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The Chick Embryo Test As Used in the Study of the Toxicity of Certain Dithiocarbamates[1]

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The Chick Embryo Test As Used in the Study of the Toxicity of Certain Dithiocarbamates. GEBHARDT, D. O. E., and VAN LOGTEN, M. J. (1968). *Toxicol. Appl. Pharmacol.* **13**, 316–324. The toxicities of six dithiocarbamates: bis(dimethyl thiocarbamoyl) disulfide (thiram), zinc dimethyldithiocarbamate (ziram), ferric dimethyldithiocarbamate (ferbam), bis(dimethyl thiocarbamoyl) ethylene bis(dithiocarbamate) (triaram), sodium diethyl dithiocarbamate (NaDEDC), and sodium ethylene bis(dithiocarbamate) (nabam) were determined in the chick embryo. The substances were dissolved in propylene glycol and injected in the air chamber prior to incubation. Injection in the yolk sac on any day of development or in the air chamber after the first day was unsuitable for the assessment of an LD50. With the exception of nabam, the dithiocarbamates were extremely toxic for the early chick embryo. Cysteine injected simultaneously in the air chamber protected the embryos from the toxic effect of thiram or ziram but not from the action of triaram. It is suggested that the former two dithiocarbamates are reduced by cysteine to less toxic compounds.

In recent years more attention has been paid to the methodology of testing the toxicity of chemical substances in the developing chick. Ridgway and Karnofsky (1952) studied the effect of various metals on the chick embryo. They injected these substances in the yolk on the day 4 or 8 or on the chorioallantois on day 8 and discovered that the place and time of injection were important factors in determining the responsiveness of the embryo. A difference in sensitivity depending on the site of injection was also found by Verrett *et al.* (1964), who showed that the toxicity of aflatoxin when injected into the air chamber was twice as high as when injected into the yolk.

Other comprehensive studies on the injection of chemicals into eggs were performed by McLaughlin and co-workers (McLaughlin *et al.*, 1963, 1964, 1965). Marliac *et al.* (1964, 1965) administered 21 pesticides in the yolk sac of unincubated eggs and found that, in general, these substances were no more toxic in the chick embryo than in the rat. Lack of toxicity of various insecticides for the hen's egg was also reported by Dunachie and Fletcher (1966). However, the fungicide tetramethylthiuram disulfide, a dithiocarbamate, was highly toxic to chicks when it was included in their diet (Waibel *et al.*, 1957). This discovery made it of interest to determine the sensitivity of

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the chick embryo to various dithiocarbamates used extensively in agriculture today. It is well known that the latter have high residues in fruit and vegetables.

Before a study of these compounds could be undertaken, it was necessary to find a suitable solvent. Most dithiocarbamates are insoluble in water, and Williamson *et al.* (1963) have shown that nonspecific malformations will arise when insoluble products are introduced into the amniotic cavity of the chick embryo. Furthermore, a toxic compound insoluble in the albumin or yolk will not have any effect on the embryo simply because it cannot diffuse into the developing organism. Propylene glycol was chosen for the work to be described here because Verrett *et al.* (1964) had already shown that it was nontoxic to the chick embryo when injected in the yolk of unincubated eggs in quantities of 0.05 ml. Besides this solvent is miscible with the albumin and can dissolve up to 1 mg/ml of most of the dithiocarbamates.

Prior to working with the combination of fungicide and solvent it seemed nevertheless advisable to study more systematically the effect of propylene glycol on the embryo. In the first place it was decided to determine whether the solvent remained nontoxic when injected either into the yolk or the air chamber on different days of development. When it was found that propylene glycol could be injected in the air chamber before incubation without loss of embryos, this method was used to establish the LD50 of each of the dithiocarbamates. A comparison was then made between the LD50 of a dithiocarbamate injected in the air chamber and in the yolk, respectively. Furthermore, the change in LD50 with the stage of development when the injection took place was investigated. It was necessary in this case to dissolve the dithiocarbamate in water since propylene glycol was toxic when injected in the air chamber after the first day of incubation (see Results). (Use was made of one of the few water-soluble dithiocarbamates, sodium diethyl dithiocarbamate, in the latter experiment.)

Finally an attempt was made to protect the embryo from the toxic action of the dithiocarbamates. Sisler and Cox (1955) [see Thorn and Ludwig (1962)], found that yeast cells could be protected from the toxic effect of tetramethyl thiuram disulfide (thiram) by adding cysteine to the medium; therefore, a study was also made of the effect on the chick embryo of a combination of dithiocarbamate and cysteine dissolved in propylene glycol.

METHODS

The eggs of known high fertility were derived from White Leghorn pullets. They had an average weight of 50 g with a standard deviation of 4 g. Before incubation, the eggs were randomized. In all comparative experiments eggs of one batch only were used. For an LD50 determination 0.05 ml of a solution of a dithiocarbamate was injected into the yolk or the air chamber. (The dithiocarbamates were readily dissolved in propylene glycol by ultrasonic disintegration.) All solutions were sterilized by filtration through an L3 Chamberland candle. About 20 eggs were used per dose. The needle of the injection syringe was 2 cm long and had an external diameter of 0.5 mm. The air chamber was located by candling with a high-intensity light source. A hole was then drilled in the middle of the shell surface above the air chamber with a dental drill. Yolk sac injections were performed through this hole with the egg and syringe in horizontal position. When the substances were injected in the air chamber, the eggs were held vertically with the large end pointing upward. Only the point of the

needle was inserted in the cavity. The eggs were closed with paraffin and placed, with the air chamber pointing upward, in an automatic rotating incubator at 38°.

On days 4 and 6 the eggs were candled to determine the number of unfertilized eggs and dead embryos. They were opened on day 14, and the mortality occurring within the first 2 weeks was used for the calculation of the LD50 values.

Bis(dimethylthiocarbamoyl)disulfide (thiram) of 95% purity and ferric dimethyldithiocarbamate (ferbam) of 91% purity were obtained from the "Chemische Industrie van Hasselt," Amersfoort. Zinc dimethyldithiocarbamate 6 H_2O (ziram) of 99% purity and sodium ethylene bis(dithiocarbamate) (nabam) of 91% purity were bought from "Wiersum Chemie," Groningen. Bis(dimethylthiocarbamoyl) ethylene bis-(dithiocarbamate) (triaram) of 87% purity came from "Vondelingenplaat," Rotterdam, and sodium diethyldithiocarbamate 3 H_2O (NaDEDC) of 99% purity from Merck, Darmstadt. The solvent propylene glycol (propanediol-1,2) was also obtained from Merck and had a purity of 99.4%.

RESULTS

Toxicity of Propylene Glycol

The embryonic mortality of three control groups was determined after injection in the *yolk sac* on different days of development. The groups consisted of (a) untreated eggs, (b) eggs in which a needle had been inserted, and (c) eggs injected with 0.05 ml of propylene glycol. The results shown in Table 1 indicate that (1) propylene glycol

TABLE 1

INFLUENCE OF 0.05 ML OF PROPYLENE GLYCOL ON EMBRYONIC MORTALITY, WHEN INJECTED INTO THE YOLK SAC ON DIFFERENT DAYS OF DEVELOPMENT

Day of incubation	Treatment	Number of eggs	Mortality[a] (%)
0	None	30	7
0	Needle inserted[b]	38	55
0	Propylene glycol, 0.05 ml	40	45
1	Needle inserted[b]	20	20
1	Propylene glycol, 0.05 ml	28	14
2	Needle inserted[b]	20	10
2	Propylene glycol, 0.05 ml	20	5
3	Needle inserted[b]	19	5
3	Propylene glycol, 0.05 ml	25	0
4	Needle inserted[b]	18	5
4	Propylene glycol, 0.05 ml	27	11

[a] Mortality is defined as the percentage of embryos that died within the first 14 days of incubation.
[b] The needle was inserted in the yolk for 2 seconds without injection of any substance.

is not toxic when injected into the yolk, and (2) the high mortality in eggs of groups b and c, injected prior to incubation, is due to the great sensitivity of the young embryo to mechanical interference. The difference in mortality between groups a and b or a

and c (but not between b and c) is highly significant ($P<0.0005$, χ^2 test) for eggs injected before the first day. An evaluation of an LD50 at this early and most sensitive stage, therefore is not feasible.

Figure 1 shows the mortality found when 0.05 ml propylene glycol was injected in the *air chamber* on different days of development. There is a highly significant increase

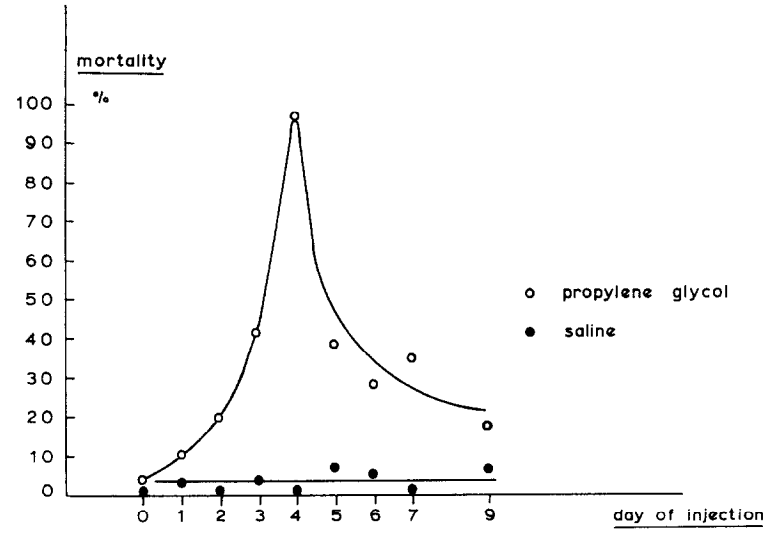

FIG. 1. The mortality of chick embryos when 0.05 ml propylene glycol (open circles) or 0.05 ml saline (filled circles) was injected in the air chamber on different days of incubation. Each point represents mortality among twenty eggs.

in mortality when the solvent is introduced after the second day ($P<0.0005$, χ^2 test). In the controls of this series, injected with 0.05 ml of saline, the mortality never exceeded 10%. It follows from Fig. 1 that the toxicity of a dithiocarbamate in propylene glycol can be determined only if the solution is injected during very early development. The toxic action of the solvent would otherwise interfere with any accurate evaluation of a dose-effect curve.

Toxicity of Certain Dithiocarbamates

The LD50 of a dithiocarbamate injected in the air chamber, prior to incubation, was calculated according to the method of Litchfield and Wilcoxon (1949). In determining the mortality of eggs treated with dithiocarbamates, it was necessary to introduce a correction factor (Emmens, 1948) for the deaths occurring among controls. The adjusted percentage response P is then given by the formula

$$P = \frac{100\,(P_o - P_c)}{100 - P_c}$$

in which P_c is the observed control percentage response (mortality among eggs treated with propylene glycol only), and P_o the observed experimental percentage response. In Table 2 the LD50 of six dithiocarbamates with the 95% confidence limits are shown.

TABLE 2

LD50 VALUES OF SIX DITHIOCARBAMATES DISSOLVED IN PROPYLENE GLYCOL AND INJECTED IN A VOLUME OF 0.05 ML INTO THE AIR CHAMBER BEFORE INCUBATION

Dithiocarbamate	LD50 (μg per egg)[a]
Thiram	1.9 (1.7–2.2)
Ziram	2.1 (1.8–2.4)
Ferbam	2.2 (1.8–2.6)
Triaram	4.8 (3.9–5.9)
NaDEDC	5.8 (4.7–7.2)
Nabam	140.0 (98.0–200.0)

[a] The 95% confidence limits in parentheses.

The comparison between the LD50 of a dithiocarbamate, injected in the yolk and in the air chamber, took place on the first day of incubation. Any other day would have been unsuitable because of the fragility of the vitelline membrane before incubation (Table 1) and the toxicity of the solvent in the air chamber from the second day onward (Fig. 1). The LD50 for thiram in the air chamber on the first day was 1.1 μg (95% confidence limits: 0.6–2.0 μg). The LD50 of thiram in the yolk was much higher viz. 18 μg (95% confidence limits: 10.6–30.6 μg).

By using the water-soluble dithiocarbamate NaDEDC it was possible to study the change in LD50 when the substance was injected on different days of development (Table 3).

TABLE 3

LD50 OF NaDEDC DISSOLVED IN WATER AND INJECTED IN THE AIR CHAMBER ON DIFFERENT DAYS OF DEVELOPMENT

Day of development	LD50 (μg per egg)[a]
0	6.3 (4.9–8.1)
1	8.6 (5.9–12.3)
2	10.4 (8.1–13.3)
3	50.0 (36.2–69.0)
4	440.0 (341.0–568.0)

[a] the 95% confidence limits in parentheses.

Influence of Cysteine on the Toxicity of Dithiocarbamates

Table 4 presents the results of the protection experiments with cysteine. It is shown that cysteine can only protect the chick embryo from the toxic influence of ziram or thiram when it is injected simultaneously with these fungicides. This observation was an indication that cysteine reacted directly *in vitro* with ziram or thiram or thiram in propylene glycol. Further support of such a direct reaction came from a study of the

TABLE 4

ACTION OF CYSTEINE ON THE TOXICITY OF ZIRAM, THIRAM, AND TRIARAM[a]

Substance(s) injected	Mode of injection	Number of eggs	Mortality (%)
Propylene glycol, 0.05 ml	—	20	5
Ziram, 4 μg	—	23	100
Ziram, 4 μg + cysteine, 4μg	Simultaneously	23	96
Ziram, 4 μg + cysteine, 40 μg	Simultaneously	20	10[b]
Ziram, 4 μg + cysteine, 50 μg	Cysteine 3 minutes before ziram	20	90
Ziram, 4 μg + cysteine, 40 μg	Cysteine 3 minutes after ziram	18	89
Thiram, 4 μg	—	29	100
Thiram, 4 μg + cysteine, 40 μg	Simultaneously	15	20[b]
Triaram, 8 μg	—	20	80
Triaram, 8 μg + cysteine, 50 μg	Simultaneously	20	80

[a] The substances were dissolved in propylene glycol and injected in the air chamber prior to incubation.

[b] Reduction in mortality highly significant, $P < 0.0005$ (χ^2 test).

ultraviolet spectra of the various compounds (Figs. 2 and 3). It appears that when cysteine suppresses the ultraviolet absorption of a dithiocarbamate (Fig. 2), it also protects the chick embryo from the action of the fungicide. On the other hand, when

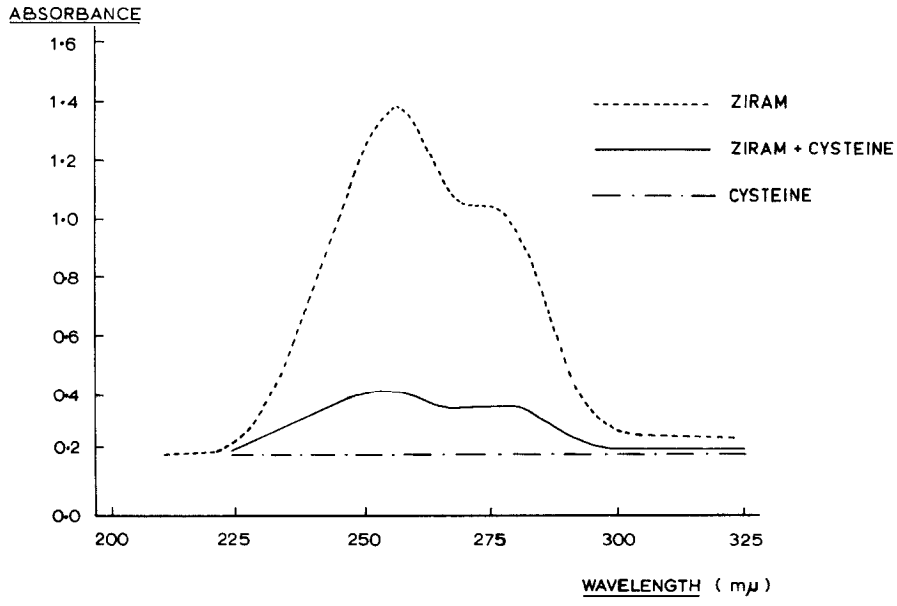

FIG. 2. The ultraviolet spectrum of ziram (0.065 mM) or of the combination of ziram (0.065 mM) and cysteine (1.6 mM). The substances were dissolved in propylene glycol and the spectra were measured in a Unicam SP.800 spectrophotometer.

cysteine does not change the ultraviolet spectrum of a dithiocarbamate (Fig. 3), it has no protective value for the chick embryo.

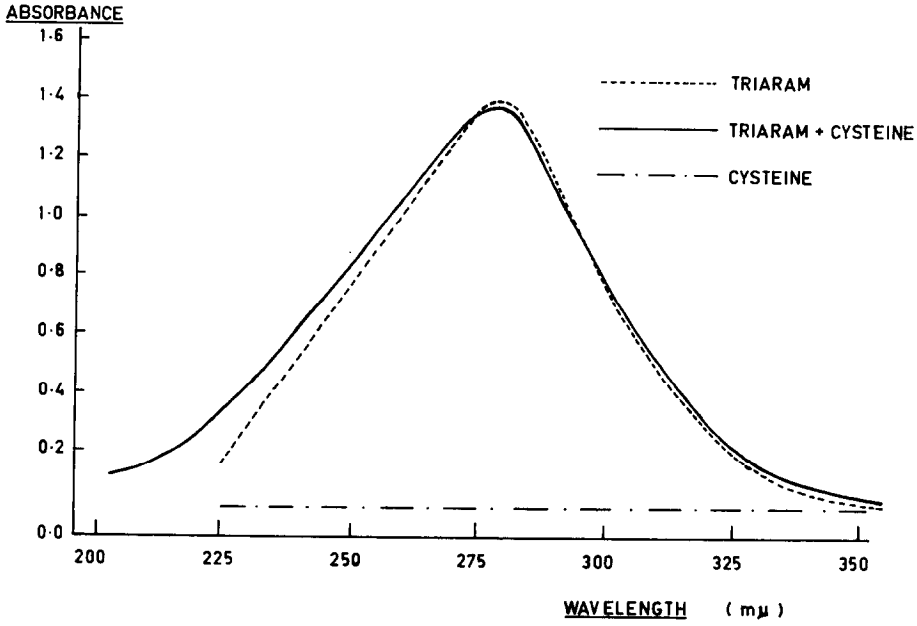

FIG. 3. The ultraviolet spectrum of triaram (0.065 mM) or of the combination of triaram (0.065 mM) and cysteine (1.6 mM). Solvent and measurements as in Fig. 2 legend.

DISCUSSION

A high sensitivity of unincubated eggs to the insertion of a needle in the yolk (Table 1) has also been reported by Carter (1964). It is possible that the death of the embryos is caused by leakage of yolk following the piercing of the vitelline membrane. This membrane is known to compress the yolk during early development (Silver, 1957). On the other hand, McLaughlin *et al.* (1963) injected many thousands of eggs before incubation without any loss among controls. The difference between the present results and those of McLaughlin *et al.* may be attributed to the use of eggs with a more resilient vitelline membrane by the latter.

An explanation must also be sought for the change in toxicity of propylene glycol when injected in the air chamber on different days of development (Fig. 1). Gebhardt (1968) injected various glycols in the air chamber and found that the chick embryo was most sensitive to the lethal action of these solvents on day 4 of incubation. It was suggested that the embryos died because the glycols destroyed the extraembryonic blood vessels. The latter do not appear until after day 2 of incubation. On the other hand, the lack of toxicity of propylene glycol in the yolk (Table 1) may be explained by assuming that the solvent is unable to reach the extraembryonic circulation by this route. From Table 2 it can be concluded that the dithiocarbamates (with the exception of nabam, a bisdithiocarbamate) are very toxic for the early chick embryo. Therefore, the question arises whether the hatchability of eggs from hens feeding on seeds treated with dithiocarbamates is reduced. Also the localization of the fungicides in

such eggs is a question of some importance (Koeman *et al.*, 1967). Further experimental work along these lines of inquiry should yield interesting results. The rapid rise in LD50 of NaDEDC with development (Table 3) is not surprising when the enormous weight increase of the embryo during the first 4 days is considered.

The much higher LD50 of thiram injected in the yolk as compared to that in the air chamber is probably caused by a difference in solubility of propylene glycol in the yolk and the albumin. When the solvent is injected in the air chamber, it dissolves completely in the albumin and can be transported with ease to the embryo (unpublished results). On the other hand, as shown by Walker (1967), propylene glycol does not readily diffuse through the yolk of unincubated eggs.

The above considerations demonstrate some of the difficulties of testing water-insoluble substances in the chick embryo. Nevertheless it should be stressed that the chick embryo test has many important applications. In the present investigation, it has been shown that the young embryo reacts to very low doses of the dithiocarbamates and that their LD50's can be determined with great precision. Furthermore the method is simple and relatively inexpensive. In addition the chick embryo can be used to study the mechanism by which the dithiocarbamates exert their lethal action.

Until now very little information was available on the subject of protecting biological systems from the action of dithiocarbamates, Nygaard and Sumner (1952) studied the inhibition of D-glyceraldehyde-3-phosphate dehydrogenase by tetraethyl thiuramdisulfide (TETD). They discovered that cysteine was effective in preventing the inhibition of this enzyme and concluded that TETD affects certain SH-groups essential for the attachment of the substrate to the enzyme. Kaars Sypensteyn and van der Kerk (1954) found that the fungitoxic action of dithiocarbamates was antagonized by adding compounds with sulfhydryl groups such as cysteine or thioglycolic acid to the culture medium. They suggested that the dithiocarbamates with a free hydrogen atom at the nitrogen were first transformed to isothiocyanates. These substances can combine chemically with the thiol group of cysteine to form stable inactive reaction products; however, the reaction mechanism proposed by these authors cannot explain the inactivation of alkyldithiocarbamates by cysteine. From the present study it follows that inactivation does not occur *in situ* but takes place before the solution of dithiocarbamate and cysteine is injected into the eggs. The reaction between cysteine and ziram or thiram *in vitro* is accompanied by a suppression of the absorption spectrum in the ultraviolet. This makes it plausible that inactivation is due to the reduction of the dithiocarbamates by cysteine. In fact Knegtel (1968) found that another reducing agent, hydroxylamine, was also able to protect the embryo and suppress the ultraviolet absorption of ziram. On the other hand, it had no effect on the toxicity or spectrum of triaram. These results suggest that cysteine protects the embryo only from those dithiocarbamates which are easily reduced *in vitro*.

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