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.

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

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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

dried shell of 1 or more eggs was dissolved in a solvent composed of 4 parts methanol to 3 parts concentrated HCl. The concentration of the crude solutions was never less than 30 mg shell/ml, but no attempt was made to hold equal concentrations between different samples. All samples were clarified by centrifugation at 3440 × *g* for 45 minutes. The color of some wild-type solutions was so intense it was necessary to dilute them 9:1 with the methanol-HCl solvent in order to reduce their optical density to within the absorption scale of the spectrophotometer. The light absorption curves of solution aliquots in 1 cm thick silica glass cells were plotted between wavelength 360-800 mμ in a Beckman DU spectrophotometer. The methanol-HCl solvent was used as the control blank.

The procedures for extraction of quail uterine tissue were adapted from the methods of Polin(5). One gram of uterine tissue was homogenized in 15 ml of 3 N HCl and allowed to stand overnight in the dark at 4°C. Particulate matter was removed from these crude extracts by centrifugation at 3440 × *g* for 45 minutes. Subsequently, 1.5 ml aliquots of extract were diluted with 1.75 ml 3 N HCl and their light absorption curves were plotted by the same procedure used for eggshell solutions except that 3 N HCl was used as the control blank.

In addition, uterine extracts were prepared in methanol-HCl and eggshells were dissolved in 3 N HCl to determine the effect of the different solvents on the wavelength positions of optical density peaks.

Finally, 3 drops of quail bile were mixed in 5 ml methanol-HCl and the light absorption curve was plotted for the other solutions and extracts.

All solutions and extracts except those from bile were examined for fluorescence in ultraviolet light.

*I. Pigment identification.* Extracts or solutions were prepared of the following materials. *Hens laying wild-type eggs:* (A) Eggshell before superficial pigment deposition, pronounced green inner shell color; (B) eggshell before superficial pigment deposition, tan inner shell color; (C) superficial pigment alone, scraped from shell of uterine egg approximately 1 hour prior to oviposition; (D) uterine tissue. *Mutant hens laying white eggs:* (E) Eggshell of newly laid eggs; (F) uterine tissue. The foregoing materials, except for (C) and (E) were taken from hens decapitated 4 hours prior to oviposition.

*II. Timing of deposition.* At hourly intervals, beginning 6 hours prior to oviposition, hens were autopsied and the presence or absence of superficial pigment on the eggshell surface was recorded. Simultaneously, a 1 g sample of uterine tissue was taken from each of 3-5 hens (except at the fifth hour) and extracted for pigment. The 410 mμ optical density of each extract was recorded.

*Results. I. Pigment identification.* Typical absorption curves are illustrated in Fig. 1 and the wavelength position of absorption peaks for each of the types of extracts and solutions are shown in Table I.

*Wild-type solutions and extracts.* Solutions of normally pigmented eggshell without

TABLE I. Absorption Maxima of Eggshell Solutions and Uterine Tissue Extracts from Mutant White-Egg and Wild-Type Japanese Quail.

| Origin | Position of peaks—mμ | | | |
|---|---|---|---|---|
| | 380 | (410) 415 | 560 | 680 |
| Wild-type hens | | | | |
| A. Shell without superficial pigment—green inner color | + | + | + | + |
| B. *Idem* tan " " | — | + | + | + |
| C. Superficial pigment alone | — | + | + | — |
| D. Uterine tissue—4 hr before oviposition | — | (+) | + | — |
| Mutant hens | | | | |
| E. Shell of newly laid eggs | — | + | +* | — |
| F. Uterine tissue—4 hr before oviposition | — | (+) | +* | — |

* Concentration of absorbing substances apparently were so low these peaks were barely detectable.

superficial pigment and green at the inner shell surface (A) had peaks at 380, 415, 560, and 680 mμ. Solutions for shells with tan inner shell color (B) had peaks at 415, 560, and 680 mμ. The 380 mμ peak was not detected in the latter type solutions and the peak at 680 mμ was not as pronounced as in the former type solution (Fig. 1). Only the 415 and 560 mμ peaks were detected in solutions of superficial pigment alone (C). Extracts of uterine tissue had peaks at 410 and 560 mμ (D).

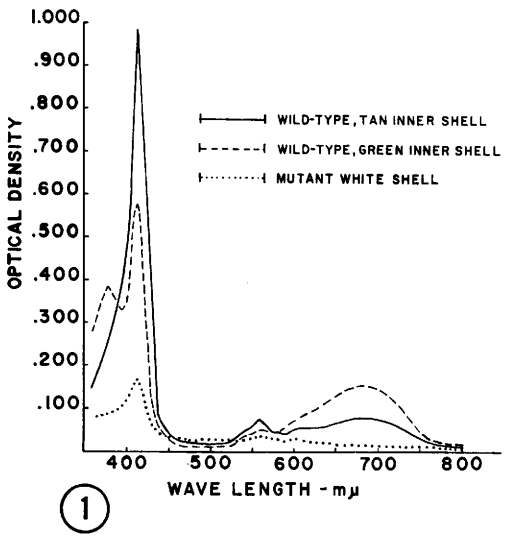
*Mutant white solutions and extracts.* Solutions of white eggshells (E) had low peaks only at 415 and 560 mμ and extracts of uterine tissue had low peaks only at 410 and 560 mμ (F). The 560 mμ peak was barely detectable in mutant samples (Fig. 1).

All of the foregoing types of solutions and extracts gave off red fluorescence when exposed to ultraviolet light.

Solutions of bile in 3 N HCl had absorption peaks at 377 and 680 mμ.

Dissolution of eggshell in 3 N HCl shifted the principal absorption peak from 415 to 410 mμ and extraction of uterine tissue in methanol-HCl shifted the same peak from 410 to 415 mμ. Other peaks did not show this shift in relation to the solvent used. In view of this, it seems safe to assume that the 410 and 415 mμ peaks represent the same light absorbing substance in both eggshell solutions and uterine tissue extracts.

*II. Timing of deposition.* No superficial pigment was observed on the shell of 24 eggs removed from the uterus 4-6 hours prior to oviposition (Table II). Likewise, 12 of 17 eggs removed 3 hours prior to oviposition had no indication of superficial pigment, although the remaining 5 did show a very slight punctate darkening in the shell pores. These



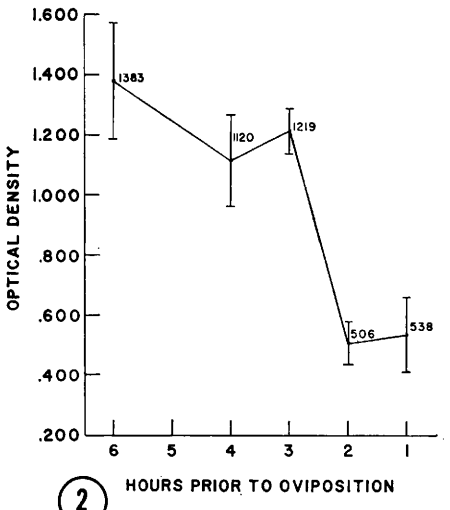




FIG. 1. Typical absorption curves of solutions of Japanese quail eggshells in a mixture of 4 parts methanol to 3 parts conc. HCl. Wild-type eggs removed from uterus before superficial pigment deposition. Conc. = 100 mg shell/ml. Mutant white eggs were newly laid and had some superficial pigment stippling. Conc. = 85 mg shell/ml.

FIG. 2. Relationship between mean optical density at 410 mμ of 3 N HCl extracts of uterine tissue and time of oviposition in Japanese quail. Vertical bars indicate ± 1 standard error of the means.

TABLE II. Relationship Between Time of Superficial Eggshell Pigment Deposition and Time of Oviposition in Japanese Quail.

| Hr prior to oviposition | No. of hens autopsied | No. of eggs with superficial pigment | No. of eggs without superficial pigment |
|---|---|---|---|
| 6 | 3 | 0 | 3 |
| 5 | 8 | 0 | 8 |
| 4 | 13 | 0 | 13 |
| 3 | 17 | 5* | 12 |
| 2 | 13 | 13 | 0 |
| 1 | 5 | 5 | 0 |

* Amount of pigment slight, indicating deposition had just begun.

darker points were assumed to be very early stages of superficial pigment deposition. By 2 hours prior to oviposition 13 of 13 eggs were superficially pigmented, 12 with heavy depositions and 1 with moderately heavy deposition. Similarly, 5 of 5 eggs removed 1 hour prior to oviposition were heavily pigmented.

The results of optical density measurements of uterine tissue extracts are in good agreement with the foregoing. The high mean optical density of extracts prepared 3-6 hours prior to oviposition is seen in Fig. 2. However, between the 3rd and 2nd hours there was an abrupt decrease so that the mean optical density of extracts at 2 hours prior to oviposition was only 41.5% of that at 3 hours. The time of this decrease coincides closely with the time of superficial pigment deposition (Table II).

Free pigment masses were not observed in the uterine lumen of any hen at any time. Three to 6 hours prior to oviposition the uterine wall was a very dark brown color and opaque; but by 2 or less hours prior to oviposition the wall had become so transparent that the superficially pigmented egg could be seen very clearly.

*Discussion.* The spectrophotometric data are interpreted as good evidence that both biliverdin (oöcyan) and oöporphyrin are responsible for pigmentation of the wild-type Japanese quail eggshell. In contrast, the slight pigmentation of mutant white eggshells appears to be due solely to small amounts of oöporphyrin—no evidence of biliverdin having been observed. Thus the phenotypic difference between the wild-type and mutant strains seems based on (i) a qualitative lack of biliverdin in white eggshells; and (ii) a quantitative difference in amount of oöporphyrin in and on the eggshell of both strains.

The presence of porphyrins in uterine tissue, the eggshell, and superficial pigment is indicated by red fluorescence of their extracts and solutions under ultraviolet light(2). More specifically, their observed absorption peaks at 410 and 415 and 560 mμ correspond with the 411 and 557.2 mμ absorption maxima of protoporphyrin in 25% HCl(2) and the 410 and 560 mμ absorption maxima for chicken oöporphyrin in 3 N HCl(5). The simultaneous appearance of superficial pigment on the wild-type eggshell and abrupt reduction in 410 mμ optical density in uterine extracts is further confirmation that the superficial pigment is composed mainly, if not entirely, of oöporphyrin.

The presence of biliverdin in solutions of wild-type eggshell with green inner surface color is clearly indicated by the absorption peaks at 380 and 680 mμ. These peaks correspond well with the 377 and 680 mμ absorption maxima of biliverdin in a solution of 5% HCl in methanol(2), and with the present solution of quail bile in methanol-HCl. Significance of the latter analogy is based on the fact that biliverdin is the principal pigment of avian bile(2).

Accordingly, one may assume the presence of biliverdin in solutions of wild-type eggshell with tan inner shell color even though a 380 mμ peak could not be detected. The increased optical density around 680 mμ is a strong indication that biliverdin was, in fact, present. It may be that in this type eggshell the biliverdin content is so low in relation to a high oöporphyrin content that its peak at 380 mμ is obscured by the nearby ooporphyrin peak at 410 mμ.

Woodard and Mather(6) have recently reported that superficial pigment is deposited on the Japanese quail egg about 21.5 hours after the preceding oviposition, and from this have estimated the event to occur "some 3.5 hours before lay." The fact that the present observations were based on quail regularly laying at close to 24-hour intervals may account for the somewhat closer time relationship between deposition of superficial pigment and oviposition.

The mechanism which controls timing of superficial eggshell pigment deposition in the Japanese quail (and in other birds as well) remains obscure. The fact that pigment deposition and oviposition are closely correlated in time does not justify assuming a cause and effect relationship between the two events. Since the superficial pigment is easily scrubbed from the eggshell surface(7), and since shell calcification is nearly complete at the time of pigment deposition(6), one may

consider the likelihood that the onset of superficial pigment deposition and cessation of calcium secretion are functionally related.

*Summary.* The eggshell pigments of wild-type and mutant white egg Japanese quail have been identified by spectrophotometric analysis of eggshell solutions, solutions of wild-type superficial pigment, and extracts of uterine tissue. Oöporphyrin and biliverdin both contribute to wild-type eggshell pigmentation but oöporphyrin alone seems responsible for the slight pigmentation of mutant white eggshells. In wild-type quail, deposition of superficial eggshell pigment begins between the second and third hours prior to oviposition and is accompanied by an abrupt reduction of oöporphyrin content in uterine tissue extracts.

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## Interferon Inhibition of Cytoplasmic DNA Accumulation in Vaccinia Virus Infection. A Radioautographic Study. (30235)

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While the action of the antiviral protein interferon has been extensively investigated in RNA virus infected cells, few studies on DNA virus infected cells have been published. The growth of a few DNA viruses has been found to be inhibited by interferon but no studies are available on whether virus DNA formation is also inhibited(1). Such studies would contribute to the elucidation of the mechanism of action of interferon.

In the present investigation the effect of interferon on cytoplasmic DNA incorporation in vaccinia virus infected chick embryo fibroblasts was investigated by radioautography (2). Interferon treatment markedly decreased the appearance of cytoplasmic DNA under conditions where virus synthesis was also depressed.

*Materials and methods.* Chick embryo fibroblasts (CEF) were grown on cover slips set in very lightly seeded glass plates(3). Cells were treated overnight with 10 units of chick interferon diluted in medium or with only medium and then washed. The chick interferon had been prepared by infecting CEF with chikungunya virus for 48 hours. The supernatant fluids were heat inactivated (65°C, 30 minutes) and titrated for interferon content by the method of Lindenmann and Gifford(4). The cells were then infected at a multiplicity of 5 plaque forming units per cell of a chick chorioallantoic membrane grown strain of vaccinia virus. After 2 hours for virus adsorption plates were again washed; 5 hours after infection each plate was pulsed for 1 hour with 3 $\mu$c of tritiated thymidine (3 c/mM, Radiochemical Centre, Amersham). The plates were then washed 3 times with phosphate buffered saline, the coverslips removed and the cells were fixed in acetone for 15 minutes at room temperature. The coverslips were then dried for 2 days in a vacuum desiccator and mounted on subbed slides. The slides were dipped in Ilford L-4 emulsion, diluted 1:2 in 1% gelatin, dried,

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