A Radioisotope Technic for Determining Allantoic Fluid Volume of Embryonated Eggs.* (23412)

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(Introduced by Edward S. West)
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Romanoff *et al.*(1,2) have published curves showing that the volume of allantoic fluid in a fertile chicken egg increases through the thirteenth day of incubation, then decreases through the remainder of embryonic development. Calculation of the amounts of waste products excreted by chick embryos requires in addition to the concentration figures, a knowledge of the allantoic fluid volume of fertile eggs at progressive stages of incubation. Embryonated eggs have been used extensively in this laboratory in studying experimental porphyria(3,4,5). Early in the course of these studies it was observed that porphyric eggs appeared to contain somewhat larger volumes of allantoic fluid than their controls several days after injection of the porphyria-producing drug. Thus, in order to evaluate the porphyrin outputs of porphyric chick embryos with a reasonable degree of accuracy it was necessary to determine volume of allantoic fluid present in eggs treated in this manner. Furthermore, it seemed advisable to determine in the same manner the allantoic fluid volume of normal embryos subjected to the conditions used in these experiments. For this purpose, a dilution technic, utilizing radioiodinated human serum albumin (RISA)® has been developed, and the results using this technic are reported here.

Materials and method. 1. *Radioassay*: Both gas-flow and well-type scintillation detectors have been used in this work. Instruments used were the windowless pre-flush flow counter (Mark 12 Model 3), manufactured by Radiation Counter Laboratories (well size 11/32 x 2″) attached to a Nuclear Superscaler, Model 172, and the Nuclear Model DS-3 scintillation counter attached to a Berkeley Model 2020 scaler. 2. *Eggs*: Pure strain White Leghorn eggs, obtained from a local hatchery after a week's incubation, were maintained in a forced-draft incubator at 37.5°C and 55-60% relative humidity. In this laboratory, it was found convenient to designate incubation age as follows: 24-48 hours incubation, one day old; 48-72 hours incubation, 2 days old, etc. Under this system, the egg is designated as up to one day younger than in Romanoff's studies(1,2). 3. *Experimental Porphyria*: Hepatic porphyria was induced by injecting 10.0 mg of allylisopropylacetylcarbamide (Sedormid)®(3) or 5.0 mg of allylisopropylacetamide (AIA)(5) in a volume of 0.5 ml into the yolk sacs of 8-day embryonated eggs. Control eggs received 0.5 ml of the drug vehicle at the same time. 4. *RISA*: For injection, RISA was diluted with 0.9% NaCl to give an activity of approximately 0.1 μc/ml. A volume of 0.10 ml of this diluted solution was injected into the allantoic sac as described below. The amount of radioactivity injected was determined by preparing 4 standard samples which were counted each day at the same time as the allantoic fluid samples. For the gas-flow counter, counting standards were prepared by further diluting the injected solution 1:5 with physiological saline, plating 0.10 ml of the second dilution in the center of a 2″ aluminum planchet and drying under an infra-red lamp. Counting standards for the scintillation detector were prepared by diluting 0.10 ml of the solution prepared for injection with 1.90 ml of 0.9% saline in ½ x 5 inch test tubes. The tubes were stoppered with Parafilm-wrapped corks and stored in the refrigerator until used. 5. *Experimental procedure*: Determinations were begun with 8-day embryos. Each egg was candled, its air sac outlined with

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pencil, and the position of the embryo marked. RISA was injected into the allantoic sac through a hole drilled in the shell just above the margin of the air sac and above the embryo. Injection was made from a Krogh pipette set at 0.10 ml bearing a ½″ 25 gauge hypodermic needle, the needle being inserted straight down through the hole in the shell. The eggs were then returned to the incubator during the time allowed for mixing. In some experiments, samples were withdrawn after ½ hour and again after 1 hour in the incubator. In others, samples were withdrawn after 1 hour in the incubator and again after 3 hours in the refrigerator (4 hours after RISA). Samples were withdrawn through a different hole in the top of the egg (the hole used for injecting via the yolk sac was usually used) with a 20 gauge, 1″ or 1¼″ needle, perforated in the terminal centimeter as described by Green and Freymann(6), and attached to a 1.0 ml tuberculin syringe. For the gas-flow counter, 0.20 ml of the fluid was plated in the center of a 2″ aluminum planchet, and dried under an infra-red lamp. Samples were prepared for the scintillation counter by transferring 0.20 ml of allantoic fluid to a ½″ x 5″ test tube and diluting with 1.80 ml of 0.9% NaCl. If the activity of such samples was too low, a 0.50 ml aliquot of allantoic fluid diluted with 1.50 ml of 0.9% NaCl was used. Measurement of these aliquots of allantoic fluid with the tuberculin syringes used to withdraw them proved accurate and convenient. On several occasions, 0.20 ml samples of yolk, amniotic fluid, and embryo homogenate (single embryos homogenized in water, then brought to 10 ml) were plated and counted to check for possible loss of RISA from the allantoic sac during mixing. To check the effect of allantoic fluid on the counting rate of RISA, an aliquot of the RISA solution injected was diluted 1:5 with allantoic fluid from normal 14 day embryos; then four 0.10 ml samples of this solution were plated, dried, and counted in the same manner as the counting standards diluted with saline. 6. *Calculations*: The number of counts injected was calculated from the average number of counts above background (C/M) of the 4 counting standards prepared as outlined above.

With gas-flow counter: C/M injected = Average C/M of standards × 5

With scintillation counter: C/M injected = Average C/M of standards

With both types of detector, using 0.20 ml samples of allantoic fluid for radioassay:

$$\text{Allantoic fluid volume} = \frac{\text{C/M injected}}{\text{C/M/ml}} = \text{ml.}$$

Results. 1. *Radioactivity in the embryo and embryonic fluids.* Amniotic fluid, yolk, and an homogenate of the embryo were checked for radioactivity 1 hour after the injection of RISA via the allantoic sac on 4 different days. No count exceeded background, thus excluding a loss of RISA from the allantoic sac. 2. *Effect of allantoic fluid on the counting rate of RISA*: Diluting the RISA used for injection with allantoic fluid rather than saline failed to alter the counting rate. (Sample diluted with saline = 3990 C/M; sample diluted with allantoic fluid = 4010 C/M.) 3. *Time required for adequate mixing*: The volumes calculated after allowing only ½ hour for mixing differed from those calculated after 1 hour's mixing by an average of 0.82 ml, while the volumes calculated after 4 hours' mixing varied from the 1 hour figure by an average of only 0.24 ml. Thus, it appears that ½ hour was insufficient for thorough mixing of RISA with allantoic fluid. 4. *Allantoic fluid volume of normal and porphyric chick embryos*: These data are summarized in Table I. To arrive at these figures the average of determinations carried out on the same egg after 1 and 4 hours' mixing was used as the allantoic fluid volume of that egg. Where 2 values were not obtained, 1 hour was allowed for mixing. Volumes calculated after only ½ hour's mixing were ignored. In both normal and porphyric embryos, allantoic fluid volume increased progressively up to 12 days of age, then steadily decreased. Four days after injection of the porphyria-producing drug (12-day embryos), porphyric embryos displayed a considerably larger allantoic fluid volume than the controls and this relationship persisted throughout the period studied. The large standard deviations emphasize the variability of this quantity.

TABLE I. Allantoic Fluid Volume of Normal and Porphyric Chick Embryos at Progressive Stages of Embryonic Development.

Incubation age (days)	Days after drug treatment	Avg vol (ml)*	
		Control	Porphyric
8	0	2.64 ± .43 (18)	—
9	1	4.25 ± 1.31 (17)	3.57 ± .96 (21)
10	2	5.56 ± 1.43 (18)	5.00 ± 1.02 (22)
11	3	5.78 ± 1.10 (16)	6.65 ± 1.57 (21)
12	4	7.01 ± 2.26 (16)	8.60 ± 2.40 (20)
13	5	6.99 ± 1.18 (15)	8.37 ± 1.77 (20)
14	6	6.45 ± 1.16 (14)	7.50 ± 1.68 (19)
15	7	5.66 ± 1.17 (17)	6.56 ± 1.64 (20)
16	8	4.62 ± 1.53 (17)	5.43 ± 1.42 (20)
17	9	3.72 ± 1.11 (12)	4.65 ± 1.73 (13)

* Figures in parentheses indicate No. of observations from which avg value was derived.

Discussion. When the average values are plotted, both sets of data give smooth curves (Fig. 1) except for the value for 11-day normal embryos. However, if this figure be ignored, the curve outlined by the other 9 points, closely resembles the allantoic fluid volume curve presented by Romanoff(2). As pointed out above, these volumes vary widely at a given time. In view of this great variability, it seems possible that the number of eggs used (16) to obtain this value was not large enough, statistically, to provide a really good estimate of the average. None of Romanoff's curves(1,2) exhibit a break at this point and his data were obtained from hundreds of eggs. Thus, it seems reasonable to read the allantoic fluid volume (6.45 ml) for this point from the curve outlined by the other 9 points.

In these studies, maximum allantoic fluid volume was found to occur at 12 days incubation age by the convention adopted in this laboratory, or 13 days by Romanoff's system. This coincides exactly with Romanoff's observations(1,2). The average maximum volume of 7.01 ml for normal embryos reported here agrees well with the value of approximately 6.5 ml reported by Romanoff(2) especially when the 0.5 ml of physiological saline injected into the yolk sacs of the control embryos is taken into account.

Retarded growth of porphyric embryos has been reported previously(3). The observation of a considerably larger volume of allantoic fluid in porphyric eggs 4 days after injection of the porphyria-producing drug coincides with(7) or just precedes(3) the appearance of a statistically significant difference in the weights of porphyric and control embryos. Elimination of metabolites which they are unable to utilize for growth probably accounts for the increased volume of allantoic fluid found in porphyric eggs. Differences between allantoic fluid volumes of normal and porphyric embryos were not significant statistically, but this probably resulted from too few observations on a highly variable figure. Gross observations on a much larger number of eggs leave little doubt that the allantoic fluid volumes of porphyric eggs consistently deviate from normal in the manner indicated.

Summary. A method for determining the

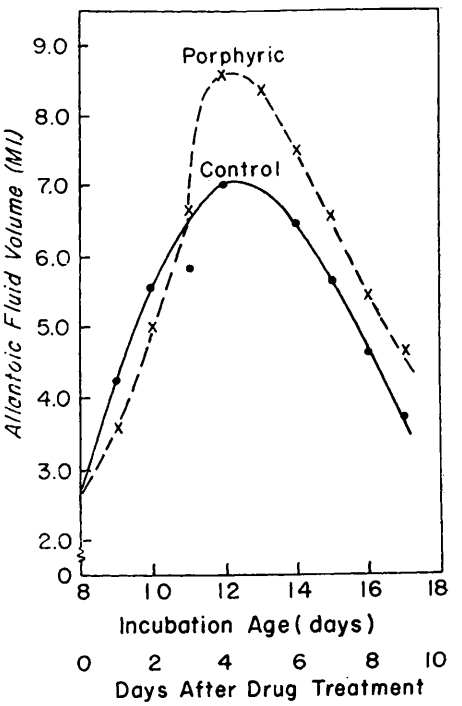

FIG. 1. Allantoic fluid vol of normal and porphyric chick embryos.

allantoic fluid volume of embryonated eggs by dilution of RISA is described. Experimental porphyria in chick embryos results in a marked increase in allantoic fluid volume.

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Studies on Propagation of the Col SK Group of Viruses in Various Tissue Culture Media.* (23413)

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Cultivation of Col SK virus *in vitro* on various cell substrates began with the early work of Jungeblut and Sanders(1,2). Explants of minced embryonic tissues, suspended under controlled oxygen-carbon dioxide tension in ox serum ultrafiltrate, were used for this purpose. The virus multiplied, to varying extent, on brain from mice and guinea pigs or on whole chick material; non-nervous embryonic tissue also supported a basic level of neurotropic virus growth. The virus yield was approximately the same from cells as from culture fluid. While the procedure did not permit demonstration of cytopathogenic effects, Huang(3) discovered that there was less glycolysis and acid production in the infected medium than in non-infected controls. The above results were confirmed by Shean and Schultz(4) who studied the mouse explant requirements of Col SK and MM virus in comparison with those of Theiler's GD VII and FA virus. Growth of MM virus in various extraneural tissues of mice or hamsters was later reported by Chambers, Smith and Evans(5,6), by Evans and Chambers(7) and by Fabiyi(8).

Only sporadic attempts have been made to grow viruses of the Col SK group in tissue cultures of embryonic or adult cells from man or monkey, utilizing modern methods of tissue cultivation. Col SK virus has been reported as growing on cynomolgus monkey testicular cell cultures(9) but the virus apparently does not multiply on cynomolgus monkey kidney (10); growth of MM virus has also been obtained in cultures of testicular cells from rhesus monkeys(11) or from man(6) and, in addition, on human fetal fibroblasts(12). Barski and Lamy(13) reported that Mengo virus multiplies abundantly in tissue cultures of kynocephalus monkey kidney cells, inducing characteristic cytopathogenic effects after 2 days and causing complete destruction of the culture within 4 days; the virus also grew on embryonic human kidney tissue.

Little is known about growth of viruses of the Col SK group on tumor cells. Among malignant mouse cell cultures, L (Earl) cells are said to support non-cytopathogenic growth of EMC virus(14), but Col SK virus failed to multiply on 2 transplantable mouse carcinoma or sarcoma cells studied by Kret (9). On the other hand, mice bearing the Ehrlich ascites tumor are apparently more susceptible to infection with Mengo virus than are normal mice(15); growth of EMC virus in Ehrlich ascites tumor transplants, with marked oncolysis, was reported by Levy and Snellbaker(16). Quite recently, multiplication, with cytopathogenic effects, was described for EMC virus by Eagle *et al.*(17) in KB cell tissue cultures, a human epider-

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