

anaphylactic reactions. They indicate that a sudden drop in anti-ferment is associated with the initiation of shock, and that a significant increase in antitryptic elements occurs during reactions, so that the serum of surviving, antianaphylactic animals possesses an unusually high antitryptic power.

13452

### Evaluation of Bactericides by Egg Injection Method with Special Reference to Development of Technic.\*

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Tissue culture methods<sup>1</sup> have been used to measure the toxic action of bactericides through their influence on groups of cells *in vitro*. Such technics, however, do not properly involve the complexity of organ and tissue interrelation which exists in the intact animal, adult or embryonic. The avian embryo was chosen principally for these experiments because manipulation with the whole animal is afforded *in vivo* and because large amounts of albuminous material with which the bactericidal agent might come in contact are present.

*General Method.* Immediately preceding injection, fertilized eggs of known origin<sup>†</sup> were candled to assure vitality. The area on the shell opposite the embryo was treated with tincture of iodine and a small hole was drilled<sup>‡</sup> through the marked area to expose the *membranae putaminis* (the membrane lining the egg shell). Throughout the manipulation the egg was kept on its side with the marked area uppermost. Using sterile technic, dilutions of the antiseptic made with sterile distilled water were rapidly injected into the egg through a ¼ inch, 27 gauge needle. The hole was then sealed with a drop of sterile paraffin at solidifying temperature.

The eggs were incubated at 38.5°C in an oblique position, with

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<sup>1</sup> Salle, A. J., and Lazarus, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1935 and 1936, **32**, 265, 937, 1057, 1119, 1481; **33**, 8, 393; **34**, 371.

<sup>†</sup> Blood-tested White Leghorns.

<sup>‡</sup> Dremel "Moto-Tool" Model No. 1, using burrs No. 107. Manufactured by the Dremel Tool Company, Racine, Wisconsin.

the blunt end up, turned twice daily and then examined for vitality by candling; confirmation of vitality was made by opening the egg.<sup>§</sup>

*Site of Injection.* Employing 9-day-old embryos it was found that consistent results were obtained when the medicaments were placed on the chorio-allantoic membrane, whether this was accomplished by the method of Goodpasture,<sup>2</sup> or Brandly,<sup>3</sup> or of Burnett.<sup>4</sup> On the other hand, no degree of consistency was obtained when the injection was made into the albuminous material surrounding the embryonic tissues.

*Determining the Volume Capacity of an Embryonic Egg.* The injection volume was without influence on the viability of 9-day-old embryos when volumes up to 2.0 cc of saline (0.85%), Tyrode's solution, ethyl alcohol (70%), or F.D.A. broth<sup>5</sup> were injected onto the chorio-allantoic membrane.

*Toxicity of Bactericidal Agents.* (A) Increasing the Dose by Varying the Volume. A series of 9-day-old fertilized eggs was injected by various methods<sup>2, 3, 4</sup> with stock solutions of the bactericidal agents and their controls. In the first series the volume was increased from 0.1 cc of the medicament to 2.0 cc by 0.1 cc quantities. After preliminary findings were obtained another series was run between the approximate end-points and the sensitivity narrowed down to 0.01 cc. Readings were made after 24 hours' incubation at 38.5°C. Results are shown in Table I.

(B) Increasing the Dose by Varying the Concentration (Volume Constant). Varying amounts of medicaments were diluted to volumes of 2.0 cc with distilled water and injected into 9-day-old avian embryos. Results were obtained identical with those found when the dose was increased by varying the volume.

*Bactericidal Efficiency Tests.* The bactericidal agents in question were tested by the Food and Drug Administration Technic (F.D.A.).<sup>5</sup> The dilutions of germicide capable of inhibiting the growth of *Staphylococcus aureus*<sup>5</sup> in 10 but not in 5 minutes were noted. All tubes were subcultured to prevent bacteriostatic action by removing one standard loopful<sup>5</sup> from the transplant tube and

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§ Vitality is evidenced during candling by movement of the embryo and pulsation of vitelline artery branches. When vitality is doubtful, eggs are opened and the embryo examined for heart-beat.

<sup>2</sup> Goodpasture, E. W., *Southern Med. J.*, 1933, **26**, 418.

<sup>3</sup> Brandly, C. A., *Veterinary Med.*, 1940, **35**, 98.

<sup>4</sup> Burnett, F. M., H. M. Stationery Office Special Report—Series 220 Universal Decimal Classification Number 576, 809, 429:611,013,3/6.

<sup>5</sup> U. S. Food and Drug Administration Bulletin No. 198, 1931.

TABLE I.  
Relative Toxicity of Bactericides to 9-day-old Chick-embryos.

Bactericide	Dilution killing <i>S. aureus</i> in 10 but not in 5 min. 37°C	A. Actual conc. of bactericide g/cc	9-day chick embryo MLD cc stock soln.	B. grams of bactericide per MLD	A — = K B Toxicity index
Phenol 1:20 aqueous	1:85	.01176	.12	.006	2.000
Hexylresorcinol 1:1000 30% glycerine aqueous solution	1:8,000	.00012	.16	.00016	0.800
Mercuric bichloride U.S.P. 1:1000 aqueous	1:16,000	.00006	.02	.00002	3.125
A mixture of ortho hydroxy phenyl mercuric chloride and 5 isomeric amyl ortho cresols 1:1000 tincture	1:18,000	.00005	.02	.00002	2.750
Sodium hydroxymercuri- nitrophenolate 1:1000 aqueous	1:1,100	.0009	.04	.00004	22.750
Sodium ethyl mercuri thio- salicylate 1:1000 tincture	1:3,000	.0003	.02	.00002	16.500
4-nitro-anhydro hydroxy mer- curi ortho cresol 1:200 tinc- ture	1:1,100	.0009	.014	.00007	13.000
Disodium dibrom-4-hydroxy mercuri fluorescein 1:50 aqueous	1:12.5*	.080*	.04	.0008	100.000
Iodine, Tincture U.S.P. XI (7% iodine)	1:6430	.00015	.02	.0014	0.11
Sodium hypochlorite solution	1:240	.00416	.04	.0004	10.4
Mild silver protein A 1:20 aqueous	1:100	.010	.9	.045	0.22
Mild silver protein B 1:20 aqueous	1:90	.011	.6	.030	0.366

\*Stock solution would not kill test organism under conditions of test.

subculturing into another tube of F.D.A. broth.<sup>5</sup> The results are shown in Tables I and II.

*Varying the Age of the Embryo.* The effect of the age of the embryo was determined by injecting control fluids (2.0 cc) onto the chorio-allantoic membrane of 12-day-old embryos. In all cases embryos survived after 48 hours' incubation at 38.5°C. Injecting onto the chorio-allantoic membrane by any of the 3 methods,<sup>2, 3, 4</sup> was found to be satisfactory and reproducible results were obtained.

The minimum lethal doses of the germicidal agents for the 12-day-old embryos are recorded in Table II.

*Toxicity Index or Coefficient of Relative Toxicity.* A comparison was made of the resistance of *Staphylococcus aureus* and of chick-embryos to germicidal agents. The bactericidal efficiencies of the germicides employed were determined by the Food and Drug Administration technic.<sup>5</sup> The highest dilution killing the test organism in 10, but not in 5 minutes, at 37°C after subculturing, was accepted

TABLE II.  
Relative Toxicity of Bactericides to 12-day-old Chick-embryos.

Bactericide	Dilution killing <i>S. aureus</i> in 10 but not in 5 min 37°C	A. Actual conc. of bactericide g/cc	12-day chick embryo MLD cc stock soln.	B. grams of bactericide per MLD	A — = K B Toxicity index
Phenol 1:20 aqueous	1:85	.01176	.2	.01	1.1764
Hexylresorcinol 1:1000 30% glycerine aqueous solution	1:8,000	.00012	.25	.00025	0.5
Mercuric bichloride U.S.P. 1:1000 aqueous	1:16,000	.00006	.1	.0001	0.625
A mixture of ortho hydroxy phenyl mercuric chloride and 5 isomeric amyl ortho cresols 1:1000 tincture	1:18,000	.00005	.1	.0001	0.55
Sodium hydroxymercuri-o- nitrophenolate 1:1000 aqueous	1:1,100	.00091	.1	.0001	9.1
Sodium ethyl mercuri thio- salicylate 1:1000 tincture	1:3,000	.00033	.1	.0001	3.3
4-nitro-anhydro-hydroxy mercuri ortho cresol 1:200 tincture	1:1,100	.00091	.02	.0001	9.1
Disodium dibrom-4-hydroxy mercuri fluorescein 1:50 aqueous	1:12.5	.08	.3	.006	13.3
Iodine, Tincture U.S.P. XI (7% iodine)	1:6430	.00015	.05	.0035	0.0445
Sodium hypochlorite sol.	1:240	.00416	.3	.003	1.3886
Mild silver protein A 1:20 aqueous	1:100	.010	>2.0*	>0.1*	<0.1
Mild silver protein B 1:20 aqueous	1:90	.011	>2.0*	>0.1*	<0.1

\* = No end point with solution used.

> = Greater than.

< = Less than.

as the dilution of the chemical required to kill *Staphylococcus aureus*. The actual amount of germicidal agent present in each cubic centimeter of the highest dilution lethal to the test organism under these conditions was calculated.

The minimum lethal dose (M.L.D.) of the germicidal agents to the embryo of the chick was determined by averaging the results of 3 tests and the actual amount of the germicidal agent present in the minimum lethal dose of the bactericide was calculated.

A number known as the "Toxicity Index" was then determined. It may be defined as the ratio of the actual amount of the germicidal agent contained in each cubic centimeter of the highest dilution required to kill *Staphylococcus aureus* in 10, but not in 5 minutes, at 37°C, to the actual amount of the germicidal agent required to kill a chick-embryo within 24 hours. Theoretically, the smaller the toxicity index the more practical the germicide.

$$\text{Toxicity Index} = \frac{\text{Actual amount of germicidal agent in one cubic centimeter of the highest dilution killing } \textit{Staphylococcus aureus} \text{ in 10, but not in 5 minutes at } 37^{\circ}\text{C}}{\text{Minimal amount of germicidal agent killing the avian-embryo within 24 hours.}}$$

*Discussion.* The experiments described include an evaluation of toxicity for the chick-embryo with one application of the antiseptic in question. This of course has a valid criticism in looking at the problem from a practical standpoint. In attempting to select an antiseptic on a basis of bactericidal action and relative freedom from tissue toxicity, one must take into consideration either frequent administration of the antiseptic or long continued contact such as is encountered in wet-dressings, irrigations, etc. For this reason both the bactericidal efficiency tests and the embryonic tissue toxicity studies may be considered as acute tests; in addition, tests of a chronic nature would be desirable. The results of such studies might alter the evaluation of the antiseptics studied in this report, particularly with reference to the heavy metals and to the halogens.

*Summary.* 1. A comparison of various methods for the injection of fertilized and incubated avian eggs has been conducted. The results were similar in all instances wherein the injection was made upon the chorio-allantoic membrane, regardless of the method used to approach the tissue membrane. 2. It was found that large quantities of distilled water, physiological saline, Tyrode's solution and F.D.A. broth could be injected into the egg without affecting the embryo's vitality. Eggs were shown to be capable of sustaining injections of at least 2 cc with maintenance of vitality during at least 48 hours' incubation at  $38.5^{\circ}\text{C}$  following injection. 3. Minimum lethal doses for the avian-embryo were established for the bactericidal agents under test, employing 9- and 12-day-old chick-embryos. The older embryos displayed higher resistance to the toxic substances than did those of 9 days age. 4. Bactericidal efficiencies of the bactericides under test were conducted by the Food and Drug Administration technic, against *Staphylococcus aureus*. In addition, all dilutions of the bactericidal agents were subcultured into another test tube of F.D.A. broth to test for bacteriostasis. 5. A number known as the "Toxicity Index" was determined from a comparison of the bactericidal efficiency against *Staphylococcus aureus*, with the toxicity of the bactericide for chick-embryos.