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The Injection of Chemicals into the Yolk Sac of Fertile Eggs prior to Incubation as a Toxicity Test

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The increasingly large number of food additive chemicals introduced into the market each year has necessitated the development of rapid and reliable methods for the evaluation of their toxicity. Toxicologic studies of all these chemicals by the usual methods using animals are very difficult, and such studies sometimes give inconclusive results.

The toxicity of some chemicals, and especially of food additives, may be determined by injection of the chemical into the yolk sac of fertile eggs prior to incubation and subsequent observation of the effects of the chemical on the embryonic development of the chick. This appears to be a promising method in that it may be carried out much more economically in terms of money and space than would be possible with larger animals. Hundreds of chicken embryos may be observed in a minimum of space, and over a comparatively short period of time. The feasibility of using such large numbers is valuable also in the statistical evaluation of toxicity data.

A review of the literature shows how little work has been done in this field except in a fragmentary way on isolated cases. Most of the reports refer to injections of chemicals made after the fourth or eighth day of incubation and examination of the embryos killed before they hatch.

The earliest work that we have found in the literature was by Féré (1893). During ten years after this date he published about sixty-seven papers; a review article (Féré, 1899) contains a summary of many of his studies. His work consisted mainly of injection before incubation, but the eggs were usually opened on the third day of incubation. His interest was mainly in the teratogenic effect of chemicals.

Since 1960 other articles have appeared in the literature, but most of them concern the effects of one or two chemicals on a small number of embryos. One of the most informative papers is that of Ridgway and Karnofsky (1952) in which they report experiments on compounds containing fifty-five of the elements, mainly the metallic ones, generally injected at either the fourth or eighth day of embryonic development to study toxicity and teratogenic effects. Hamburger and Hamilton (1951) have described an elegant method for determining the stages of development of the chick embryo. More recent investigations employing the chick embryo to study the toxicity of various chemicals have been reported by Kemper (1962), Platt *et al.* (1962), and Goerttler (1962). Finally, the classic books of Romanoff and Romanoff (1949) and Romanoff (1960) contain a wealth of information on the avian embryo.

Preliminary reports of this investigation have been presented by Marliac (1962) and McLaughlin and Mutchler (1962).

EXPERIMENTAL

The fertility and hatchability of eggs and the livability of chicks are dependent on a complex interrelationship of ecological factors, among which are the genetic background and the age of the mated birds, the nutritional status and general management of the flock, and seasonal variations. In view of this the initial phase of our work, which started in 1959, was devoted to a study of the hatchability of our supply of White Leghorn eggs[1] under conditions existing in our laboratories. The data accumulated during two years for control eggs showed that the hatchability of these eggs was, in fact, consistent, reproducible, and very high. The possibility of a seasonal variation occurring in responses to compounds introduced into the eggs was also examined by repeated testing of several chemicals at all seasons of the year. No important variation was detected.

Selection of eggs. Eggs to be injected are first candled in order to discard those that are defective and to outline with a pencil the exact location of the air cell. In our laboratory 9% of 5000 eggs had to be discarded: 2% cracked, 4% with improperly calcified shells, 1.5% with a tremulous air cell, 1% with the air cell in the wrong place, and 0.5% with blood clots. After the elimination of such defective eggs, the hatch of control eggs averages 95%. A further restriction is based on the weight of the eggs: all those weighing less than 52 g or more than 63 g are rejected. After

[1] *Truslow Eggs, Chestertown, Maryland.*

candling, the eggs are randomized in order to avoid series of infertile eggs in any one experiment.

The initial experiment with a given chemical is for range-finding and is performed at two or more concentrations of the chemical with 10 eggs per level. On the basis of this information, 20 or more eggs are injected with the appropriate amount of the chemical.

If the chemical proves to be nontoxic, the experiment is repeated with the minimum number of eggs that will give a reliable and reproducible value for the hatchability. In the case of a toxic chemical, additional eggs are injected to determine the specific effects of the chemical. The total number of eggs used for a chemical depends upon the data obtained initially and upon the kind of information desired. Hence, data for some chemicals are based on less than one hundred eggs, whereas data for others are based on several hundred eggs.

Technique of injection. The injections of pure chemicals, chemical solutions, or suspensions are made at volumes up to 0.10 ml. When necessary, dilutions are made with solvents such as water, propylene glycol, corn oil, peanut oil, or other nontoxic solvents.

In order to avoid contamination, the injections are carried out in an Isolator Box[2] with a sterile atmosphere created by using formaldehyde vapors (produced by mixing 2 g of potassium permanganate and 50 ml of 37% formalin). During the period of a year, more than fifteen hundred noninjected eggs were exposed to formaldehyde vapors; no toxic effect was noticed. After exposure to these vapors for 30 minutes, the eggs are ready for injection.

The large end of the egg is wiped with a sterile gauze pad moistened with a 70% alcohol solution, and a hole is drilled in the shell in the center of the surface over the air cell (Fig. 1). Care must be taken not to damage the shell membrane with the point of the drill[3]; this is to avoid, if possible, contact of the air with the egg membrane. Fine particles of shell are removed with an aspirator to prevent the needle from carrying them into the yolk.

Immediately before the injection, each egg is shaken with a quick twist of the wrist. Since the germinal disc occasionally sticks to the air cell and it is possible to damage it with the needle, this movement will allow the disc to float free in the egg.

[2] Kewaunee Scientific Company, Adrian, Michigan.
[3] Burgess Vibrocrafters Inc., Grayslake, Illinois.

The needle (hypodermic, 1 inch long, either no. 22 or no. 27, depending on the viscosity of the liquid to be injected) is inserted horizontally through the air cell into the yolk (Fig. 1). Care must be taken in withdrawing the needle to avoid damaging the vitelline membrane since such damage could cause the yolk to spread out in the albumen. If the end of the needle has some yolk on it, the injection is not satisfactory. The needle should be wiped with a sterile gauze pad between each injection. As soon as the egg has been injected the hole in the shell is covered with a small piece of Scotch tape, care being taken not to cover the entire air cell.

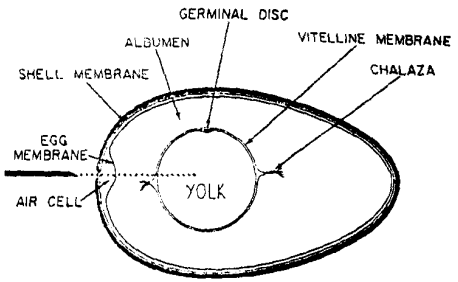

FIG. 1. Diagram of egg and position for injection.

Incubation and hatching. The injected eggs are put into the incubator trays with the large end up; the trays are placed in the incubator,[4] which automatically rotates hourly and is maintained at an optimum temperature of 38°C and a relative humidity of 60%. The eggs are candled on the fifth day of incubation and every day thereafter. Clear eggs and dead embryos are removed for examination. On the seventeenth day of incubation the fertile eggs are transferred to the hatcher[5] and kept at a temperature of 37°C until they hatch.

EVALUATION OF DATA

The injection of the chemical into the egg may produce one of four possible results: (1) the chemical is highly toxic at the level injected, and

[4] Humidaire Incubator Co., New Madison, Ohio; model no. 50, capacity 450 eggs.
[5] Brower Manufacturing Co., Quincy, Illinois.

all the embryos are killed during the first 20 hours of incubation (before the two-somite stage); (2) the chemical is toxic but allows a number of embryos to develop only up to a certain point, and some possibly even to hatch; (3) the chemical has little effect on the hatch; and (4) the chemical has no effect on the hatch or on the posthatch development of the chick ("no effect" level).

If the chemical appears to be highly toxic and all the embryos are killed, the experiment is repeated with smaller doses of the chemical until some hatch is obtained. If the chemical is toxic, but allows the embryos to develop for a longer period of time, dead embryos are examined pathologically and the chicks that do hatch are examined for eye damage, color of the feathers, weight, length of the legs, form of the beak and of the rump, hematologic changes, and condition of the internal organs (liver, kidneys, heart, gall bladder, and spleen).

It is advisable in all cases to observe the chicks for a period of a few weeks in order to detect any delayed effects. Since as much as 30% of the yolk remains at the time of hatching and is absorbed during the first 7 days thereafter, effects of a toxic chemical may first be observed at this time. There may be weight retardation, death during the first week, or the appearance of nerve damage occurring as late as 2–6 weeks after hatching.

The toxicity of a chemical is evaluated mainly from the percentage of hatch at varying dosages of the chemical as compared to noninjected (control) eggs, from a study of the embryonic development of the eggs that fail to hatch, and from a study of the appearance and development of the chicks that do hatch. However, several other factors must also be considered in this evaluation: these are specific gravity, solubility, coagulating effect, pH, and the ionic concentration of the chemical tested.

If the chemical has a high specific gravity, there is the possibility of its settling out in the bottom of the egg and thereby giving a value of apparently low toxicity.

The solubility is quite important since the availability of the chemical for utilization in the chick embryo is partially dependent on its solubility in the egg. However, since egg yolk is an emulsion, solubility problems are somewhat minimized. In the case of insoluble chemicals that are injected as suspensions, it is also necessary to consider particle size in the evaluation of toxicity data.

In order to have a toxic effect, the chemical must come in contact with the embryo either directly or indirectly through the bloodstream. Chemicals such as the lower aliphatic alcohols have a coagulating effect on the pro-

tein, and this coagulation may decrease the availability of the chemical as well as some yolk nutrients, and thereby alter the response of the embryo.

If the pH is highly acidic or basic, a pH effect differing from the true toxic effect of the chemical may be obtained due to interference with the normal acid-base equilibrium in the egg.

The ionic concentration is also important for a similar reason. The observation of increasing toxicity with increasing concentration of a chemical should be interpreted cautiously, since highly concentrated solutions may upset the physical equilibrium of the yolk by causing osmotic effects.

Finally, the introduction of a chemical into the yolk may cause a special type of toxicity because it destroys, alters, or combines with essential nutrients such as vitamins and minerals.

EXPERIMENTAL DATA

Twenty-five thousand eggs have been used in our laboratory during the past three years to test more than 100 chemicals with the following results: (1) nontoxic chemicals injected at an appropriate level allowed the embryo to develop and to hatch as did the controls; (2) toxic chemicals produced effects at dose levels which may be compared to those producing effects in feeding experiments using animals; and (3) this technique often provided toxicologic information which had not been shown by conventional methods.

TABLE 1
NONTOXIC CHEMICALS

Chemical	Solution injected		Per cent hatch
	Concentration	Quantity (ml)	
Water (boiled)	—	0.05	95
Propylene glycol	Undiluted	0.05	95
Corn oil	Undiluted	0.05	90
Peanut oil	Undiluted	0.05	90
Sodium chloride	0.9% in water	0.05	90
	5.0% in water	0.05	70
	5.0% in water	0.10	30
	10.0% in water	0.05	20
Dextrose	5.0% in water	0.05	90

Table 1 lists the results obtained with some chemicals in common use in food and shows the dosages used and the percentages of hatched chicks.

TABLE 2

CHEMICALS WITH A HIGH ORDER OF TOXICITY

Chemical	Solution injected: Concentration	Quantity (ml)	Per cent hatch	Remarks
Lead acetate	10% in water	0.05	0	—
	2% in water	0.05	0	Hydrocephalus in embryos that failed to hatch
	0.5% in water	0.05	20	—
Mercuric chloride	1% in water	0.05	0	All embryos died at the beginning of incubation
Thiourea	10% in water	0.05	0	Some embryos lived up to 34 days
	5% in water	0.05	20	Hatched on the 28th day of incubation; growth retardation
Triorthocresyl phosphate	Undiluted	0.02	0	Most of the developing embryos died on the 15th day of incubation
	Undiluted	0.01	40	Hatched chicks showed growth retardation and developed paralysis 2–6 weeks after hatching
p,p'-Diaminodiphenylmethane	10% in ethanol	0.05	30	Leg damage; beak deformity (short mandible)
Sodium selenite	0.2% in water	0.05	0	—
	0.1% in water	0.05	20	—
	0.1% in water	0.02	80	—
Dibutyl-tin-dilaurate	Undiluted	0.01	0	Beak deformity
Aroclor 1242 (chlorinated polyphenyls)	Undiluted	0.025	0	—
	Undiluted	0.01	5	Beak deformity (short upper beak); edema; growth retardation

These data, supplemented by an examination of the nonviable eggs and an autopsy of the chicks that hatched, have not indicated any hazard from their use in food. However, it must be pointed out that any chemical, added at a sufficiently high concentration, may have some toxic effect. Since our data and those reported in the literature indicated safety, this phase of the study was not carried beyond a preliminary examination to ascertain that nontoxicity also was shown for these chemicals by the chick embryo technique. This is of theoretical as well as practical importance for the evaluation of any new technique to be used in toxicologic studies.

Table 2 lists our results with several chemicals which have been shown to be highly toxic to animals. In each case there is not only the low percentage of hatch at a low level of the chemical tested, but there are also congenital abnormalities and other responses that raised extremely serious questions as to the safety to the consumers of any food contaminated with these chemicals.

Lead acetate resulted in no hatch at a level of 1 mg per egg. Autopsy of the dead embryos showed extensive brain damage, as has been reported by de Franciscis and Bocalatte (1962) and by Karnofsky and Ridgway (1952).

Mercuric chloride showed no hatch even at a level of 0.5 mg per egg.

Thiourea is a known carcinogen with a basic effect on the thyroid gland. The hatch time was delayed with increasing amounts of this chemical. At a level of 5 mg per egg no chicks hatched and the embryo required 35 days to develop to the stage normally attained at 20 days. At 2.5 mg per egg some chicks hatched, but most of them had to be helped out of the shell. This effect has been reported also by Yushok (1950).

Cavanagh (1954) reported that triorthocresyl phosphate (TOCP), a well-known plasticizer for nonfood use, causes paralysis when fed to adult chickens. We observed this paralysis in some chicks which hatched from eggs injected with 10 mg of the undiluted chemical.

The compound *p,p'*-diaminodiphenylmethane has been reported by Zylberszac (1951) to cause cirrhosis of the liver in rats. We found this chemical to be extremely teratogenic at a level of 5 mg per egg; more than 90% of the chicks had a short mandible and leg damage consisting of a severe bending of the tibia and a general shortening of the bones of the leg.

Sodium selenite proved to be highly toxic. At a level of 0.1 mg per egg, no embryos developed to more than the 5-day stage.

Dibutyl-tin-dilaurate showed no hatch at a level of 10 mg per egg. The majority of the embryos, which did not live more than 15 days at this

TABLE 3

CHEMICALS WITH AN INTERMEDIATE ORDER OF TOXICITY

Chemical	Solution injected: Concentration	Quantity (ml)	Per cent hatch	Remarks
Acetone	Undiluted	0.05	70	
n-Butanol	Undiluted	0.01	95	* At these levels: corneal opacity, cataract, cleft palate, nerve and kidney damage
		0.02*	70	
		0.03*	20	
		0.04	0	
Carbon tetrachloride	Undiluted	0.05	40	
		0.10	0	
Diethylene glycol	Undiluted	0.05	80	
		0.10	55	
Ethyl acetate	Undiluted	0.05	35	
		0.10	15	
Ethyl alcohol	Undiluted	0.05	95	
		0.10	60	
		0.30	0	
Ethylene glycol	Undiluted	0.05	80	
		0.10	65	
Di-2-ethylhexyl phthalate	Undiluted	0.05	95	
Heptachlor	1% in propylene glycol	0.05	75	
		0.10	65	
Hydrochloric acid	4% in water	0.05	80	
Isopropanol	Undiluted	0.05	35	
		0.10	15	
Malathion	Undiluted	0.05	60	Short legs; bleaching effect on chick down
		0.10	0	
Methanol	Undiluted	0.05	90	
		0.10	65	
Styrene	5% in 80% ethanol	0.05	95	

level, had a short and/or flexed mandible; in addition, some embryos showed subcutaneous edema.

Aroclor 1242 gave no hatch at a level of 25 mg per egg. At a level of 10 mg per egg, one chick hatched out of 20 injected eggs, but died 2 days later. Some embryos, which were examined after they died, showed beak deformities (often a short upper beak), edema, and growth retardation.

Table 3 lists preliminary data on the toxicity of some chemicals which have been shown to be toxic in some degree to animals, and which may be found in some processed foods. Included in this group are various solvents, plasticizers, and insecticides. Some of the chemicals on this list require further study, including observation of the chicks until they reach maturity, before they may be classified as to low or high toxicity.

DISCUSSION

The injection of chemicals into the yolk of fertile eggs prior to incubation is a method which can be advantageously used as an element in the evaluation of the safety of food additive chemicals and drugs, and which could be used to screen new products and eventually to correlate their toxicity with that of similar products already tested.

If one considers that a chemical injected into the yolk may be compared to a substance which has the power to cross the placental barrier, this technique, in addition to being an embryonic feeding study, assumes further importance in that it is also a reproduction study. The unfortunate experiences recently suffered with chemicals that have teratogenic effects in humans, and the failure of conventional testing methods to produce this effect in animals, emphasize the urgent necessity for new methods of analysis. Preliminary work that we have done in this area has given satisfactory results (Verrett and McLaughlin, 1963).

Since this represents a system in which the chemical is in direct contact with the embryo throughout development, it is more than likely that any toxic or teratogenic effects would be readily observed. However, there is always the possibility that the chicken will not be a species susceptible to a particular compound, just as it has been shown that the other commonly used species of animals do not respond to all chemicals in a similar manner. It is also possible for the chicken to be more sensitive to a chemical than other species.

Finally, this technique may be applied also to the study of the synergistic effects of chemicals. Results of experiments in our laboratory on

the potentiation of a few pesticides have been very encouraging (Marliac and Mutchler, 1963).

SUMMARY

An evaluation of toxicity by injection of the chemical into the yolk sac of fertile eggs prior to incubation gave the following results:

Water, propylene glycol, corn oil, peanut oil, isotonic saline solution, and isotonic glucose solution showed no toxicity or a very low order of toxicity.

Mercuric chloride, lead acetate, selenium, triorthocresyl phosphate, *p,p'*-diaminodiphenylmethane, thiourea, Aroclor 1242, and dibutyl-tin-dilaurate showed a high order of toxicity and/or teratogenic effects at certain levels.

Acetone, methanol, ethanol, *n*-butanol, diethylene glycol, ethylene glycol, isopropanol, di-2-ethylhexyl phthalate, hydrochloric acid, carbon tetrachloride, ethyl acetate, malathion, heptachlor, and styrene showed an intermediate order of toxicity.

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