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Toxicity of rubber chemicals towards three-day chicken embryos

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KORHONEN A, HEMMINKI K, VAINIO H. Toxicity of rubber chemicals towards three-day chicken embryos. *Scand j work environ health* 9 (1983) 115—119. Three-day chicken embryos were exposed to 80 different rubber chemicals dissolved in either acetone or water. The following classes of chemicals were studied: thiurams, dithiocarbamates, thioureas, benzothiazoles, benzenesulfohydrazide, dithiodimorpholine, amines, acrylates, guanidines, resorcinol, phthalates, adipates, phosphates, oils, peroxides, heavy metal salts, and sodium nitrite. Dose-response curves and median lethal doses (LD_{50}) or median effective doses (ED_{50}) were calculated for mortality in 2 d after the treatment, for total mortality in 11 d after the treatment, and for the total effect, including deaths and malformations 11 d after the treatment, when the test was finished. Sixteen of the chemicals had no effect on the embryos when injected into the air chamber. Incomplete and irregular dose-response curves for the total effect were obtained with 13 of the chemicals. For them, the ED_{50} could not be calculated. Among the remaining 51 chemicals, the most potent were the dithiocarbamates and cadmium and copper acetates, with the total effect ED_{50} from 2.4 to 160 nmol/egg. Other chemicals that had an ED_{50} smaller than 100 nmol/egg were thiurams, cyclohexylthiophthalimide, acrolein, and dithiodimorpholine. The majority of the chemicals had an ED_{50} between 100 nmol/egg and 10 μmol/egg. The least potent were sodium nitrite and methylmethacrylate with an ED_{50} of 22 μmol/egg. All the 64 embryotoxic chemicals caused malformations with only one exception (dibutylthiourea). The maximum observed frequencies of malformed embryos varied from 3 to 100 % of the treated embryos.

Key terms: adipates, dithiocarbamates, embryotoxicity, methacrylates, peroxides, phthalates, teratogenicity, thioureas, thiurams.

During 1979—1982 a three-year project, supported by the Swedish Work Environment Fund, was carried out at the Institute of Occupational Health to evaluate the toxicity and occupational hazards of chemicals used in the manufacturing of rubber. The toxicity of these chemicals to the chicken embryo was investigated as a part of this project. The results from the testing of each group of chemicals have been published as separate papers (2, 3, 4, 5, 6, 7). In the present communication a summary of all the results is given.

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Material and methods

Most of the chemicals were of technical grade; they were obtained from the rubber factory of Oy Nokia Ab, Nokia, Finland. The remaining chemicals were obtained from several suppliers. The names of the chemicals and the abbreviations used are given in table 1. The solvent for the chemicals was acetone (analytical grade) or water. As a solvent control, ten eggs of each batch were injected with 5 μl of acetone.

Three-day White Leghorn chicken embryos were injected by the dropping of the chemical into the air chamber of the egg. Repeated injections were used with some of the chemicals. Care was taken to focus the drop exactly on the embryo visible under the inner shell membrane. Two days later dead embryos were scored and discarded. The remaining embryos were checked for deaths and malformations up

Table 1. Median effective doses (ED_{50}) and the slopes of the dose-response curves for total effect consisting of deaths and malformations caused by various rubber chemicals.

Chemical	Abbreviation	Total effect		Maximum percentage [a] of malformed embryos
		ED_{50} (dose/egg)	(tan α)	
Tetramethylthiuramdisulfide	TMTD	18 nmol	1.3	40
Tetramethylthiurammonosulfide	TMTM	34 nmol	1.3	15
Tetraethylthiuramdisulfide	TETD	91 nmol	1.3	34
Cadmium-diethyldithiocarbamate	CdDE	2.5 nmol	1.2	55 [b]
Zinc-diethyldithiocarbamate	ZnDE	3.6 nmol	1.4	70
Zinc-ethylphenyldithiocarbamate	ZnEP	4.3 nmol	2.1	63
Zinc-dibutyldithiocarbamate	ZnDB	6.2 nmol	1.1	61
Copper-dimethyldithiocarbamate	CuDM	15 nmol	1.2	43
Tellurium-diethyldithiocarbamate	TeDE	26 nmol	1.8	53 [b]
Piperidinepentamethylenedithiocarbamate	PPM	160 nmol [c]	—	33 [b]
1,3-Dibutylthiourea	DBTU	0.28 μmol	1.5	0
1,3-Diphenylthiourea	DPTU	0.41 μmol	1.8	100 [b]
Tetramethylthiourea	TEMTU	0.80 μmol	0.83	47
Trimethylthiourea	TRIMTU	1.6 μmol	1.0	47
1,3-Diethylthiourea	DETU	3.1 μmol	1.3	7
1,3-Ethylenethiourea	ETU	4.5 μmol	0.79	33 [b]
4,4'-Dithiodimorpholine	DDM	0.09 μmol	1.2	67
N-t-butylbenzothiazylsulfenamide	BBS	0.30 μmol	0.99	13
N-oxydiethylene-2-benzothiazylsulfenamide	ODEBS	0.39 μmol	0.74	27
N-cyclohexyl-2-benzothiazylsulfenamide	CBS	1.0 μmol [d]	—	13 [b]
2-Mercaptobenzothiazole	MBT	2.0 μmol [d]	—	20 [b]
Benzenesulfohydrazide	BSH	2.6 μmol	1.1	43 [b]
N-phenyl-N'-isopropyl-p-phenylenediamine	IPPD	0.11 μmol	0.93	50
N,N,N-triethylethylenediamine	EED	0.36 μmol	1.4	50
N,N'-dicyclohexyl-p-phenylenediamine	DCPD	0.6 μmol [c]	—	83 [b]
Triethylamine	TREA	0.9 μmol	0.98	43
Triethylenetetramine	TRITE	1.1 μmol	1.5	40
N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine	DBPD	1.5 μmol [c]	—	40 [b]
Triethanolamine	TROL	2.6 μmol	1.6	7
N-phenyl-2-naphthylamine	PBN	3.0 μmol [c]	—	30 [b]
Aniline	ANI	3.9 μmol	1.3	50 [b]
N-nitrosodiphenylamine	NDPA	7.0 μmol [c]	—	13 [b]
Resorcinol	RES	2.4 μmol	2.4	10
N,N'-di-o-tolylguanidine	DOTG	0.17 μmol	1.3	13
N,N'-diphenylguanidine	DPG	0.20 μmol	1.4	23
Acrolein	ACR	0.10 μmol	1.4	33
1,3-Butyleneglycoldimethacrylate	DGDMA	2.5 μmol	1.3	3
Tetrahydrofurfuryldimethacrylate	THFMA	3.0 μmol	1.5	23
Trimethylolpropanetrimethacrylate	TPTMA	7.8 μmol	1.4	30
Methylmethacrylate	MMA	22 μmol	0.45	23 [b]
Cyclohexylthiophthalimide [e]	CPHI	0.04 μmol	0.71	30
Phthalic anhydride [e]	PHTA	0.38 μmol	1.6	20
Butylbenzylphthalate [e]	BBPH	27 μmol [c]	—	37 [b]
Dibutylphthalate [e]	DBPH	33 μmol [c]	—	3 [b]
Polypropyleneglycoladipate [e]	PGAD	4.5 mg [c]	—	7 [b]
Tricresylphosphate [e]	TKP	7.0 μmol	2.0	87 [b]
Synthetic aryl phosphate [e]	AP	9.5 mg [c]	—	23 [b]
Highly aromatic oils [e]	HIAO	31 μg	1.4	55 [b]
Low aromatic, paraffin base oils [e]	LOAO	87 μg	1.1	30 [b]
Naphthenic oils [e]	NAPO	480 μg	1.5	40 [b]
Cyclohexanone peroxide [f]	CHOP	0.13 μmol	1.7	27
Cumolhydroperoxide [f]	CHP	0.16 μmol	1.3	30
Ethylmethylketoneperoxide [f]	EMKOX	0.19 μmol	4.3	47
Dibenzoylperoxide [f]	DBP	0.27 μmol	0.48	40
Acetylacetoneperoxide [f]	AAP	0.34 μmol	1.2	23

Table 1. (Continued)

Chemical	Abbreviation	Total effect		Maximum percentage [a] of malformed embryos
		ED_{50} (dose/egg)	(tan α)	
Perbenzoic acid tert butylester [f]	PBB	0.42 μmol	1.5	17
Dicumylperoxide [f]	DICUP	1.2 μmol	0.94	57
Dilauroylperoxide [f]	DLP	1.8 μmol [1]	—	25 [b]
Hydrogen peroxide [f]	H2O2	2.7 μmol	4.6	27
Cadmium-acetate	CdAC	2.4 nmol	1.1	10
Copper (II)-acetate	CuAC	96 nmol	1.5	11
Zinc-acetate	ZnAC	1.0 μmol [c]	—	20 [b]
Sodium dihydrogenphosphate	NaP	11 μmol	1.9	10
Sodium nitrite	NaN	22 μmol	3.1	27
Dicyclohexyl-2-benzothiazylsulfenamide	DCBS	—	—	—
2,2'Dibenzothiazyldisulfide	MBTS	—	—	—
Zinc-2-mercaptobenzothiazole	ZMBT	—	—	—
Pentachlorothiophenol	PCTP	—	—	—
Alkylphenoldisulfides	APD	—	—	—
Alkylphenolformaldehyde resin	AFR	—	—	—
2-Mercaptobenzimidazole	MBI	—	—	—
Hexamethylenetetramine	HMTA	—	—	—
N,N'-dinitrosopentamethylenetetramine	DNPT	—	—	—
Di-β-naphthyl-p-phenylenediamine	DNPD	—	—	—
N,N'-diphenyl-p-phenylenediamine	DPPD	—	—	—
Octylated diphenylamine	OCT	—	—	—
Aldol-α-naphthylamine	ANA	—	—	—
Di-2-ethylhexyladipate [e]	DOAD	—	—	—
Ethylhexylphthalate [e]	EHPH	—	—	—
Dioctylphthalate [e]	DOPH	—	—	—

[a] Percentage of treated embryos.
[b] Incomplete, irregular or flat dose-response curve for early death.
[c] Approximated for incomplete dose-response curve.
[d] Dose of maximum effect.
[e] Results taken from Korhonen et al (8).
[f] Results taken from Korhonen et al (9).

to day 14 of the incubation, 11 d after the treatment.

The median effective doses (ED_{50}) and the median lethal doses (LD_{50}) and the slopes of the dose-response curves were calculated (11) for the total effect on day 14 of the incubation, at the end of the test; for total mortality on day 14; and for early deaths on day 5, 2 d after the treatment.

When incomplete or irregular dose-response curves were obtained, the ED_{50} was approximated on probit paper. When no approximations could be made, the maximum effective dose is given instead of the ED_{50}.

Dose-response curves were also plotted for the percentage of treated malformed embryos. The maximum percentage of malformed embryos obtained on this curve was used to indicate the relative amount of malformations caused by each chemical.

Results

As the solvent background, a total of 600 embryos were injected with 5 μl of acetone along with the test series. Of these 600, five (0.8 %) died within 2 d of the treatment, ten (1.7 %) died between days 6 and 14 of the incubation, and five (0.8 %) were malformed. Four embryos (0.7 %) were alive with malformations on day 14. The total number of malformed embryos, dead or alive, was 9 (1.5 %), and the total number of affected embryos was 19 (3.2 %).

A total of 80 chemicals was tested, 16 of which did not give any effect above the solvent background (table 1). The 64 effective chemicals produced four different sets of dose-response curves for total

effect and early death. These were: (i) incomplete curves produced by chemicals that were not fully effective at doses introduced in the volume of 5 μl; (ii) irregular or very flat curves for all measured effects, including the total effect [Some of these curves reached the level of 50 % effect; some stayed below it or even turned downwards at the highest doses. Precipitate was often found in the air chamber on day 14. Only approximate ED_{50} values were obtained for the 13 chemicals belonging to groups 1 and 2 (table 1).]; (iii) complete, regular and relatively steep (tan $\alpha > 0.5$) dose-response curves for total effect, with irregular or very flat curves for early death, or no early deaths at any dose [Twelve chemicals belonged to this group (table 1).]; and (iv) complete, regular, and relatively steep (tan $\alpha > 0.5$) dose-response curves for all measured effects, including early death [There were 39 chemicals in this group.].

The ED_{50} and the slopes of the dose-response curves are given in table 1. Malformations, as well as deaths, were caused by 63 of the 64 effective chemicals. Only DBTU had no other effect but early death, with a complete (tan $\alpha = 1.5$) dose-response curve. The maximum percentages of malformed treated embryos caused by the remaining 63 chemicals ranged from the 3 % with DBPH and BGDMA to 100 % with DPTU (table 1).

Five to seven classes of malformations predominated in all treatments. There were two different and obviously independent eye defects, one affecting the eye cup and the other the lids and the cornea; defects of the wings; defects of the backbone; defects of the coelomic wall; encephalocele or lesions of the head skin and skull bone; and edema or enlarged lymph sacks on the rump. The first five defect types were almost always present.

Specific defect types were observed too. DPTU caused considerable retardation in the growth of the head. DCPD had a somewhat similar effect, concerning mainly the eyes. TKP and, to some extent, AP retarded the growth of the long bones of the legs. DICUP inhibited the metatarsal and metacarpal bones. EMKOX caused some cleft palates, as did H202 and TMTD. CuDM and CuAC affected the upper beak.

Considerable amounts of late deaths, after day 5 of the incubation, were produced by the thiurams, the dithiocarbamates, DPTU, TKP, and the aromatic oils. Several chemicals caused no late deaths at all. Most of the embryos that died later than 2 d after the treatment were malformed, but CuDM and the oils killed equal amounts of nonmalformed and malformed embryos.

Discussion

This review summarizes the experimental results of a chicken embryo test applied in a uniform way to 80 industrial chemicals.

The doses of the chemicals injected into the eggs were limited by the volume of the solution, which was 5 μl in the majority of the tests. When no effect was produced, there was no way to discern whether the dose had been inefficient or whether the chemical had failed to reach the embryos. The albumen water layer forms a strong barrier against rubber chemicals, most of which are insoluble in water.

Slow diffusion from the air chamber to the embryo can explain all the irregular and flat dose-response curves obtained. Poor diffusion was indicated by the occurrence of precipitates in the air chamber and the turning downwards of many dose-response curves at high doses. Many chemicals caused increasing numbers of malformations, but few or no early deaths at increasing doses. Obviously only a teratogenic, but not a lethal dose, was able to penetrate the embryos during the first two days. Dose-response curves that were much flatter for the early deaths than for the total effect may also indicate poor diffusion of high doses. There may be other reasons, like a different mechanism for death and malformation, for the different slopes of the respective dose-response curves, but poor diffusion cannot be excluded. Even the differences between the slopes of the dose-response curves of different chemicals may be explainable by solubility factors. Different equilibrium states may have existed between the chemical, the acetone, and the albumin water, which determined the penetration of the chemical inside the

vitelline membrane.

The teratogenic dose range is limited in the upper end by doses which cause rapid death of the embryos before the malformations become manifest. For a long teratogenic dose range, high percentages of malformations are observed before the early deaths begin to decrease their amounts. Poor diffusion of the lethal doses extends the teratogenic dose range and produces false high percentages of malformed embryos. Diffusion through the albumen and the vitelline membrane is specific for this method only. Consequently, to exclude strictly methodological effects, one should consider only the malformation percentages when regular and relatively parallel dose-response curves are obtained for the total effect and the early deaths.

Another result of poor diffusion may be the late deaths. Very often chemicals that did not cause early deaths killed the embryos later, before day 14. The exposure of the embryos in the egg is usually chronic, and the death of embryos at advanced stages may well have resulted from prolonged exposure.

An interesting feature was the high potency of the metal-containing dithiocarbamates with an ED_{50} from 2.5 to 26 nmol/egg. As a comparison, mitomycin C and cyclophosphamide had an ED_{50} of 4.2 and 10.8 nmol/egg, respectively, tested with this method (personal communication from Sipo Vanhanen).

Another interesting aspect was the universal occurrence of malformations. This was probably a result of the use of doses close to the LD_{50}. This occurrence is seldom possible in mammalian tests, where toxicity to the dam may mask effects on the embryo.

Unfortunately, only a few of the present compounds have been tested in mammals. The following teratogenic doses have been published (1): cadmium (sulfate, hamster) at 2 mg/kg, cadmium (chloride, rat) at 6 mg/kg, copper (sulfate, hamster) at 7 mg/kg, ETU (rat) at 40 mg/kg, TETD (hamster) at 125 mg/kg, TMTD (hamster, rat) at 250 mg/kg, TMTD (mouse) 300 — 500 mg/kg, and cyclophosphamide (rabbit) at 2—50 mg/kg. Other chemicals which were not tested in the present work but which are chemically related are methacrylate esters (rat) at 5—700 mg/kg, phthalate esters (rat) at 300—1,000 mg/kg (1), and dithiocarbamates (rat) at 1,000—2,000 mg/kg (10). Except for the high potency of cadmium and cyclophosphamide, no congruence can be found in comparison to table 1. In addition, the comparison is difficult because the median doses are seldom calculated in teratologic tests. More exact comparisons of the chicken embryo and the mammalian tests are needed before further extrapolation from the present results can be made.

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