nitrosation of strongly basic secondary amines (Challis, 1973). *p*-Nitrosophenol could also be formed in the digestive tract by reaction of phenol with nitrite ingested in the saliva.

Comparison of eq 1 and 2 gives a measure of the relative rates of formation in the catalyzed and uncatalyzed reactions at about pH 4 which would apply to anacid stomach conditions during digestion:

$$\frac{\text{rate (catalyzed)}}{\text{rate (uncatalyzed)}} = 1 + \frac{k'[p\text{-nitrosophenol}]}{k[\text{nitrite}]} = 1 + \frac{70[p\text{-nitrosophenol}]}{[\text{nitrite}]} \quad (3)$$

Although there is no information on the levels of *p*-nitrosophenol present in bacon nor the proportion of phenol nitrosated in the stomach, if we assumed complete nitrosation of the phenol by the nitrite which is continually ingested in the saliva using the values given above, this would indicate a maximum potential increase in rate of formation of NDEA under in vivo conditions by a factor of about 140. Whilst such a calculation based on a model system is obviously an oversimplification since there would be inhibiting factors as well as possible additional catalytic effects by other nitrosophenols, it illustrates a potential promoting effect on in vivo formation of nitrosamines due to constituents in food itself. This could be important to the evaluation of environmental exposure to these compounds.

Work is currently in progress to extend this study to some polyhydric and other phenols which constitute an essential part of the structure of naturally occurring polyphenols.

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Received for review June 20, 1978. Accepted October 25, 1978. This work was undertaken with the support of the National Institute on Alcohol Abuse and Alcoholism, National Institute of Health (United States) under contract number HSM 42-73-116. The TEA detector was provided on loan by the National Cancer Institute of the United States under contract NCI CP 55715.

Comparative Embryotoxicity of Dimethylnitrosamine in the Chick Embryo

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The LD_{50} values of dimethylnitrosamine (DMN) for the chick embryo were estimated in four strains of the domestic fowl (*Gallus domesticus*), namely, White Leghorn, White Rock, Rhode Island red, and a local breed. The White Leghorn embryo was most susceptible to DMN poisoning, with an LD_{50} of 3.3 μg of DMN/50 g egg weight, when compared with the others which had LD_{50} values of 14.8, 5.7, and 8.4 μg of DMN/50 g egg weight, respectively. The main liver lesion observed in moribund embryos was a massive congestion of the lobule. No centrilobular cell necrosis of the tissue, characteristic of DMN acute poisoning, was evident. It would appear from the results that eggs from the White Leghorn flock are suitable for the bioassay of nitrosamines.

There is relatively very little information on the toxicological effects of *N*-nitrosamines on chick embryos. Aleksandrov (1967) showed that dimethylnitrosamine is lethal to and inhibits growth of the chick embryo. No teratogenic effect due to the compound was, however, observed.

The fertile avian egg is very sensitive to minute changes affecting its milieu interieur. Such minute changes, like

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0021-8561/79/1427-0396$01.00/0

those arising from the presence of foreign compounds, could very adversely hamper the normal development of the embryo. As a result of this hypersensitivity of the developing embryo, the fertile avian egg is very suitable for the bioassay of toxic compounds. In most cases, however, fertile chicken eggs are employed in the bioassay because of the relative ease with which the eggs are obtained locally.

Apart from the deliberate introduction of a toxin into the fertile chicken egg, such a toxin may as well find its way into the environment of the developing embryo via the chicken feed and/or drinking water or as a result of in vivo nitrosation reaction between suitable precursors ingested in the diet. In the case of nitrosamines, which are toxic (Barnes and Magee, 1954) and carcinogenic (Magee and Barnes, 1956), there are reports of their ability to affect the progeny of animals treated during pregnancy (Mohr and Althoff, 1965; Mohr et al., 1965a,b). Nitrosamines have been found in animal feed (Ender et al., 1964) and can be formed in soil from suitable precursors (Ayanaba et al., 1973) or the herbicide glyphosate (Khan and Young, 1977).

A situation in which widespread poisoning of fertile chicken eggs, or those of any other domesticated avian species for that matter, occurs as a result of the ingestion, or in vivo formation, of nitrosamines could consequently pose serious economic problems for the poultry industry. In this regard, the report by Juszkiewicz and Kowalski, at the fifth Meeting on Analysis and Formation of *N*-Nitroso Compounds held in Durham, N.H., in 1977, that DMN fed to New Hampshire laying hens was transferred to the eggs is very pertinent. About 31 and 0.08% of an initial dose of 30 mg of DMN/kg body weight were detected in the eggs after 1 and 14 days, respectively. When a much smaller dose of 0.1 mg/kg body weight was given by them, about 28 and 3% were detected after 1 and 6 days, respectively.

The LD_{50} values of dimethylnitrosamine for the embryos of four strains of the domestic fowl (*Gallus domesticus*), namely, the White Leghorn, White Rock, Rhode Island red, and a local breed, have therefore been estimated in this study. The livers of affected and unaffected embryos have also been examined for histological lesions due to dimethylnitrosamine poisoning. The range of DMN doses adopted in the present investigation have been selected to cover levels that have been reported in food, including animal feed (Ender et al., 1964), and to which poultry are likely to be exposed.

MATERIALS AND METHODS

Reagents and Egg Supply. Dimethylnitrosamine (DMN) was prepared, purified, and characterized in our laboratory by the procedures which have been reported elsewhere (Bassir and Maduagwu, 1978). Standard test solutions of the compound were prepared in sterile distilled water. These ranged between 0 mg/mL, which produced no toxic effect, and 2.25 mg/mL, which caused virtually 100% mortality in White Leghorns on dosing. The following toxin concentrations were employed; 0.0, 2.0, 7.8, 31.3, 125.0, 500.0, and 2000 μg of DMN/mL (care should be taken in handling these solutions because DMN is volatile, toxic, and carcinogenic).

Fertile medium-sized eggs (each approximately 50 g in weight) from inbred White Rock, White Leghorn, Rhode Island red flocks, and a local breed were obtained from the University Farm. The eggs were selected from birds with no overt signs of disease. The eggs were candled, according to the technique of Lennette and Schmidt (1962), to eliminate misplaced and tremulous air cells, blood spots, hair-line cracks, or other shell imperfections and any other abnormalities. The eggs were transferred from a Robbins farm incubator (The Robbins Incubator Co., Denver, Col.) on the eighth day to a laboratory incubator (Western Curfew Incubators Ltd., East Hanningfield, Chelmsford, Essex, England).

Inoculation of Eggs. The eggs were inoculated with the standard solutions of dimethylnitrosamine on the tenth day, which is half-way through embryo development (Gablicks et al., 1965). Aliquots of 0.1 mL of each test DMN solution, which had been selected after a preliminary experiment, was administered. By random selection, eggs belonging to each strain of chicken were shared into groups of 33 for each level of dose of DMN to be administered. This number is in agreement with the recommendation of Russell (1966). Eggs receiving only sterile distilled water (0 μg of DMN) served as controls.

All eggs were candled prior to inoculation and the location of each air cell outlined with a pencil mark. This area was sterilized by cleaning with a swab of denatured alcohol. A pin hole was drilled in the center of the air cell with a sharp sterile needle, and bits of broken shell membranes were removed with a pair of forceps. Using a sterile syringe and needle, the required amount of a sample was dispensed onto the egg membrane while care was taken not to penetrate the membrane. The egg was placed on the palm of the left hand and the needle directed into the pin hole horizontally.

After inoculation, the pin holes were sealed with vacuum grease and the eggs were placed undisturbed in a vertical position (with air cell up) for 1 h to disperse the toxin. The eggs were then loaded into the laboratory incubator, and the incubator temperature and relative humidity were maintained at optimum values of 99.8 °F and 60%, respectively. Control eggs, 33 in number, were inoculated with 0.1 mL of sterile distilled water only.

Postinoculation Management of Eggs. The eggs were turned, by hand, twice daily (morning and evening) and records of dead embryos, detected by candling, for each dose level of toxin and for controls were made at the same time. The livers of dead embryos were immediately dissected out for histological examination. The eggs hatched by the 21st–23rd day and those failing to hatch were opened and the embryonic livers also sampled for histological examination.

Histology. General methods described in standard practical histology books were employed in the histological examination of the embryonic livers. Each tissue sample was fixed in 10% formol saline and, after dehydration, was embedded in molten paraffin wax. Sections of this were mounted on clean slides after melting the wax and using egg albumen as an adhesive. Staining was done in hematoxylin. Permanent mounts, prepared with Canada balsam, were examined under the microscope using preparations from control embryos for comparison.

Statistical Estimation of LD_{50} Values. The LD_{50} value of DMN for each of the four test strains of *Gallus domesticus* was estimated by the probit analysis as recommended by Finney (1971) and Fisher and Yates (1964). Dose–response curves (percent mortality, expressed as probit units/log of dose concentration) were obtained by regressing doses on probit values. The LD_{50} doses were then calculated, at probit 5.0 (corresponding to 50% mortality), by solving the respective regression equations. The standard error of each LD_{50} value was estimated according to the method of Miller and Tainter (1944).

Table I. Derivation of LD_{50} Values of Dimethylnitrosamine (DMN) for *Gallus domesticus* Embryos

strain	no. of eggs/ DMN dose	no. of dead embryos/DMN dose: DMN dose, (μg/50 g egg wt) 0.0[e]	0.2	0.8	3.1	12.5	50.0	200.0	regression equation[f]	correlation factor	LD_{50} dose = (X/CL) ± SE[d] (μg of DMN/50 g egg wt)
White Rock	33	2	4	6	10	16	22	25	$x = -5.10 + 1.46y$	0.79	14.8 ± 3.4[a]
White Leghorn	33	2	7	11	17	20	28	31	$x = -4.19 + 1.14y$	0.77	3.3 ± 0.7[b]
Rhode Island red	33	2	5	11	14	17	26	29	$x = -4.57 + 1.27y$	0.77	5.7 ± 1.4[c]
Local breed	33	1	2	5	9	15	26	29	$x = -3.84 + 1.15y$	0.78	8.4 ± 0.7[c]

[a] Significantly different from *b*, *c*; $p = 0.001$. [b] Significantly different from *a*, *c*; $p = 0.001$. [c] Not significantly different; $p = 0.001$. [d] SE = standard error. [e] Control. [f] y (dependent variable) = embryo mortality expressed as probit values; X (independent variable) = dosage of DMN expressed as log DMN concentration/50 g egg wt; CL (confidence level) = 95%.

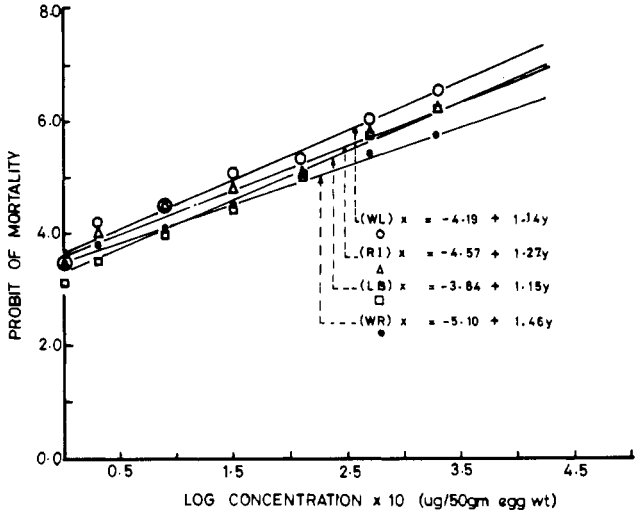

Figure 1. The relationship between dosage of dimethylnitrosamine and probit of mortality of the chick embryo, showing probit regression lines, for White Rock (WR), Rhode Island (RI), White Leghorn (WL), and a local breed (LB).

RESULTS AND DISCUSSION

The LD_{50} doses of dimethylnitrosamine for the various strains of *Gallus domesticus* investigated and the regression equations from which these values were computed are shown in Table I. The White Leghorn embryo was most susceptible and that of the White Rock the least susceptible to dimethylnitrosamine poisoning; and there was good positive correlation between dose concentrations of dimethylnitrosamine employed and the mortality of the test chick embryos (Figure 1).

A histological examination of the livers of affected embryos revealed a general severe congestion of the lobules due to the toxic effect of dimethylnitrosamine. This lesion was not localized and no centrilobular cell necrosis of the liver, characteristic of acute dimethylnitrosamine poisoning, was observed. No lobule congestion was detected in livers of either dead control embryos or controls that hatched. Dead embryos from different strains were compared for incidence of liver lobule congestion. Differences in the frequency distribution of this lesion between strains were very marginal. In general, the occurrence of the lesion was between 90 and 100%. Minimum survival times were not recorded.

The indication from this study is that dimethylnitrosamine is lethal to the chick embryo and strain differences exist in the susceptibility of the embryo to nitrosamine poisoning. White Leghorn embryos would appear to be particularly suitable for the bioassay of nitrosamines.

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Received for review March 24, 1978. Accepted September 11, 1978.