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Effects of Certain Triazenes on Chick Embryos and on Tumors Explanted to the Chorioallantois.* (22074)

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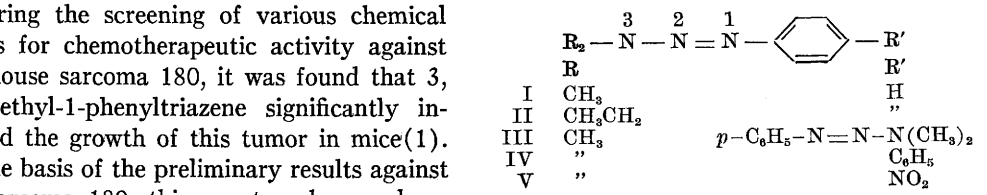
During the screening of various chemical agents for chemotherapeutic activity against the mouse sarcoma 180, it was found that 3, 3-dimethyl-1-phenyltriazene significantly inhibited the growth of this tumor in mice(1). On the basis of the preliminary results against the sarcoma 180, this agent and several related compounds were selected for more extensive study in secondary systems used in the tumor screening program(2-4).

This report is concerned with the effects of certain triazenes on the development of the chick embryo and on tumors growing on the chorioallantois. In the studies reported here it was found that the anomalies induced in the chick embryo by triazenes were similar to those caused by other, unrelated, teratogens whose effects are preventable or reversible by nicotinamide(5-9). Consequently, nicotinamide was tested for its ability to prevent the teratogenic effects of the triazenes.

Materials and methods. Commercially obtained White Leghorn eggs, incubated at 38°, were used for all experiments. The compounds tested for teratogenic activity against the chick embryo have the following structures:



	R	R′
I	CH_3	H
II	CH_3CH_2	"
III	CH_3	p-C_6H_5-N=N-N$(CH_3)_2$
IV	"	C_6H_5
V	"	NO_2

I: 3,3-dimethyl-1-phenyltriazene
II: 3,3-diethyl-1-phenyltriazene
III: 1,1′-(4-4′-biphenylene) bis [3,3-dimethyltriazene]
IV: 1-(4-biphenylyl)-3,3-dimethyltriazene
V: 3,3-dimethyl-1-*p*-nitrophenyltriazene

I is an amber-colored liquid with a specific gravity of 1.02. II is also a liquid, while III, IV, and V are solids. Because of their low solubility, these compounds were prepared as suspensions in isotonic saline or in 0.5% sodium carboxymethylcellulose in isotonic saline. The freshly prepared suspensions were injected into the yolk sac of 4-day-old embryos. The eggs were sacrificed after the 18th day of incubation. The embryos were weighed, examined for gross abnormalities, and selected embryos were cleared and stained with alizarin to show skeletal defects. In the protection experiments, nicotinamide in isotonic saline was injected immediately following the triazene. The tumors used were mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3 (H.Ep.#3)(10,11). Preparatory to chemotherapy trials, triazene-I was injected into the yolk sac of 12-day-old embryos to determine tolerated dose levels

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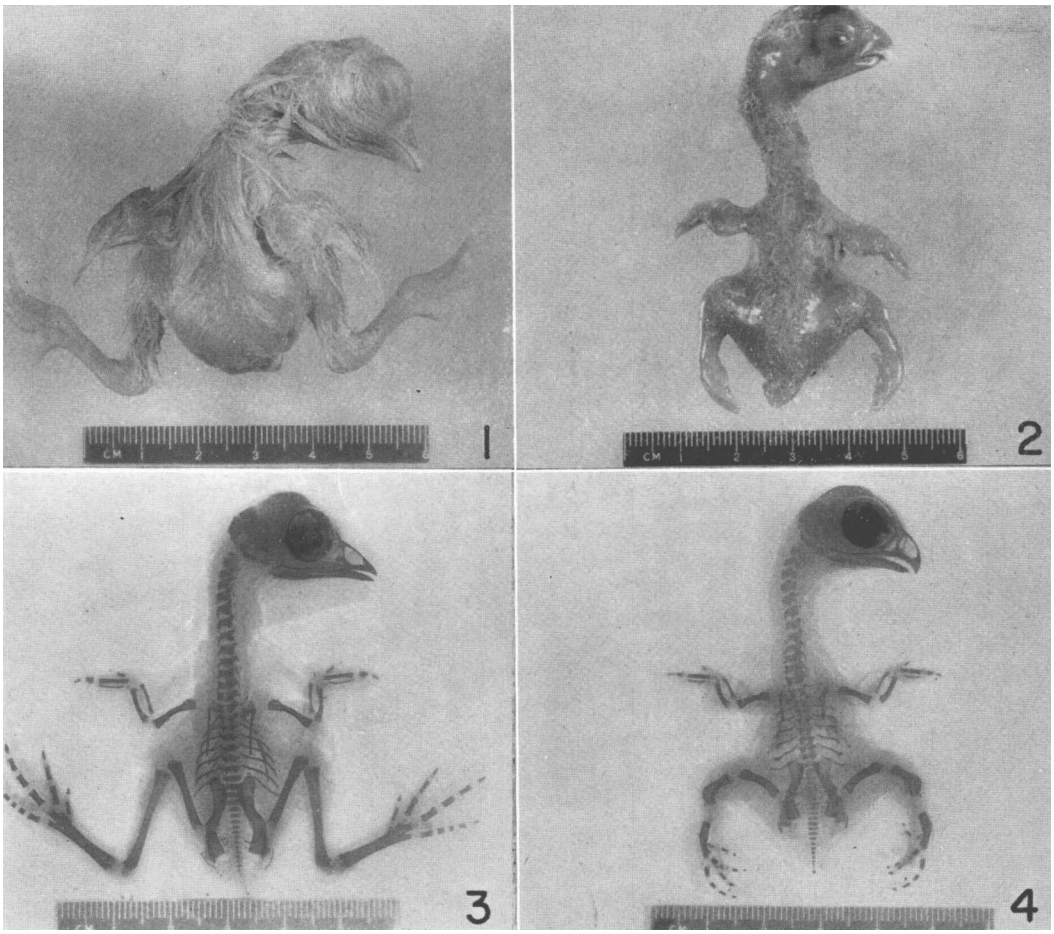

PLATE I

FIG. 1. 18-day control embryo.
FIG. 2. 18-day embryo treated with triazene-I at 4 days.
FIG. 3. Skeleton of 18-day control embryo.
FIG. 4. Skeleton of 18-day embryo treated with triazene-I at 4 days.

and the morphological alterations in the surviving embryos.

Single fragments of tumor were explanted to the chorioallantois on the 8th day of incubation. Four days later, after those eggs with implanted and apparently viable tumors had been selected, triazene-I was injected into the yolk sac. On the 16th day of incubation the eggs were sacrificed; the tumors were weighed and portions saved for microscopic examination. Whenever possible the tumors were bioassayed. Fragments of the H.Ep.#3 were pooled in 100-150 mg lots in each experimental group and inoculated into irradiated-cortisonized rats. Individual tumors of the S180 were assayed by subcutaneous implantation into mice.

Results. Teratogenic activity of 3,3-dimethyl-1-phenyltriazene: Triazene-I, when injected on the 4th day of incubation, produced consistent and characteristic abnormalities in development, clearly seen in embryos surviving to the 18th day of incubation (Fig. 1-4). The LD_{50} was 0.5-1.0 cmm/egg and the teratogenic effects were produced over this range (Table I). The abnormal embryos showed weight inhibition associated with skeletal deformities of the beak and appendages. Edema and feather growth inhibition, with clubbing, occasionally occurred.

TABLE I. Toxicity and Incidence of Abnormal Embryos in Eggs Injected with Triazenes on 4th Day of Incubation and Sacrificed after 18th Day. Protection against teratogenic effects of triazenes with nicotinamide.

	Dose, mm³/egg	No. inj.	Mortality, %	Abnormal survivors, %
T*-I	.25	20	40	25
	.5	47	30	79
	1.0	25	76	100
	2.0	15	93	100
T-II	.25	20	35	69
	.5	45	49	100
	1.0	20	45	100
	2.0	15	67	100
T-I + N*	.5 5.0	45	62	0
T-II + N	.5 5.0	43	86	0
T-II + N	.5 1.25	49	78	0

* T = Triazene; N = Nicotinamide.

The principal beak defect was parrot-beak (Fig. 2 and 4), although less severe effects such as shortening of the upper and lower beak, or the lower beak alone, were sometimes seen. Abnormalities of the appendicular skeleton included micromelia, bending of the tibiotarsus, and occasionally, bending of the tarsometatarsus. No abnormalities were found in the axial skeleton or in the pelvic and shoulder girdles.

Following injections into the 12-day yolk sac, no gross skeletal abnormalities were produced at tolerated doses. Doses of 5 to 10 cmm/egg led to stunting, inhibition of feather growth with clubbing, and edema in a large percentage of the embryos. Another consistent effect in the 12-day embryo was a reduction in spleen weight. Thus, at 16 days the control spleens weighed 13.6 mg, on the average, as compared to 4.5 mg for the affected embryos.

Teratogenic activity of other triazenes: The tolerated doses and the estimated LD_{50}'s of triazenes II, III, IV, and V in the 4-day embryo did not appear to be appreciably different from triazene-I. In this series, only 3,3-dimethyl-1-p-nitrophenyltriazene failed to produce any abnormalities. Triazene-II induced the same pattern of embryonic malformations seen with triazene-I and at approximately the same doses. Triazene-III, however, was effective only at much higher dose levels and affected fewer of the surviving embryos. The overall teratogenic effects of triazene-IV were different from those of the other active compounds in that they occurred less consistently and the only abnormality observed was a bending of the tibiotarsus.

Protection with nicotinamide: Nicotinamide was tested against doses of triazene-I and II which, alone, produced a high incidence of abnormalities in the surviving embryos (Table I). Under these conditions nicotinamide protected against the teratogenic effects but did not restore the weight to normal nor did it protect against the lethality of triazene-I and II. The minimal effective ratio of nicotinamide to triazene has not been determined.

Tumor inhibiting effect of triazene-I: In the chemotherapy tests, triazene-I inhibited the growth of the S180 and H.Ep#3 (Table II). Histological sections of the tumor

TABLE II. Effect of Triazene-I on Mouse Sarcoma 180 and Toolan's H.Ep. #3 Growing on Chorioallantois of the Chick Embryo.

Group	Dose, mm³/egg	No. inj.	No. survivors	Tumor wt, mg	Embryo wt, g
		Mouse sarcoma 180			
Control	0	8	7	172 ± 72	14.4 ± 1.9
Triazene-I	5.0	10	6	76 ± 44	12.9 ± 1.1
"	7.5	10	6	38 ± 39	10.0 ± 1.9
		Toolan's H.Ep. #3			
Control	0	29	26	229 ± 48	12.7 ± 1.3
Triazene-I	5.0	34	27	42 ± 30	10.0 ± 1.2
"	7.5	24	15	49 ± 37	9.1 ± .7
"	10.0	15	2	19 & 38	8.0 & 8.0

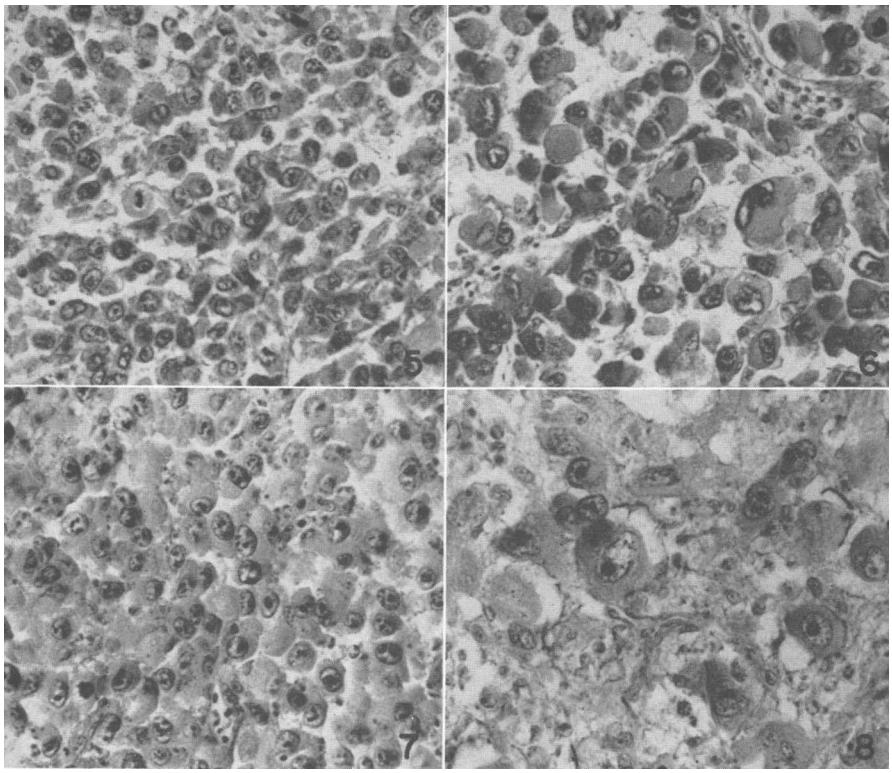

PLATE II

FIG. 5. Untreated mouse sarcoma 180 growing on the chorioallantois. × 200.

FIG. 6. Mouse sarcoma 180 growing on the chorioallantois, treated with triazene-I, 5.0 mm³ per egg. × 200.

FIG. 7. Untreated Toolan's H.Ep. #3 growing on the chorioallantois. × 200.

FIG. 8. Toolan's H.Ep. #3 growing on the chorioallantois treated with triazene-I, 7.5 mm³ per egg. × 200.

showed cellular enlargement, nuclear changes indicative of cellular injury, and a decrease in number of cells (Fig. 5-8). Histologically, the sarcoma 180 appeared to be more sensitive than the H.Ep.#3, since greater cellular damage was observed at the lower dose (5 cmm) in the sarcoma 180. When the sarcoma 180's were assayed in mice, the untreated tumors grew rapidly, but, in no case did any of the triazene-treated tumors show any evidence of growth during a 2-week observation period. This indicated that the triazene destroyed the viability of sarcoma 180 as measured by transplantation. In spite of the marked histological effects produced by triazene in the egg-grown H.Ep.#3, the tumors gave positive bioassays in rats. It is noted, however, that in order to prepare a satisfactory inoculum, several human tumors were pooled for bioassay, and therefore, the inclusion of a slightly damaged tumor in the pooled tumor inoculum could produce a positive bioassay. Thus, it is concluded that the viability of every one of the treated H.Ep. #3's was not destroyed by triazene.

Discussion. The effects of triazenes on chick embryos and on explanted tumors have interesting correlations with other agents. Of major significance is the observation that triazene-I causes distinctive and consistent effects on the mouse sarcoma 180 and Toolan's H.Ep.#3 explanted to the chorioallantois. The most important criteria of effect are the histological changes in the tumor and viability on bioassay. The histological changes of cellular enlargement and nuclear distortions with a decrease in cell numbers are similar to those engendered by nitrogen mus-

tard and related polyfunctional alkylating agents and by x-rays(12). The greater sensitivity of the sarcoma 180 as compared to H.Ep.#3 to triazene parallels the results seen with nitrogen mustard(11). A further similarity between the two types of compounds is that both the polyfunctional alkylating agents and triazene-I retard the growth of the embryonic spleen.

Quantitative dissimilarities between triazenes and polyfunctional alkylating agents are apparent in their comparative effects on 12-day-old chick embryos and egg-grown tumors. Doses of nitrogen mustard causing relatively slight disturbances in the embryo are markedly injurious to the tumors and affect their viability as indicated by bioassay (12,13). In contrast, the dose of triazene-I required to obtain an equivalent degree of tumor inhibition and cellular damage will induce definite injury to the embryo.

Despite certain similarities in the biological actions of nitrogen mustards and the triazenes, parallel information on the chemical reactivity of triazenes in biological systems is not available. It has been shown that among biologically active alkylating agents, compounds possessing 2 functional groups are far more active than the monofunctional agents (one-armed mustards)(14,15). While the amount of triazene necessary to produce effects in the explanted tumors in the egg are much greater than that required for most polyfunctional alkylating agents, 0.1 mg for nitrogen mustard as compared to 5 mg (5 cmm) for triazene-I, neither altering the structure of the triazenes (cf. triazene-I, II, IV, V) nor producing a two-armed compound (triazene-III) has enhanced the biological activity.

The triazenes are of further interest since certain members of this series produce a definite syndrome of malformations when injected into 4-day-old embryos. The effects on the facial and appendicular skeleton are similar, if not identical, to those following treatment by insulin(6,7), sulfanilamide (5,9), and eserine(5,8). Furthermore, as in the case of the latter compounds, nicotinamide will prevent the teratogenic action of triazenes in the 4-day embryo. This teratogenic property distinguishes triazenes from the polyfunctional alkylating agents which do not produce specific or consistent teratogenic effects. The biochemical mechanism for the production of the parrot beak-micromelia-bent tibiotarsus syndrome has not been definitely established, although Landauer(7) and Zwilling(9) have related it to interference with carbohydrate metabolism.

Since the effects of triazene-I on tumors and 12-day embryos are similar to those produced by certain polyfunctional alkylating agents while the effects on younger embryos resemble those following treatment with insulin, sulfanilamide and eserine, it is possible that the molecule is pluripotent or that it is converted in the egg into 2 or more metabolites with dissociated activity. In acid solutions triazene-I is broken to the diazonium salt and dimethylamine. Diazonium salts have been shown to polymerize *in vitro* to form diazoaminobenzene, a compound that is toxic to the liver, spleen, and kidneys in mice, and is carcinogenic in mice on local application to the skin(16). When injected into the 4-day-old embryo, *p*-diphenylamine diazonium fluoroborate produces a syndrome of abnormalities which is similar to that caused by triazenes-I and II(3).

Because of the wide range of biological effects of the polyfunctional alkylating agents, the discovery of a tumor-inhibiting drug with somewhat similar but also distinct effects, and acting by means of a considerably different chemical structure, becomes of interest.

Summary. 1. A new compound that inhibits the growth of the sarcoma 180 in mice, 3,3-dimethyl-1-phenyltriazene, "triazene," also exhibits teratogenic activity in the chick embryo and damages mouse and human tumors explanted to the chorioallantois of the chick embryo. 2. The teratogenic syndrome produced by the injection of "triazene" into the yolk sac of the 4-day embryo consists of parrot-beak, micromelia, bent tibiotarsus, edema, and feather growth inhibition. This effect can be prevented by the injection of nicotinamide. Several analogs of "triazene"

have been tested for teratogenic properties. 3. When injected into the yolk sac of embryos carrying chorioallantoic grafts of the mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3, "triazene" causes inhibition of tumor growth and cellular injury. This effect is similar to that produced by nitrogen mustard and other polyfunctional alkylating agents. Because "triazene" shows certain similarities in tumor-inhibiting activity when compared to nitrogen mustard and because of its distinctly different chemical structure, it warrants consideration as a candidate agent for clinical cancer chemotherapy.

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Serum Glycoprotein Concentrations in Rats Infected with the Cestode, *Hymenolepis diminuta*. (22075)

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(Introduced by Charles M. Carpenter.)

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The serum protein response in a large number of infectious diseases has been studied by many investigators employing electrophoretic or salting-out methods. Gutman(1), Fisher(2) and Stauber(3) have summarized the significant data in their reviews. In contrast, few reports are available on effects of infectious diseases on carbohydrate-containing proteins of serum. Studies concerned with the influence of infection on protein-bound carbohydrates of serum have been briefly reviewed by Weimer *et al.*(4). Diseases in which serum glycoproteins have been determined are characterized by invasion of tissues by the etiologic agent and marked, systemic host reactions. It was of interest to investigate the serum glycoproteins in an experimental infection in which the parasite is localized and invasion of the tissues does not occur.

The present investigation was undertaken to study the effects of experimental infection with *Hymenolepis diminuta* on serum concentrations of total glycoprotein, seromucoid (mucoprotein), γ-globulin polysaccharide, total protein, and γ-globulin protein in rats.