# Relation of Oxygen and Yolk to Retinal Pigmentation in Chick Embryos Subjected to Yolk-Sac Perfusion.* (30233)

C. R. GRAU, B. W. WILSON AND G. C. MATTESON
*Department of Poultry Husbandry, University of California, Davis*

A method of perfusing the yolk sac of the chick embryo with nutrient solutions has been used to study nutritional factors influencing embryonic growth and development(1). Survival of embryos was extended and development was enhanced when egg white was added to a yolk-sac perfusion fluid containing salts and glucose(2). In experiments in which the amount of yolk remaining in the yolk sac was minimized, the eyes of some embryos were almost devoid of pigment. Survival time was shortened, with many embryos dying at 6 days of incubation. The experiments reported here were designed to find some of the causes of the developmental abnormalities.

*Methods.* The first experiment was carried out by methods similar to those already reported(2) except that great care was taken to reduce residual yolk to a minimum. After 3 days of incubation, embryos were prepared for yolk-sac perfusion by forming a canal of coagulated egg white extending from outside the shell to the interior of the yolk sac. Then most of the yolk was replaced by a solution of salts, glucose, and antibiotics (salts 26)(2), and the eggs were returned to the incubator until the next day, when the small amount of yolk remaining was flushed out of the yolk sac. Following this treatment, the eggs were attached to the perfusion apparatus and every 8 hours, 30 ml of salts 26 were perfused through the yolk sacs during a 20-minute feeding period. Comparisons were then begun (at 5 days of incubation) among embryos receiving media containing 80 ml white per l, 20 ml yolk per l, or certain other nutrients. In some later experiments, such intermittent perfusion was begun at 4 days.

In most of the experiments reported here, a peristaltic pump was attached to the apparatus and the medium was continuously perfused through the yolk sac at a rate of 90 to 100 ml per day. Continuous perfusion was begun either when the embryos were first prepared (after 3 days of incubation) or a day later.

Oxygen tensions were measured by means of a Clark polarographic electrode (Yellow Springs Instrument Co.) attached by an adapter circuit(3) to a Beckman Model G pH meter.

Observations were made every few hours, and dead embryos were preserved in 10% formalin for wet-weight measurement and for gross and histological examination.

*Results.* The first experiments, in which embryos were perfused intermittently with nutrient solutions, yielded ambiguous results. Some embryos receiving solutions containing egg white had eyes almost devoid of pigment (Fig. 1B); however, embryos receiving yolk-containing solutions or media containing amino acids also had abnormal retinal pigmentation (Fig. 1C). Many embryos died at 6 days of incubation; these and older embryos often exhibited hematomas, pericardial edema, connective tissue weakness, and mouth deformity, in addition to a marked reduction of the retinal epithelium pigment (Fig. 2B), folding of the pigment layer (Fig. 2C), and a degeneration of the neural retina (Fig. 2C). Allantois development was retarded. Under these conditions no beneficial effects were obtained by varying such components of the medium as copper, iron, manganese, magnesium, sulfate, reducing compounds, antibiotics, pH, and white and yolk fractions.

Because it was believed that the pressures associated with intermittent perfusion might have adversely affected development, a peristaltic pump was used to deliver, at a uniform rate, 90 to 100 ml of medium per day. The medium was composed of salts 26 or of salts 26 plus 20 ml of yolk per liter. Contrary to

* This research was aided by Grant GB 1487 of National Science Foundation. The skilled technical assistance of K. Cherry and M. Farris is gratefully acknowledged.

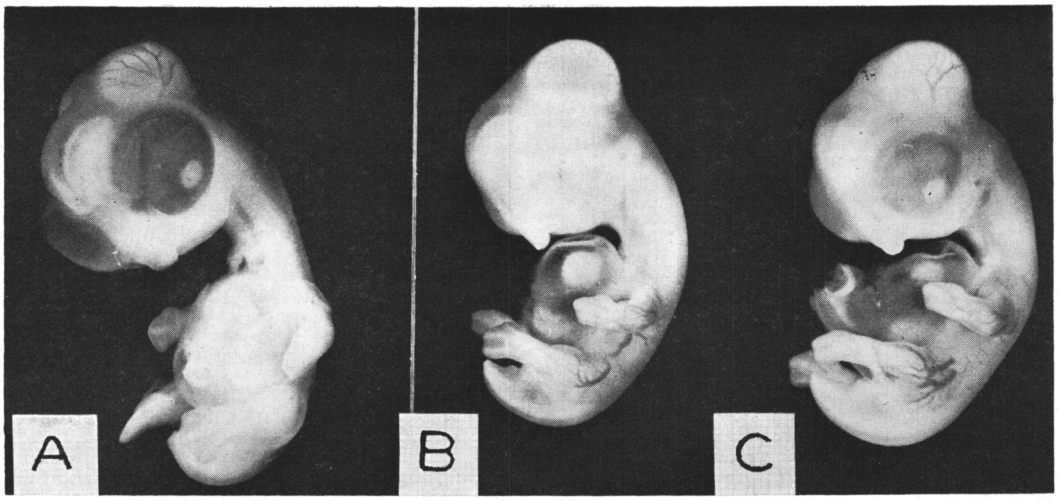


FIG. 1. Appearance of chick embryos after 7 days of incubation. A, control (not perfused); B, perfused with 40 ml/l egg white in salts-glucose solution; C, perfused with 20 ml/l yolk. Pigmentation of the eyes of embryo B was virtually nil. Eyes of embryo C were paler than the control. Both B and C exhibited pericardial edema.

expectation, continuous feeding appeared to be much more damaging than intermittent perfusion. Survival of the embryos was reduced, and all eyes were abnormally pale.

The possibility that death resulted from insufficient oxygen being transported by the blood vessels of the yolk sac to the embryo was studied by placing the egg and part of the perfusion apparatus inside a small, airtight chamber into which oxygen was introduced. The oxygen content of the atmosphere was maintained at about 40%, and was monitored by using the polarographic "oxygen" electrode. The medium consisted of salts 26 or salts 26 plus 10 ml of fresh yolk per liter. Continuous perfusion was begun when the embryos were first prepared (at 3 days) or a day later.

When perfusion was begun at 3 days, 16 of the 19 experimental embryos survived to 5 days of age. When the perfusion was begun at 4 days, 16 of the 17 perfused embryos

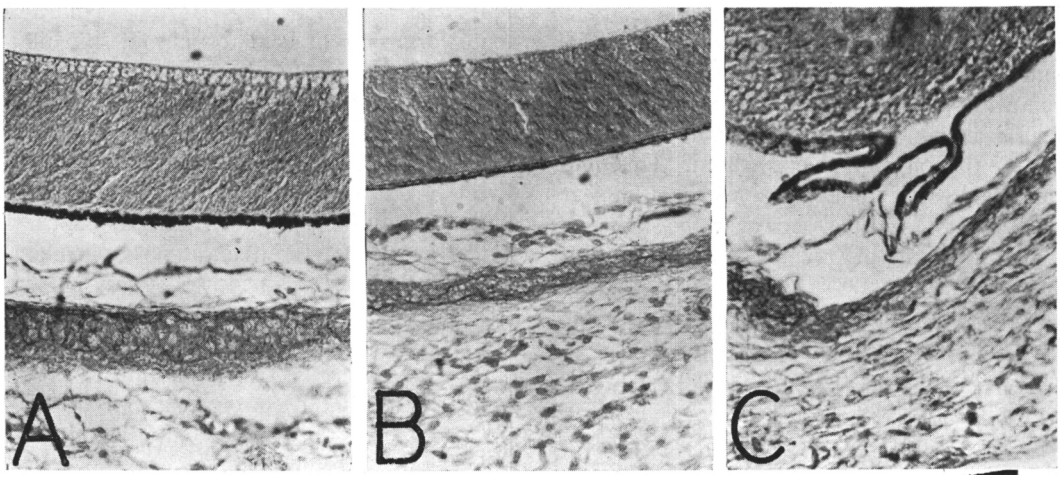


FIG. 2. Sections of the retinas of normal (A) and pale (B) eyes. (C) shows reduced pigmentation and folding of the retinal epithelium. The lumen of the eye is at top of picture. Stained for melanin by the ferrous iron technique; counterstained with Alcian Blue. These sections are not from the embryos of Fig. 1.

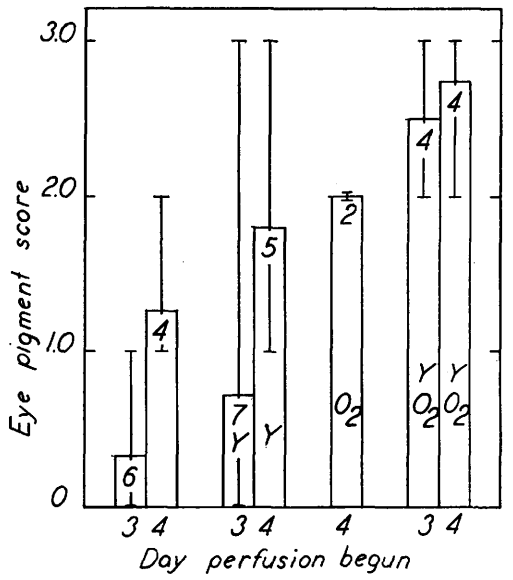


FIG. 3. Effect on eye pigmentation of yolk in the perfusion medium and of incubation in 40% oxygen. Perfusion was begun on incubation day noted at the bottom. Total numbers of embryos are noted at top of each bar. 0 = no pigment observable by gross observation, 3 = normal pigmentation by visual observation, Y = yolk present (10 ml/l), $O_2$ = atmosphere containing 40% $O_2$, 60% $N_2$. Ranges of pigmentation are shown by vertical lines.

survived to 6 days of age. The results presented in Fig. 3 show clearly that when embryos did not receive yolk and additional oxygen, very little pigment was present in the eye, in contrast to embryos given yolk and kept in an oxygen-rich atmosphere. Some embryos that had been subjected to perfusion and had received no additional oxygen in the atmosphere exhibited degeneration of the neural retina, and some also exhibited a folding of the pigmented epithelium. This folding resulted in a splotchy external appearance. Most marked, however, was the reduced amount of pigment present in the retinal epithelium.

*Discussion.* The experiments reported here indicate that embryos undergoing yolk-sac perfusion suffer from an oxygen deficiency which can be overcome by incubating them in an elevated oxygen atmosphere. The data also show that the development of the neural and the pigment layers of the eye of the chick embryo are particularly sensitive to lack of oxygen and that an adequate supply of oxygen plus some factor or factors present in the yolk are needed to obtain normal development of these structures. One of the factors required for pigment formation is tyrosine(4).

The time at which these embryos were first exposed to a lack of oxygen corresponds to the onset of melanin formation. Melanin is first detected in the epithelial layer of the chick retina at stage 20 of Hamburger and Hamilton(5), that is, after 3½ days of incubation(6,7). Tyrosinase activity in the pigment layer continues to increase until the 10th day of incubation, then falls sharply to zero by the 13th day(8). In the studies on the development of the eye in culture(9,10, 11,12,13) the roles of environmental factors have not been clearly shown, but Harrison and Berry(14) observed some differences in pigmentation in relation to glucose and some other factors.

Previous studies of the effects of oxygen deficiency on chick embryos have shown the importance of oxygen for normal development. Taylor and coworkers observed both immediate and delayed effects on survival when eggs were subjected to reduced oxygen atmospheres during the first 4 days of incubation(15), or during the fifth through the eighth days(16) and then returned to normal atmospheric conditions. Grabowski found that after a 6-hour exposure to reduced oxygen concentration, 4-day embryos exhibited acute formation of hematomas and skin blister, and chronic effects on viscera, on eye and brain, and on general rate of development(17).

The results obtained here indicate that a direct link exists between oxygen supply and melanin formation. That this link was not observed in earlier studies of hypoxia in chick embryos may be explained by the very narrow range of oxygen levels combined with the precise timing required to obtain the effect. The time of onset of the conditions must correspond to the beginning of pigmentation if melanin formation is to be prevented. Hypoxia and yolk deprivation must be severe enough to halt melanin formation yet not so severe that the embryo dies before exhibiting the effect. Pigmentation that has been halted

may, under some conditions, begin again after restoring the absent factors(18).

The effect of oxygen supply on retinal pigmentation is easily seen and may permit further study of the several factors which are known to be involved in melanin formation.

*Summary.* Constant perfusion of a salts-glucose solution through the yolk sacs of 3- or 4-day chick embryos resulted in poor survival and abnormally pale eyes. Incubation in a 40% oxygen atmosphere in the presence of a yolk-supplemented medium permitted virtually normal pigmentation, and greatly enhanced survival. It is concluded that both yolk and oxygen must be present for normal development of retinal pigmentation.

1. Grau, C. R., Fritz, H. I., Walker, N. E., Klein, N. W., J. Exp. Zool., 1962, v150, 185.

2. Grau, C. R., Walker, N. E., Fritz, H. I., Peters, S. M., Nature, 1963, v197, 257.

3. Bulletin 3019, Beckman Instruments, Inc., Fullerton, Calif.

4. Grau, C. R., Austic, R. E., Matteson, G. C., Science, 1965, in press.

5. Hamburger, V., Hamilton, H. L., J. Morph., 1951, v88, 49.

6. Smith, D. T., Bull. Johns Hopkins Hosp., 1920, v31, 239.

7. O'Rahilly, R., Meyer, O. B., Acta Anat., 1959, v36, 20.

8. Miyamoto, M., Fitzpatrick, T. B., Science, 1957, v126, 449.

9. Strangeways, T. S. P., Fell, H. B., Proc. Roy. Soc., Ser. B., 1926, v100, 273.

10. Dorris, F., J. Exp. Zool., 1938, v78, 385.

11. Harrison, J. R., ibid., 1951, v118, 209.

12. Lucas, D. R., Trowell, O. A., J. Embryol. Exp. Morph., 1958, v6, 178.

13. Sidman, R. L., Diseases of the Nervous System, Monograph Supplement, 1961, v22, 14.

14. Harrison, J. R., Berry, M. A., Physiol. Zool., 1959, v32, 115.

15. Taylor, L. W., Sjodin, R. A., Gunns, C. A., Poultry Sci., 1956, v35, 1206.

16. Taylor, L. W., Kreutziger, G. O., ibid., 1965, v44, 98.

17. Grabowski, C. T., J. Exp. Zool., 1964, v157, 307.

18. Austic, R. E., Grau, C. R., unpublished data.

Received March 19, 1965. P.S.E.B.M., 1965, v119.

## Spectrophotometric Identification of Eggshell Pigments and Timing of Superficial Pigment Deposition in the Japanese Quail. (30234)

H. K. POOLE (Introduced by M. W. Olsen)

*U. S. Department of Agriculture, Animal Husbandry Research Division, ARS, Beltsville, Md.*

Although there is general agreement that bile pigments and porphyrins (oöporphyrin) are the major pigments of birds' eggshells, the physiological processes by which they are produced and deposited are not clearly understood(1,2). A study of these processes has begun in Japanese quail using a recessive, mutant, white-egg strain whose eggshell contains very little pigment(3) and a dominant, wild-type, heavily pigmented egg strain. The necessary first steps were to identify the eggshell pigments of this particular species and to determine the time of deposition of superficial pigment on the wild-type eggshell surface.

*Materials and methods.* Our quail hens, housed in individual laying cages within a light-tight room, are exposed daily to 14 hours of fluorescent light (2 A.M.-4 P.M.). Under this regimen all but a few eggs are laid between 8 A.M. and 4 P.M., during which time an attendant maintains an hourly record of lay. Although the hour varies between individuals, many hens lay at the same hour each day in long uninterrupted sequences. For any of these with a hard-shelled egg in the uterus 18 hours following one oviposition, the time of oviposition of the uterine egg can be fairly accurately predicted.

The technique of Warren and Conrad(4) was adapted for preparation of eggshell solutions. Quail eggs were broken out, their contents and shell membranes discarded, and the shells allowed to dry. Subsequently, the