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Location: Industrial Hygiene and
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Subject: Teratogenicity

Methylene Chloride
Trichloroethane
Trichloroethylene
Tetrachloroethylene
Toluene

Please note the attached article from "Toxicology" on this subject. I would appreciate your evaluation of these findings in relation to our discussions on fetal protection programs.

A handwritten signature in dark ink, appearing to be 'LW Keller', written over the typed name.

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EFFECTS OF METHYLENE CHLORIDE, TRICHLOROETHANE, TRICHLOROETHYLENE, TETRACHLOROETHYLENE AND TOLUENE ON THE DEVELOPMENT OF CHICK EMBRYOS

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SUMMARY

Toluene and 5 aliphatic chlorinated hydrocarbons of wide industrial use were injected into the air space of fertilized chicken eggs at 2, 3 and 6 days of incubation. The embryotoxicity was evaluated as survival and death incidences after 14 days of incubation, and also the weights and lengths of the embryos were recorded. The approximate LD₅₀ value for trichloroethylene and trichloroethanes varied between 50 and 100 µmol/egg while for toluene, tetrachloroethylene and methylene chloride it was over 100 µmol/egg. Macroscopic malformations of various kinds were produced with doses of 5-100 µmol/egg. The teratogenic potential of the tested compounds decreased in the following order: 1,1,1-trichloroethane > trichloroethylene > methylene chloride, tetrachloroethylene, 1,1,2-trichloroethane > toluene > olive oil control.

INTRODUCTION

Chlorinated hydrocarbons are a widely used group of chemicals to which thousands of people are exposed either at the workplace or in the environment where the compounds are found as pollutants. However, an increasing body of evidence is pointing to both acute and chronic toxicity of the compounds [1-3]. Among the saturated hydrocarbons, methylene chloride, is reported to be mutagenic to *Salmonella typhimurium* without an activation system [4]. Teratogenic studies of methylene chloride in rat and mouse produced no clear evidence on teratogenicity [5]. Other chlorinated hydrocarbons, 1,1,1-trichloroethane and 1,1,2-trichloroethane have been assayed in a long-term animal test in rat and mouse, and the latter was reported to

CH_2Cl_2	Methylene chloride	<u>85</u>
$\text{Cl}_3\text{C}-\text{CH}_3$	1,1,1-Trichloroethane	133
$\text{Cl}_2\text{HC}-\text{CH}_2\text{Cl}$	1,1,2-Trichloroethane	133
$\text{ClHC}=\text{CCl}_2$	Trichloroethylene	131
$\text{Cl}_2\text{C}=\text{CCl}_2$	Tetrachloroethylene	166
 -CH ₃	Toluene	92

Fig. 1. Chemical formulas of the compounds under study.

cause hepatocellular carcinomas in mouse [3]. 1,1,1-Trichloroethane appeared not to be carcinogenic in the 2 species [3,6]. Of the unsaturated hydrocarbons, data on both trichloroethylene and tetrachloroethylene suggest that the compounds are carcinogenic in long-term animal studies [7-10]. In addition trichloroethylene, but not tetrachloroethylene, has been found to be mutagenic to bacteria [7,11]. Long-term toxicity of another widely used solvent, the aromatic hydrocarbon, toluene have not been well established. Toxicity has been described on developing chick embryos [12,13] but results on chronic toxicity in exposed human subjects have been conflicting [14-17].

In the present work the possible toxicity and teratogenicity of methylene chloride, 1,1,1-trichloroethane, 1,1,2-trichloroethane, trichloroethylene, tetrachloroethylene and toluene was studied with chick embryos. The chemical formulas of the tested compounds are shown in Fig. 1.

MATERIALS AND METHODS

Chicken eggs of White Leghorn SK 12 strain (supplied by a hatcherie, Siipikarjanhoitajain liitto r.y., Hameenlinna, Finland) were incubated in an incubator (Tu 40b Memmert-Oven, 854 Schabach, F.R.G.) with a relative humidity of about 60% and access to fresh air. During incubation, the eggs were rotated once a day.

The compounds used in our tests and their sources were the following: Methylene chloride, GC grade, from J.T. Baker Chemical Co.; 1,1,1-trichloroethane, p.a. grade, 1,1,2-trichloroethane, purity 99% and tetrachloroethylene, purity > 99%, from Fluka AG, Buchs, Switzerland; trichloroethylene, extra pure grade, and toluene, p.a. grade, from Merck AG, Darmstadt, F.R.G.

The tested compounds were dissolved in olive oil to the desired concentration. Different dosages were injected at developmental stages (described in ref. 18) of 2, 3 and 6 days of incubation in a total volume of 25 μl through a small hole into the air space of the eggs ($N = 520$); the hole was then covered with a piece of surgical tape. Control eggs received 25 μl of olive oil ($N = 71$). The eggs were examined after 14 days of incubation. The number of treated eggs, the percentages of live and dead embryos calculated per treated eggs, and the incidence of gross external malformations among survivors were recorded. The embryos were also weighted and measured

from crown to rump for a description of the effects on embryonic growth; however, for dead (resorbed) embryos only the length was measured.

RESULTS

The experimental dosages used in this study were chosen for a more detailed evaluation of the teratogenic effects of tested substances from initial data obtained from pilot studies based on wide logarithmic scales. An approximate estimation of the LD₅₀ values varied between 50 and 100 $\mu\text{mol/egg}$ for trichloroethylene and trichloroethanes while the LD₅₀ of toluene, tetrachloroethylene and methylene chloride was over 100 $\mu\text{mol/egg}$ (Tables I and II).

The administration of 1,1,1-trichloroethane and 1,1,2-trichloroethane exhibited a clear dose-response relationship in regard to the survival of the embryos irrespective whether the eggs were treated on the 3rd or the 6th day of development. Methylene chloride showed an anomalous dose-response in regard to the survival of chicken embryos (Table I). Trichloroethylene, tetrachloroethylene and toluene administered on the 6th day at doses of

*~ 1 mmole/kg egg
= 133 $\mu\text{g/kg}$ for trichloro
Big dose
causing dose +
not white are
+ trapped inside of
egg!*

TABLE I

EFFECTS OF DIFFERENT DOSES OF METHYLENE CHLORIDE, 1,1,1-TRICHLOROETHANE AND 1,1,2-TRICHLOROETHANE ON CHICK EMBRYO DEVELOPMENT EXAMINED ON THE 14TH DAY OF INCUBATION

Treatment	No. of eggs	Age at injection	Live embryos (%)	Weight (g)	Length (cm)	Dead embryos (%)	Length (cm)	Malformed of survivors (%)
Methylene chloride								
100 $\mu\text{mol/egg}$	10	3 d	40	7.6	5.4	50	1.2	25
100 $\mu\text{mol/egg}$	9	6 d	78	8.9	6.0	22	2.3	0
50 $\mu\text{mol/egg}$	10	3 d	100	8.6	5.8	0	—	20
50 $\mu\text{mol/egg}$	10	6 d	80	9.8	5.7	20	4.0	13
25 $\mu\text{mol/egg}$	10	3 d	60	7.7	5.7	40	2.4	0
25 $\mu\text{mol/egg}$	10	6 d	70	9.6	5.7	30	2.5	14
5 $\mu\text{mol/egg}$	10	3 d	60	8.3	5.7	40	2.9	0
5 $\mu\text{mol/egg}$	10	6 d	60	10.2	5.7	40	3.0	0
1,1,1-Trichloroethane								
100 $\mu\text{mol/egg}$	10	3 d	0	—	—	70	0.8	0
100 $\mu\text{mol/egg}$	10	6 d	10	8.9	5.9	90	1.2	—
50 $\mu\text{mol/egg}$	10	3 d	50	9.2	5.8	20	0.8	20
50 $\mu\text{mol/egg}$	10	6 d	20	10.5	6.1	70	1.9	0
25 $\mu\text{mol/egg}$	10	3 d	80	8.6	5.6	20	0.8	38
25 $\mu\text{mol/egg}$	10	6 d	80	9.8	5.9	20	3.0	13
5 $\mu\text{mol/egg}$	10	3 d	60	9.6	5.9	20	3.0	0
5 $\mu\text{mol/egg}$	10	6 d	80	9.1	5.7	10	0.2	25
1,1,2-Trichloroethane								
100 $\mu\text{mol/egg}$	10	3 d	10	9.6	6.0	80	0.4	0
100 $\mu\text{mol/egg}$	10	6 d	0	—	—	80	1.0	—
50 $\mu\text{mol/egg}$	10	3 d	70	8.7	5.7	20	0.4	0
50 $\mu\text{mol/egg}$	10	6 d	60	8.3	6.1	20	1.1	0
25 $\mu\text{mol/egg}$	10	3 d	90	8.9	5.9	10	1.0	11
25 $\mu\text{mol/egg}$	10	6 d	60	9.0	5.6	30	2.0	17
5 $\mu\text{mol/egg}$	10	3 d	70	8.4	5.5	10	3.5	14
5 $\mu\text{mol/egg}$	10	6 d	80	9.9	6.1	20	3.5	0
Olive oil								
25 $\mu\text{l/egg}$	15	3 d	73	8.8	5.9	13	3.2	0
25 $\mu\text{l/egg}$	14	6 d	79	8.5	5.6	21	3.1	0

TABLE II

EFFECTS OF DIFFERENT DOSES OF TRICHLOROETHYLENE, TETRACHLOROETHYLENE AND TOLUENE ON CHICK EMBRYO DEVELOPMENT EXAMINED ON THE 14TH DAY OF INCUBATION

Treatment	No. of eggs	Age at injection	Live embryos (%)	Weight (g)	Length (cm)	Dead embryos (%)	Length (cm)	Malformed of survivors (%)
Trichloroethylene								
100 μ mol/egg	9	2 d	22	6.5	5.5	56	1.3	0
100 μ mol/egg	10	6 d	40	7.6	5.5	50	1.7	50
50 μ mol/egg	10	2 d	80	11.7	6.0	20	1.3	13
50 μ mol/egg	10	6 d	80	9.2	5.9	0	—	0
25 μ mol/egg	10	2 d	80	10.4	5.9	20	0.9	25
25 μ mol/egg	10	6 d	80	8.6	6.0	0	—	13
5 μ mol/egg	10	2 d	60	10.3	5.9	30	3.2	33
Tetrachloroethylene								
100 μ mol/egg	8	2 d	88	7.7	5.9	12	1.5	14
100 μ mol/egg	10	6 d	30	7.9	5.5	60	1.5	0
50 μ mol/egg	10	2 d	60	10.5	6.0	20	3.1	0
50 μ mol/egg	10	6 d	80	9.4	5.9	20	2.3	13
25 μ mol/egg	10	2 d	90	11.0	6.0	10	0.1	22
25 μ mol/egg	10	6 d	100	8.6	5.6	0	—	0
5 μ mol/egg	9	2 d	78	11.0	6.8	11	4.5	29
Toluene								
100 μ mol/egg	10	2 d	60	7.2	5.5	20	0.9	17
100 μ mol/egg	10	6 d	0	—	—	100	1.5	—
50 μ mol/egg	10	2 d	70	11.6	6.2	20	0.9	14
50 μ mol/egg	10	6 d	80	8.6	5.8	10	0.5	0
25 μ mol/egg	10	2 d	70	11.9	6.3	30	1.1	0
25 μ mol/egg	10	6 d	90	9.9	5.8	10	5.0	11
5 μ mol/egg	10	2 d	90	10.7	6.0	10	5.0	0
Olive oil								
25 μ l/egg	13	2 d	77	10.7	6.2	23	1.7	0
25 μ l/egg	14	6 d	79	8.4	5.8	14	0.6	9

25–100 μ mol/eggs influenced the survival incidence while the treatment of eggs on the 2nd day was more or less ineffective in comparison with the olive oil controls (Table II). Detrimental effects of all 3 solvents, were seen, however, in the enhanced number of dead embryos roughly corresponding to the dose. An increase in the embryonic lengths of dead embryos demonstrated delayed lethal toxicity due to declining dose. Both weight and length measurements were recorded for live embryos. These parameters of growth effects were, however, affected noteworthily only with the highest doses (Tables I and II).

Malformations were registered on the basis of macroscopic examinations of 14- or 15-day-old surviving embryos. Teratogenic potential did not depend, systematically, on the day (2nd, 3rd or the 6th day) of the administration of the compounds (Tables I and II). Moreover, the teratogenic potential of the compounds was also distributed over a wide concentration range including the doses which were no more lethal (Tables I and II).

The types and incidences of malformations from Tables I and II and from additional data obtained at a concentration of 25 μ mol/egg are summarized in Table III. The teratogenic potential of the investigated compounds decreased as follows: 1,1,1-trichloroethane > trichloroethylene > methylene chloride, tetrachloroethylene, 1,1,2-trichloroethane > toluene > olive oil

TABLE III

TYPES AND INCIDENCE OF MACROSCOPIC MALFORMATIONS IN LIVE CHICK EMBRYOS

Treatment (5-100 μ mol/egg)	Incidence		Number of malformations			
	No. of malformed/ No. of survivors	(%)	Exterior- ization of viscera	Profound edema	Eye abnor- malities	Skeletal abnor- malities
Methylene chloride	7/64	(10.9)	4	2	4	2
1,1,1-trichloroethane	10/48	(20.8)	1	5	3	7
1,1,2-trichloroethane	5/55	(9.1)	2	1	1	3
Trichloroethylene	9/55	(16.4)	1	5	2	3
Tetrachloroethylene	6/61	(9.8)	1	—	2	5
Toluene	3/46	(6.5)	—	1	—	3
Olive oil (25 μ l/egg)	2/56	(3.6)	—	—	2	1

control (Table III). The types of abnormalities included absence of eye(s), upper-beak deformations, exencephaly, brain hemorrhages, paleness (anemia), profound edemas either in the rump sacs or of a more generalized nature, external viscera, and more or less severe musculoskeletal defects of lower extremities, but not of the wings. Some of the typical malformations are shown in Fig. 2. The background level of vehicle-injected controls showed a low incidence of malformations, too. However, the types included only eye and skeletal abnormalities.

DISCUSSION

Chlorinated hydrocarbons and toluene are widely used in industry as solvents and chemical intermediates. The world-wide production of trichloroethylene and tetrachloroethylene was estimated at over 1 million tons in the early 1970's [8,9,19]. The production of methylene chloride was 470 million pounds [20], and that of toluene 700 million gallons in the U.S.A. alone in the early 70's [17]. Industrial exposure of workers in the U.S.A. has been estimated at 280 000 [21] or more [7] for trichloroethylene, 70 000 for methylene chloride [20] and 100 000 for toluene [17]. However, the public at large is exposed to all these compounds through polluted air, drinking water and food. As the health hazard of some of these compounds, such as trichloroethylene and tetrachloroethylene, are emerging, consumption is being restricted, and they are being replaced by other compounds, for example 1,1,1-trichloroethane [1]. However, the biological effects and safety of 1,1,1-trichloroethane remain to be established.

In the present study the possible toxicity and teratogenicity of some chlorinated hydrocarbons and toluene was tested on developing chick

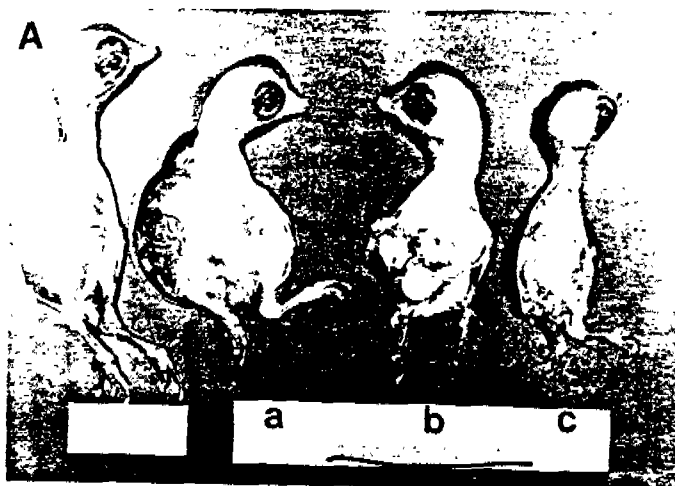


Fig. 1 (A)



Fig. 1 (B)

embryos so that more information could be gained on the biological effects of these compounds. The test doses ranged between 5 and 100 $\mu\text{mol/egg}$. For 1,1,1-trichloroethane, 1,1,2-trichloroethane and trichloroethylene a clear dose-dependent toxicity was demonstrated at 2 injection times between days 2 and 6 of embryonic development. For these compounds the apparent values for LD_{50} ranged between 50 and 100 $\mu\text{mol/egg}$. For methylene chloride, tetrachloroethylene and toluene only 1 of the largest doses appeared to be effective, with apparent LD_{50} values of more than 100 $\mu\text{mol/egg}$.



Fig. 2 (C)

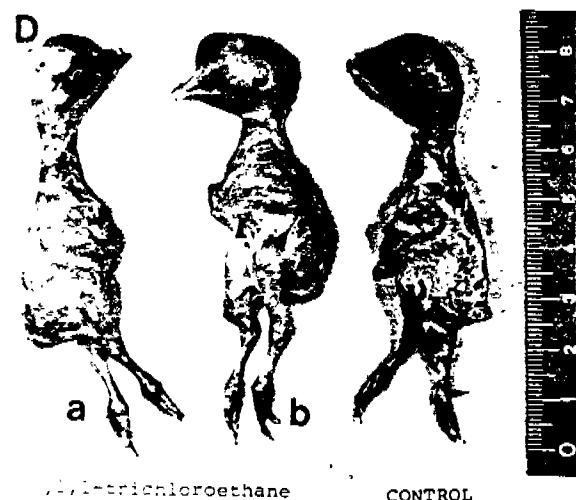


Fig. 2 (D)

Fig. 2. Types of external abnormalities observed in chick embryos treated with chlorinated hydrocarbons surviving to the 14th day: (A) Trichloroethylene treatment on the 2nd day of incubation at doses of 50 μ mol (a), 25 μ mol (b) and 5 μ mol (c) per egg produced subcutaneous edema (a), stunted lower extremities with a reduced number of digits, subcutaneous edema, external viscera and ectopia of cordis (b) and crooked toes (c). (B) Profound edematogenic effects were seen after treatment with 1,1,2-trichloroethane (a), trichloroethylene (b) and methylene chloride (c) injected at a dose of 25 μ mol/egg on the 3rd day of incubation. Note the bloody subcutaneous edema of the whole body (a) and the large edematous cysts on the rumps of the embryos (b) and (c). (C) and (D) Malformations caused by treatment on the 6th day of incubation. Methylene chloride (50 μ mol) resulted in complex maldevelopment showing microcephalus, microphthalmia, no upper beak, short neck and external viscera. 1,1,1-Trichloroethane (25 and 5 μ mol) caused exencephalia and beak abnormalities (a), and 1-eyed embryos with a crossed upper beak (b).

We have previously tested the effects of styrene and styrene oxide on chick embryos [22]. Styrene was shown to have an LD₅₀ of 40 μmol/egg, a level in agreement with that of the present compounds. However, styrene oxide had an LD₅₀ that was about 25 times lower. This difference was interpreted to reflect a low level of cytochrome P-450 linked monooxygenase activity, which is responsible for the conversion of styrene into its oxide. It is likely that at least some of the toxicity of the present compounds is mediated by metabolic activation in the monooxygenase system [23], and the still dormant embryonic enzyme activities could explain the high tolerated levels of the present compounds. In mammals methylene chloride is metabolized into carbon monoxide and reactive free chlorine radicals via homolytic or heterolytic cleavage mechanisms, just as the other chloromethanes methyl chloride, chloroform and carbon tetrachloride are (see refs. 1, 11). The metabolism of trichloroethylene and tetrachloroethylene is thought to proceed via epoxide intermediates catalyzed by the monooxygenase system [1,23]. The oxirane compounds formed are instable for trichloroethylene and more stable for tetrachloroethylene and may therefore explain the differences in mutagenicity of the 2 compounds [11]. The final metabolic products of the 2 compounds are trichloroacetic acid and trichloroethanol.

All the compounds tested produced malformations in excess of the vehicle control. In the case of methylene chloride, 1,1,2-trichloroethane, tetrachloroethylene and toluene the malformation frequency was increased 2- to 3-fold (Table III), while with 1,1,1-trichloroethane and trichloroethylene the increase was 4- to 6-fold.

The results of the present study demonstrate toxicity of the tested compounds on chick embryos. Although the mechanism of action of the tested compounds remains unexplained, the study should indicate a need for caution and further study on the adverse effects of the compounds.

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