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A Chick Embryo Technic for Intravenous and Chemotherapeutic Studies.*

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(Introduced by Werner Henle.)

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The chick embryo has become increasingly valuable in investigative work and in the production of certain biologic materials. Up to the present time no less than 20 viruses have been propagated by its use. Many of the bacteria, certain rickettsias, leptospira icterohemorrhagica, and a growing number of types of tissue cells, both benign and malignant, have been studied in the embryo or upon its membranes. Several recent studies have dealt with chemotherapeutic testing as applied to experimentally infected embryos. None of these papers has given detailed quantitative information concerning levels attained by therapeutic agents in the blood and extraembryonic fluids.[1-5] In the course of the above types of studies many technics have been devised.

Rous and Murphy were the first to use the chorio-allantoic membrane for the study of transplanted tumor fragments. They were also the first to use the chick embryo in the study of an infectious agent, the virus of the Rous Sarcoma.[6]

The majority of studies involving the exposed chorio-allantoic membrane as a site of inoculation have made use of a method of opening eggs first described by Clark.[7]

Woodruff and Goodpasture modified Clark's technic.[8] Later Goodpasture and Buddingh described improvements and their technic, with slight variations, has been used by many laboratories.[9] The technic described by Burnet involving the production of an artificial air sac has also found wide use for inoculation of the chorio-allantoic membrane and for infection by the amniotic route.[10] Taylor and Chialvo reported a simplification of Burnet's method which they found particularly valuable for intraamniotic injections.[11] A method for intravenous inoculation of the embryo was described by Eichorn.[12] Many modifications of the above technics have been devised.

Each of the technics mentioned is satisfactory for certain purposes. However in the course of experiments concerned with the chemotherapy of bacterial infections produced in chick embryos by intravenous inoculation,† it became apparent that no method previously described was entirely satisfactory. A method was required that would permit intravenous inoculation of the embryo as well as accurate injection into or aspiration from any or all of the extraembryonic sacs at the same or at separate times. It was also necessary that the method permit later sampling of the blood. The method here reported permits all of the above procedures as well as placement of

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† To be reported separately.

TABLE I.
Average Water Loss (g) 10th Through 20th Day of Incubation. Each Figure Is Average of 12 Eggs.

	Unopened eggs	Opened, membrane exposed, single layer Scotch-tape	Ditto double layer	Ditto triple layer
Wt. loss in g	4.0	7.7	5.1	4.6

TABLE II.
Volume of Blood (cc) Obtained from Embryos of Various Ages.

Embryo No.	Age in Days 10	11	12	13	15	17	18
1	.08	.20	.23	.26	.30	.39	.70
2	.05	.18		.29	.32	.42	.70
3	.10	.23		.31	.33	.40	.50
4	.09	.20		.31	.29	.33	.60
5	.12	.28		.20	.36	.43	.65
6	.12	.10	.24	.34	.36	.43	.75
Avg.	.09	.19		.28	.32	.39	.65

material upon the exposed chorio-allantoic membrane.

Technic. Eggs of the selected incubation-age are candled and the air sac margin is marked with a pencil. For intravenous work the embryo should be of at least 10 days incubation. The shell along the air sac margin is wiped off with 70% alcohol. When many eggs are handled a squat, wide-mouthed bottle filled with 70% alcohol with a pad of cotton over its mouth facilitates this step. The cotton is allowed to project into the alcohol-bearing cotton. The shell over the air sac is removed by cutting just distal to the pencil line with a thin carborundum disc rotating at high speed. The motor is fixed in a vise and the egg rotated in contact with the cutting disc. With a little practice this "decapping" procedure can be done very rapidly. It is possible to remove the shell cap entirely or to leave it attached by partially cut shell so that it serves temporarily as a cover against air-borne dust. If the cap has been left attached it is easily removed with cover-slip forceps. The egg is inverted and tapped lightly to remove shell dust from the surface of the reflected portion of the shell membrane. The rest of the work requires a satisfactory egg-holder. A low wide-mouthed bottle with its open end covered with cotton moistened with 70% alcohol is ideal for this purpose. The cotton supports the egg firmly but permits instant adjustment of position. Good lighting is also important. A light source in front of the operator has been found best, provided that a piece of Polaroid is placed between the light source and the egg. The Polaroid serves to eliminate glare from the moist membrane surfaces.

As recommended by Burnet in his description of a side-window technic about 0.1 cc of sterile saline solution is placed over a slight nick made in the shell membrane overlying the chorio-allantoic membrane. The nick is made with the fine needle which deposits the saline. The saline solution serves to initiate separation of the shell membrane from the underlying chorio-allantoic membrane. In the presence of this saline the shell membrane may then be gently picked up with fine-pointed forceps and stripped off with no resultant capillary bleeding. The entire surface of the chorio-allantoic membrane lying under the air sac may be exposed or as much of this area as is desired for the particular work being done. Injection intravenously, into the amniotic fluid, into the allantoic fluid, or into the yolk sac, may then be made under full visibility. Blood or extra-embryonic fluids may be collected or materials may be deposited upon the surface

TABLE III.
Survivals After Opening Shell, Exposing Chorio-allantoic Membrane and Sealing with Scotch Tape for the Remainder of Incubation. Three Separate Groups of Eggs.

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened group 1	18	18	18	18
" " 2	18	18	16	16
" " 3	18	18	18	18

TABLE III-A
Same as Table III Plus Injection of .1 cc of Saline Into the Yolk, Into the Amniotic Fluid, and Into the Allantoic Fluid.

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened. No injection	18	17	16	16
" Injected	18	16	15	15

TABLE IV.
Intravenous Injection.
Survival Rates with .05 cc Saline as Inoculum. Injection Time Approximately 5 Seconds. All Control Groups Opened, Membrane Exposed, Sealed.

	Age in Days					
	10	11	12	15	18	20
Controls	18	18	18	18	18	18
Large vessels, no hemostasis	48	21	21	18	17	17
Controls	18		16	16	16	16
Fine vessels selected	18		12	11	9	9
Controls	18		18	18	18	18
Fine vessels selected	18		15	14	13	13
Controls		18	18	18	18	18
Fine vessels selected		18		15	15	15
Controls		18		16	16	16
Fine vessels selected		18		14	12	12
Vessel entered with needle but no injection		18		15	14	14

of the membrane. The collection of blood is difficult before the ninth day of incubation.

The open end of the egg is then sealed with 3 layers of transparent Scotch tape. The triple layer is used since it permits the closest approximation to normal water-loss during the remainder of the incubation period as determined by loss of weight. (Table I.) Substances may later be applied to the chorio-allantoic membrane by simply passing a fine needle through the Scotch tape. At any time the tape may be partially or completely removed for further work. The tape should be applied in such lengths that it extends down over the egg for approximately ½ inch.

Intravenous Injection. Embryos show individual variations of the pattern of veins in the exposed chorio-allantoic membrane. Usually there are several venous branches available for injection. In an occasional egg no suitable vessels are present in this area. The usual situation is to find one large allantoic vein with two or more tributaries. Very small vessels may be entered with a No. 27 needle provided its point is perfectly sharp and its shaft smooth. It is preferable to use the tributaries and not the main vessel since less bleeding results upon withdrawing the needle. When a large vessel is used it is possible to control the bleeding by the use of very small "serrefine" hemostats. The open jaws of these small spring-activated hemostats are slipped onto the vessel just as the needle is withdrawn and the hemostat is held in place for 15-20 seconds before removal. Hemostasis is complete. An increase in mortality results from this clamping procedure but this increase is not regularly predictable. This step is not necessary if the small vessels are chosen for injection.

TABLE V.
Intravenous Injection of Saline. Effect of Volume and Rate of Injection.

Days old	10	11	12	13	14	15	16	17	18	19	20
				A. Effect of Rate of Injection							
Controls	18			16			16			16	16
.05 cc in 2 sec. approx.	18			9			9			9	9
.05 cc in 5 sec. approx.	18			12			11			9	9
.05 cc in 60 sec. approx.	18			11			11			8	8
				B. Effect of Volume of Injection							
Controls	18			18			18			18	
.05 cc in 5 sec. approx.	18			15			14			13	
.1 cc in 5 sec. approx.	18			9			4			3	
.2 cc in 5 sec. approx.	18			3			1			1	

TABLE VI

Compound	No. of embryos	Dose in mg per 100 g of egg wt.	% survival* after 9 days
Promin	18	60	0
	18	50	50
	18	40	80
	18	30	100
Sulfathiazole	18	20	50
	18	15	84
	18	10	100
Promizole	12	5	0
	12	3	75
	12	2	92
	12	1	100
3,3'-dimethyl-4,4'-diaminodiphenyl sulfone	12	5	0
	12	2	75
	12	1	100
3-methyl-4,4'-diaminodiphenyl sulfone	12	5	0
	12	2	59
	12	1	81
	12	.5	100
Diamino diphenyl sulfone	12	5	0
	12	2.5	0
	12	1.5	46
	12	1.	83
	12	.5	92

In all cases above compounds were injected into yolk sac on the 10th day of incubation. Regardless of route of administration it was found that *comparative* toxicity remained the same.

* All figures are corrected for mortality of controls.

Quarter cc tuberculin syringes and ½ inch No. 27 needles have been found most suitable.‡

Collection of Blood Samples. Bleeding is accomplished by one of two methods. If it is desired to obtain more than one sample from an embryo one of the branches of the allantoic vein must be used. A citrated 1.5 cc syringe and a No. 25 needle is suitable. When withdrawing blood after entering the vein, very gentle intermittent traction on the syringe plunger is employed. When it is desirable to obtain as large a blood specimen as possible, it is best to use the large main trunk of the allantoic vein as it proceeds toward the embryo in a fold of membrane. An avascular area of the chorio-allantoic membrane is selected. With two pairs of fine pointed forceps the membrane is picked

‡ Results obtained by the use of this method for intravenous inoculation with suspensions of tubercle bacilli will be the subject of a separate report.

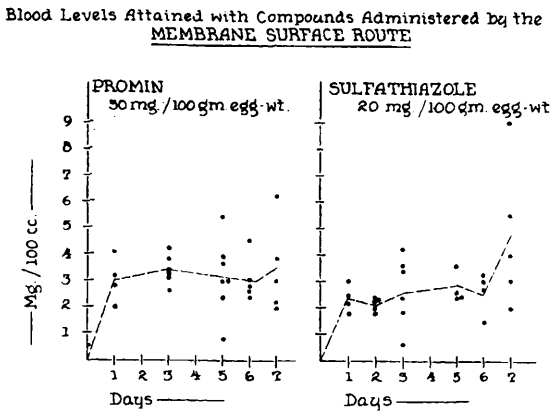

Fig. 1.

up and torn in this area. The allantoic vein is grasped with forceps and a No. 25 needle is inserted. The needle is advanced into the vein for approximately one-quarter inch. The forceps may then be removed and blood gently aspirated. The amount of blood obtainable from the embryos of various ages is shown in Table II.

Chick embryos are notably resistant to accidental infection, and it has been the experience of others that it is possible to work in an open room without any special precaution against air-borne contamination. The studies here recorded were carried out as aseptically as possible under a hood. Occasional contamination occurs under the best of conditions.

Advantages of the Technic. The principal advantages of the technic may be summarized as follows:

1. Opening and sealing of eggs is relatively rapid.

2. Adequate exposure is obtained for selective or combined intravenous, intra-yolk, intra-amniotic or intra-allantoic injection or aspiration.

3. All structures are clearly visible, thereby assuring accuracy of placement of injected materials.

4. The same egg may be used for later bleeding and/or sampling of the extra-embryonic fluids.

5. Embryo and membranes remain visible during incubation. Candling is unnecessary since death of the embryo is immediately apparent through the large transparent window.

6. The feasibility of selecting very small allantoic venous tributaries for intravenous injection minimizes bleeding.

Mortality from Procedure. When using embryos of 10 days incubation or more the mortality resulting from opening the shell and exposing the chorio-allantoic membrane is negligible. (Table III). Injection of sterile saline solution into the amniotic, allantoic, or yolk sac does not increase the mortality. (Table III, A).

Following intravenous injection of sterile saline solution the mortality rises sharply when using 10-day embryos. With the use of 11-day embryos the survival rate improves somewhat. (Table IV). Volume of injection, within limits, is more important than rate of injection. (Table V). In addition to the data given in these tables, a series of experiments involving controls receiving intravenous saline solution has demonstrated a uniformly higher survival rate when 11-day-old embryos are used as compared to 10-day-old embryos. This age factor is important principally in regard to intravenous work. For other types of work, survivals are excellent with embryos 5 days of age and up.

Quantitative Studies of Compounds Administered to Chick Embryos. Although the chick embryo is being used for pilot testing of compounds for possible chemotherapeutic value, there are few data available concerning the distribution of such compounds after their placement within the egg. It has been shown that certain sulfonamides are rapidly distributed through the egg but reports are chiefly qualitative.[2,3,4] Depending upon the route of inoculation and upon the conditions of a given experiment, information concerning levels attained in yolk, amniotic, and allantoic fluid may be of value. Experimental infection of the embryo itself may be produced by intravenous inoculation. Under such circumstances a knowledge of blood levels reached by chemotherapeutic compounds makes possible a more definitive interpretation of results.

Two compounds were chosen for quantitative studies. One, sulfathiazole (2-(p-amino-

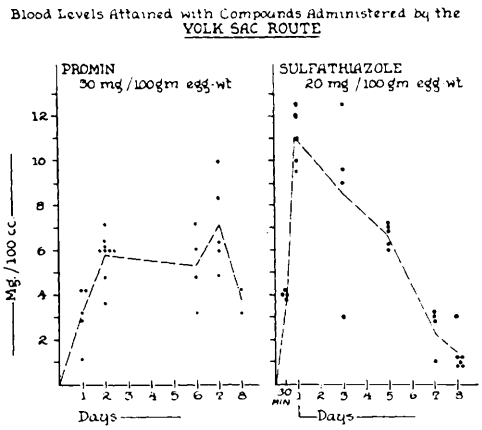

Fig. 2.

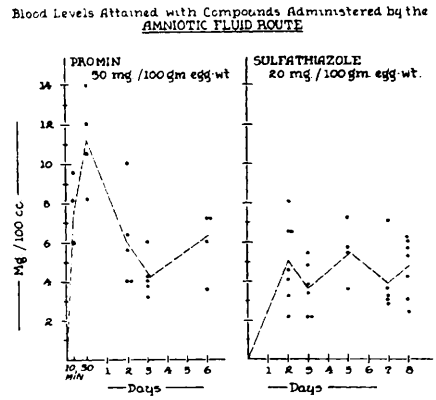

Fig. 3.

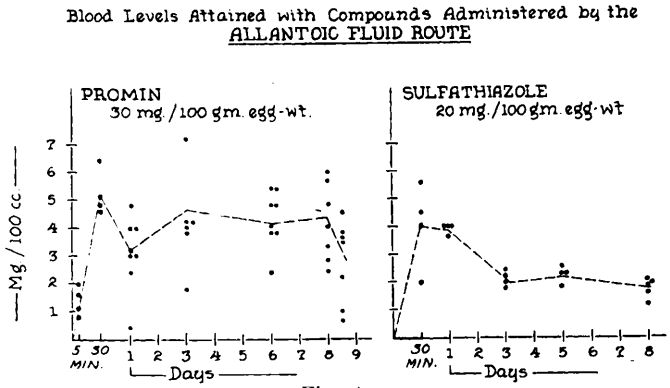

Fig. 4.

benzenesulphonamido)thiazole), is a relatively insoluble sulfonamide while the other, Promin, (sodium p-p′ diaminodiphenylsulfone N-N′ didextrose sulfonate), is a highly soluble sulfone. In addition a few streptomycin assays have been completed.

Methods. The technic described above was used throughout. The compounds were administered as a 5% solution in the case of Promin or as a 5% suspension of microcrystals in the case of sulfathiazole. Dosage was computed in terms of milligrams per 100 g of egg weight. Although the weight of the eggs used varied from 48 to 64 g, the dosage was computed on the basis of the uniform egg weight of 55 g. This introduces an error in dosage not exceeding 15%. All possible routes of administration were tested.

Toxicity studies were carried out first. Maximum tolerated doses by the yolk sac route were determined. The findings for promin, sulfathiazole, and 4 other compounds are given in Table VI. For these toxicity studies the compounds were injected into the yolk sac on the 10th day of incubation. Toxicity was determined in terms of embryo survival to the 20th day of incubation. The figures are corrected for the mortality of controls from the same group of eggs. The controls were handled exactly as the test embryos except that no material was injected. Eighteen embryos were used for each dosage group for the promin and sulfathiazole, while in the case of the other compounds 12 were used for each group.

On the basis of these toxicity studies dosages of the 2 compounds were selected for quantitative studies. The following graphs reveal the results obtained. All figures represent milligrams per 100 cc of fluid or blood. Although the emphasis was placed upon blood levels, a number of determinations in the

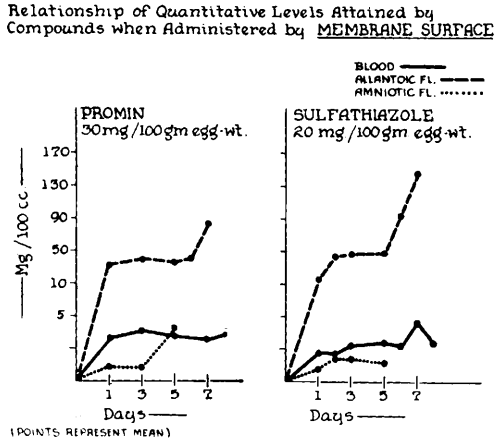

Fig. 5.

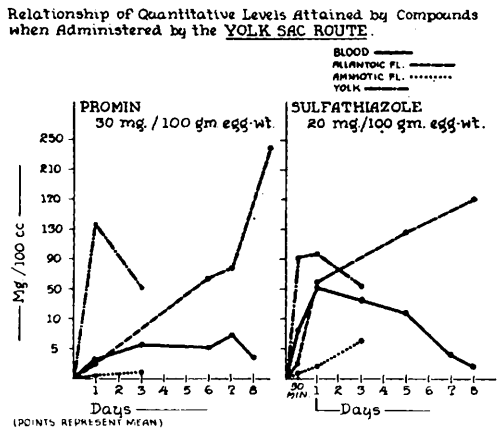

Fig. 6.

other fluids were done and suffice to give an indication of the distribution of the compounds. A total of 850 quantitative determinations were made. Studies with low doses and with toxic doses are omitted from the tables. Determinations were made by the Proom modification of the Marshall method.[13]

In all cases the figures represent single specimens from individual embryos and not serial specimens from the same embryo. It is possible, however, when such data is needed, to obtain at least 3 blood specimens at intervals from an individual embryo.

The few streptomycin determinations that have been completed indicate that this substance is present in the blood in a concentration of 6 to 10 units per cc of blood 12 hours after the placement of 1000 units on the surface of the chorio-allantoic membrane. Higher levels over longer periods are maintained in the allantoic fluid.

Discussion. Despite its limitations, which must be clearly recognized, the chick embryo is a valuable laboratory aid for screening procedures in chemotherapeutic research. It presents both simple (membrane mesenchyme) and highly complex tissues for study. Because of its immunologic status it is adaptable to certain bacteriologic problems wherein tissues are needed which are, so far as has been ascertained, free from antibody and sensitization phenomena. Within the egg one may study infection in the presence of living parenchymatous organs with their enzyme systems and metabolic processes intact. Naturally occurring infection of any sort in the chick embryo is a rare occurrence. A large number of embryos may be maintained in scarcely more space than is required for tissue culture studies. It is believed that the technic here presented may serve to facilitate such investigations as may make use of the chick embryo.

Summary. A chick embryo technic is described which possesses certain advantages over reported methods. The technic is applicable to many types of investigation. Quantitative data are presented on the distribution of sulfathiazole and promin within the fertile egg.§

[13] Proom, H., *Lancet*, 1938, **1**, 260.

§ This work was carried out in laboratory space provided by The Henry Phipps Institute of the University of Pennsylvania.