culture medium or ascites fluids appear to be identical. In addition, they are antigenically related to permeability factors produced by guinea pig line 1 or 104 C1 tumor cells. Secretion of permeability-increasing activity appears to be a common feature of tumor cells, and may contribute to the abnormal accumulation of fluid associated with neoplastic disease.

Note added in proof: The immune IgG raised against line 10 permeability factor also neutralizes the rat dermal vessel permeability-increasing activity released by Walker rat carcinoma cells in culture. Preimmune IgG has no neutralizing effect.

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2. Clearance of labeled HSA from the peritoneal cavity was not impaired in tumor-bearing animals at any interval. For example, 7 days after intraperitoneal injection of 3×10^7 line 10 tumor cells, 58.4 ± 4.2 percent (mean ± standard error) of the HSA (90 to 95 percent precipitable with trichloroacetic acid) remained in the peritoneal cavities after 2 hours; 56 ± 1.15 percent remained in the controls.
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9. Activities from both ascites fluid and serum-free culture medium bound completely to both heparin-Sepharose and hydroxylapatite. Columns were eluted with linear gradients; ascites and culture medium activities coeluted from heparin-Sepharose as a peak centered at 0.40*M* NaCl–0.01*M* PO_4 (*p*H 7.0) and from hydroxylapatite at 0.25*M* sodium phosphate (*p*H 7.0). To avoid complicating our analysis of diluted ascites fluid fractions with unmasked PF/dil (*7*), we added soybean trypsin inhibitor (20 μg/ml) to all ascites fluid column fractions before assay.
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Yolk Pigments of the Mexican Leaf Frog

Abstract. *Eggs of the Mexican leaf frog contain blue and yellow pigments identified as biliverdin and lutein, respectively. Both pigments are bound to proteins that occur in crystalline form in the yolk platelet. The major blue pigment is biliverdin IX α. The eggs vary in color from brilliant blue to pale yellow-green depending on the amount of each pigment. These pigments may provide protective coloration to the eggs.*

While studying the lipid composition of the eggs and embryos of the Mexican leaf frog, *Agalychnis dacnicolor* (*1*), we observed that their green coloration was due to the presence of two pigments, one blue and one yellow, which together produce blue, blue-green, or yellow-green eggs. We have now identified the major blue pigment as biliverdin IX α and the major yellow pigment as lutein. The presence of the latter pigment is not surprising since lutein is widely distributed among plants and animals (*2*). Biliverdin occurs less often as a pigment, although it has been found in the dog placenta, in the shells of bird eggs, in the skin of fishes and amphibians (*2, 3*), in the eggs and larvae of the tobacco hornworm (*4*), and in the serum and eggs of *Xenopus* (*5*). It seems likely that the utilization of these two pigments by *A. dacnicolor* evolved as a mechanism for producing green eggs. The green coloration of leaf frog eggs, which are laid on green vegetation, may afford camouflage to protect eggs and embryos from predation. However, as far as we can ascertain, there have been no definitive studies on the adaptive value of green eggs, although the ecological implications deriving from a two-pigment system for egg coloration are apparent.

Six different batches of *A. dacnicolor* eggs (100 to 250 eggs) varying in color from brilliant blue to yellow-green were extracted with a 1:1 mixture of chloroform and methanol and a mixture of acetone and hydrochloric acid to obtain the yellow and blue pigments. The pigments were separated by column chromatography on silicic acid. Chloroform eluted the yellow pigment, and acetone eluted the blue pigment. The pigments were further purified by preparative thin-layer chromatography (TLC). The yellow and blue pigments were localized in lipid-rich yolk platelets.

Yolk platelets, which were pale blue-green or pale yellow, were obtained by collagenase treatment of homogenized eggs, followed by differential centrifugation. Analysis by light microscopy of the isolated fresh yolk platelets revealed rounded rectangular platelets of different sizes, and electron microscopy showed that the platelets consisted of closely stacked crystalline arrays about 70 Å thick.

The ultraviolet to visible spectra of the silicic acid column–purified pigments from different eggs are given in Fig. 1, a and b. The blue pigment has major bands at 372 to 376 nm and 640 to 690 nm. The yellow pigments have major bands at 442 to 444 nm and 470 to 471 nm. The relative amount of the yellow and blue pigments in the various eggs was determined by the ratio of the absorbance at 442 nm to that at 650 nm. This ratio was correlated with the color of the egg. The ratio of brilliant blue eggs was 1.15, that of blue eggs was 1.7 to 2.3, that of green eggs was 3.4 to 3.6, and that of yellow-green eggs was 10.4.

The blue pigment has properties consistent with a biliverdin. Both the blue pigment and biliverdin (Sigma) were converted to methyl esters by treatment with methanolic HCl (Supelco). The dimethyl esters were purified by preparative TLC (Merck-Darmstadt silica gel 60 plates) using chloroform and methanol 9:1. Both had identical relative mobility (R_f) values of 0.62, and gave a purple

color reaction on exposure to iodine vapors. The ^{1}H NMR (nuclear magnetic resonance) spectra of the dimethyl esters of the TLC-purified blue pigment and biliverdin were identical. The ^{1}H NMR spectra were run in $CDCl_3$ on a Bruker 400-MHz spectrometer. The chemical shifts (parts per million) and coupling constants (J) in hertz units are as follows: C-3 methyl, 2.09; C-7 methyl, 2.18; C-13 methyl, 2.07; C-18 methyl, 1.87; C-5 methine, 6.01; C-10 methine, 6.78; C-15 methine, 6.07; C-2 vinyl, 5.43, 6.12, and 6.52 (J = 1.8, 11.5, and 17.6); C-17 vinyl, 5.63, 5.66, and 6.62 (J = 2.0, 11.6, and 17.7): α-methylenes of propionic acid side chains on C-8 and C-12, 2.55; β-methylenes of propionic acid side chains on C-8 and C-12, 2.91 and 2.93 (J = 7.6); methoxy methyls, 3.68. The ^{1}H NMR spectra were identical to the NMR spectrum of biliverdin IX α reported by Lehner *et al.* (*6*), except for the coupling constants of the β-methylene protons. We found a J value of 7.6 Hz, whereas Lehner *et al.* (*6*) reported a J value of 3 Hz. Since our spectra were run on a 400-MHz unit, we obtained a better resolution of the bands for the β-methylene protons and were able to measure these coupling constants possibly more accurately than Lehner *et al.* (*6*), who used a 300-MHz unit. On the basis of the chemical, spectral, and chromatographic properties of the TLC-purified blue pigment, it is identified as biliverdin IX α. Very small amounts of other isomers of biliverdin were present in the yolk platelets.

The yellow pigment was compared to authentic lutein (Hoffmann–La Roche). Both had identical R_F values when chromatographed on silica gel 60 plates in a mixture of ethyl acetate and hexane 1:1 (R_F 0.46) and in a mixture of chloroform and methanol 9:1 (R_F 0.64). Both compounds gave a green color reaction when exposed to iodine vapors and were separated from zeaxanthin (Hoffmann-La Roche) and α- and β-carotenes (Sigma) by TLC in a mixture of ethyl acetate and hexane 1:1. The yellow pigment had bands in hexane at 442 to 444 nm and at 470 to 471 nm that are identical to those of lutein (*2, 7*). Partitioning between hexane and 95 percent methanol showed that most of the yellow pigment stays in the hypophasic methanol layer, but a small amount is epiphasic. After mild alkaline hydrolysis, the yellow pigment is primarily free lutein, but a small amount is esterified, possibly to fatty acid. On the basis of chemical, spectral, and chromatographic analyses, the major yellow pigment is identified as lutein. Other minor yellow pigments are present.

Green coloration is common among amphibians, reptiles, and birds where it is often the result of a blending of blue and yellow colors (*2, 8*). Blues are usually structural colors arising from light scatter, diffraction, and interference, whereas yellows arise from specific yellow pigments such as pteridines and carotenoids. The green color of the eggs from the leaf frog is also due to a mixture of blue and yellow elements. However, unlike the situation in frog skin, where blue is a structural color (*8*), the blue component of the egg is a true pigment, biliverdin. The presence of a biliverdin in the *Xenopus* egg has been reported by Redshaw *et al.* (*5*), but the specific type of biliverdin was not characterized. Our study demonstrates the presence of biliverdin IX α in eggs of *A. dacnicolor*. As with *Xenopus*, biliverdin in the eggs of *A. dacnicolor* is bound to protein. In the former it is associated with the yolk proteins lipovitellin and phosvitin. It is likely that the same holds for *A. dacnicolor*.

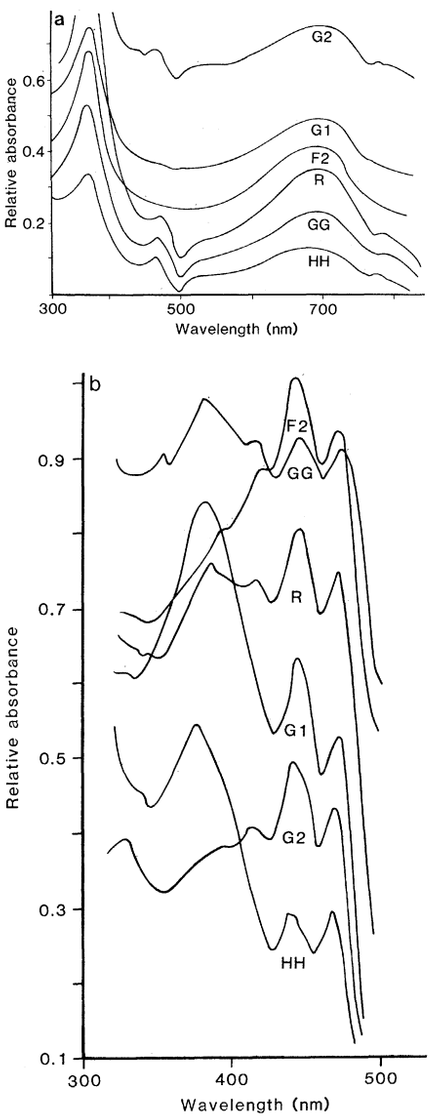

Fig. 1. Ultraviolet to visible spectra of (a) the blue pigments and (b) the yellow pigments from eggs of *A. dacnicolor*. The spectra were run in methanol on a Cary 219 spectrophotometer. The various batches of eggs have the following colors: H-H, brilliant blue; GG, blue-green; G-1, blue; R, blue; G-2, green; and F-2, green.

In both lepidopterans (*4*) and the Mexican leaf frog, a specific biliverdin isomer provides the blue component of the green color, while yellow is provided by lutein. The utilization of lutein by both species may occur because lutein is a principal carotenoid component of lepidoptera (*2, 9*), which are a major dietary item of leaf frogs. Frogs deprived of lutein source by being fed on crickets lay eggs that are blue. The development of these blue eggs is normal. The utilization of biliverdin isomers is very likely a convergent character resulting from there being relatively few blue pigments available to animals. Biliverdin is the most common of blue pigments and is readily derived from other tetrapyrroles (*2, 3*).

The presence of these egg pigments in yolk platelets reflects their relation to the process of vitellogenesis (*5, 10*). In *Xenopus*, the synthesis of both vitellogenin and biliverdin is under estrogenic control, as is their secretion into the blood. During subsequent transport to the ovary, biliverdin is bound to vitellogenin. Thus, *A. dacnicolor*, like *Xenopus*, appears to be suitable for studies of amphibian vitellogenesis (*11*). Like *Xenopus*, *A. dacnicolor* can be maintained easily and induced to breed in the laboratory out of season (*12*). Large *A. dacnicolor* females can produce more than 1000 eggs (2.5 m in diameter) per spawning.

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Ammonium Chloride Prevents Lytic Growth of Reovirus and Helps to Establish Persistent Infection in Mouse L Cells

Abstract. *Ammonium chloride, a lysosomotropic agent that raises intralysosomal pH, reduces the yield of reovirus during infection of mouse L cells. Subsequent removal of ammonium chloride results in the rapid establishment of a persistent infection.*

Viruses as well as hormones and toxins may enter cells through receptor-mediated endocytosis (*1–3*). Since this pathway involves transport of viruses into lysosomes before viral entry into the cytoplasm, altering the lysosomal contents could affect the early interaction of viruses with cells (*4*). For example, treatment of cells with lysosomotropic weak bases such as NH_4Cl raises the intralysosomal *p*H (*5*), which in turn may inhibit fusion activity of enveloped viruses and block viral replication (*6, 7*). We report here that replication of the mammalian reoviruses—icosahedral, nonenveloped viruses containing segmented, double-stranded RNA—is inhibited by NH_4Cl. In addition, if NH_4Cl is removed after treating cells for 4 days in culture, persistent infection is readily established. Thus, the effect of NH_4Cl on viral replication is not limited to enveloped viruses, and persistent, noncytocidal infections may be readily established after such treatments.

To determine the effect of NH_4Cl on reovirus replication, virus was adsorbed to mouse L cells in monolayer at 37°C for 1 hour. The cells were then maintained in medium with or without 10 m*M* NH_4Cl. After 4 days the yield of reovirus type 1 or type 3 was more than 2 $\log_{10}$ lower in NH_4Cl-treated cells than in the controls (Fig. 1). In addition, reovirus-infected cells treated with NH_4Cl did not exhibit the characteristic cytopathic effects. Four days after infection, L cells not treated with NH_4Cl were rounded and dying; the treated cells, however, showed minimal cytopathic effects (Fig. 2). To determine whether this reduced destruction of L cells is correlated with increased cell viability, we exposed cells that had survived in the presence of NH_4Cl to trypan blue. Virtually all the cells excluded the dye, indicating that they were not only morphologically intact but also fully viable.

Since NH_4Cl markedly reduced the capacity of reovirus to damage L cells, we next sought to determine whether the infected cells could continue to replicate while supporting the growth of infectious virus. L cells were infected with reovirus type 1 and type 3 in the presence of NH_4Cl, and 96 hours later the medium was removed and replaced with fresh medium without NH_4Cl. By the following day (120 hours after infection) 50 to 75 percent of the cells had been lysed. However, 25 to 50 percent of the cells survived and a persistent infection was readily established. During the initiation of these cultures, titers of infectious virus increased from 10^7 to 10^8 plaque-forming units per milliliter (Fig. 1). By day 10 the cells were doubling every 48 hours (compared to 24 hours for parental L cells). The medium for these cells contained 10^6 to 10^8 plaque-forming units of reovirus per milliliter for at least 98 days after the initiation of the persistent infection. The persistently infected cell lines are stable and have been passaged more than 30 times in 4 months. Ten different persistently infected lines have been established by means of this approach. Thus, removal of NH_4Cl after 4 days of infection allowed virus titers to increase initially and routinely resulted in persistently infected cell cultures.

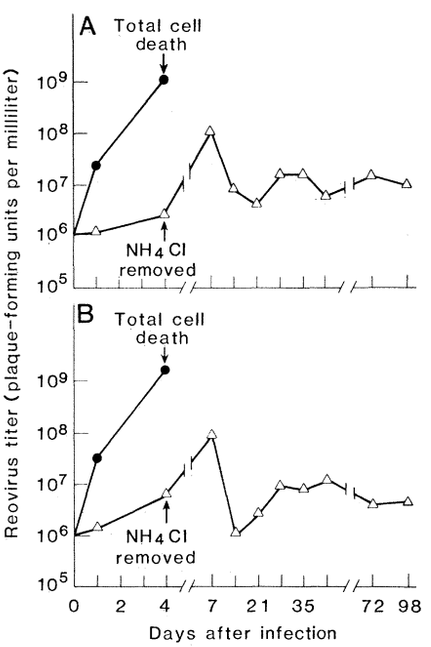

Fig. 1. Effect of NH_4Cl on the yield of reovirus type 1 (A) or type 3 (B) in mouse L cells. Virus was adsorbed to mouse L cells at a multiplicity of infection of 1 at 37°C for 1 hour in the absence of NH_4Cl. The cells were then overlaid with Joklik's modified Eagle's medium supplemented with 5 percent fetal calf serum with (△) or without (●) 10 m*M* NH_4Cl. By day 4 after infection there was complete cell lysis of cells infected in the absence of NH_4Cl. On day 4 the supernatant was removed from the treated cells and replaced with fresh medium not containing NH_4Cl. These cells were then maintained in NH_4Cl-free medium. Virus was grown, maintained, and titered in mouse L cell monolayers (*18*).

To further characterize the nature of the persistent infection, we analyzed cells by infectious center assay, immunofluorescence, and electron microscopy. Infectious center assay showed that 20 to 40 percent of the cells were producing infectious virus. Reovirus antigens were detected in a subset of the persistently infected cells by immunofluorescence (Fig. 3). Many cells expressed little or no viral antigen, while others were strongly fluorescent. Overall, about 25 percent of the cells were positive for viral antigen. An electron micrograph of representative antigen-positive cells (Fig. 3C) shows crystalline arrays of reovirus within cytoplasmic inclusions. Thus, the persistently infected cell lines that were started in cultures treated with NH_4Cl were similar to other reovirus carrier cultures in that they contained readily detectable viral inclusions and released large amounts of infectious virus (*8, 9*). The high percentage of cells containing no detectable viral antigens did, however, differ from our previous results that indicated a proportion of antigen-positive cells of 80 to 100 percent in carrier cultures established after infection with defective viruses (*9*).

Some investigators have argued that NH_4Cl, by raising intralysosomal *p*H, inhibits a low *p*H-dependent fusion activity of enveloped viruses in the lysosome, blocking viral penetration of the