) induced stomach cancer in mice and rats with EDB and dibromochloropropane. The drastic conditions of their experiments (frequent doses close to the LD_{50}) are representative of acute poisoning. These results are not relevant to our work, which deals with chronic administration of fumigant residues in feed.

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03/22/2000 10:49 5056617468 P NEWELL PAGE 07