Table 2. Release of ^{3}H-leucine-labeled vitellogenins to an incubation medium by tissues of *Periplaneta americana* (L.), as measured by precipitation of radioactive material by specific antibody.

Stage of development	Antibody-precipitated label in 0.8 ml	
	Radioactivity (10^3 disintegrations per minute ± S.D.)	Fraction of perchloric acid–insoluble label (%)
	Female fat body	
Newly emerged adult	0.19 ± 0.02	0.95
Ootheca forming	1047 ± 40	83
Day 1 after ootheca forming	891 ± 52	77
Day 2 after ootheca forming	891 ± 98	81
Day 3 after ootheca forming	841 ± 176	78
Day 4 after ootheca forming	446 ± 15	62
	Female midgut	
Ootheca forming	2.24 ± 0.28	1.60
	Male fat body	
Mature adult	2.36 ± 0.43	2.00

at the *p*H and ionic strength of the culture medium produced neither band. This result confirms the electrophoretic evidence that vitellogenin from *Periplaneta* is not readily detectable in the fat body (*7, 15*).

An additional difference between the two animals was the time in the life cycle at which the vitellogenins appeared. Vitellogenin from cecropia appears in the blood during the larval-pupal molt and is stored there at maximum concentration until yolk formation commences during the pupal-adult transformation (*1*). In contrast, the two vitellogenins from *Periplaneta* appear together approximately 5 days after the emergence of the adult, when yolk deposition begins.

Incubation of cecropia fat body, taken from animals between day 3 of the larval-pupal molt and day 4 after pupation, led to the appearance in the medium of a labeled protein precipitable by perchloric acid and antibody (Table 1). On day 5 of the molt, the label precipitable by antibody was at a maximum, accounting at that time for 69 percent of the total protein label.

After a 30- to 40-minute lag at the outset of the incubations, label precipitated by both perchloric acid and antibody increased linearly with time; both were completely inhibited by $5 \times 10^{-3} M$ cycloheximide (*16*). Therefore, the demonstrated radioactivity did not result from direct adsorption of free amino acid to carrier protein or from inadequate washing procedures. The lag period presumably encompassed the time required for synthesis and secretion as well as for cellular adaptation to the medium.

The labeling of vitellogenin in vitro resembled the synthesis and secretion of this protein in vivo in that the activity was limited to female fat body at appropriate stages of metamorphosis (Table 1). Thus, fat body from caterpillars or from females initiating the larval-pupal molt, prior to the appearance of vitellogenin in the blood, did not significantly promote the appearance of antibody-precipitated labeled protein in the incubation medium, although label insoluble in perchloric acid appeared in copious amounts. Neither midgut nor wing sacs at the time of pupation incorporated labeled leucine into vitellogenin. Finally, and what is most convincing, fat body from pupating males was also inactive.

Parallel results were obtained with *Periplaneta* (Table 2). Between 62 and 83 percent of the label secreted as protein into the incubation medium by the fat body of egg-producing females was precipitated by homologous antibody to vitellogenin. Neither male nor newly emerged adult female fat body exhibited this capacity, nor did the midgut of mature females, though all of these tissues gave rise to labeled proteins in the medium.

In both species the specificity of incorporation with regard to sex and to stage in the life cycle were consistent with well-established occurrences of vitellogenin in vivo (*1–3*). Our conclusions are in agreement with results obtained by other methods (*17*) for the wood roach, *Leucophaea maderae*, and for the moth, *Antheraea polyphemus*. Taken in conjunction with the evidence that the fat body in a variety of insects plays a metabolic role in egg production (*18*), the similarity in our data from two widely divergent species suggests that insect vitellogenins in general may prove to be secreted by the fat body. Failure to detect the protein in tissue extracts indicates either a change in solubility characteristics of the protein at the time of secretion or a fairly prompt secretion following its synthesis.

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Transplantation of Pluripotential Nuclei from Triploid Frog Tumors

Abstract. *Renal tumors were produced by injection of a cell fraction of a tumor into triploid tadpoles of* Rana pipiens *before they began feeding. Triploid tumor cells were dissociated and transplanted into activated and enucleated eggs. Pluripotency of the implanted nuclei was evidenced by the formation of swimming triploid tadpoles.*

Diploid nuclei of renal tumor cells, when transplanted to enucleated eggs of the common leopard frog *Rana pipiens*, result in a low yield of tadpoles (*1*). These experiments suggest that pro-

gression to a tumorous state has proceeded by way of epigenetic mechanisms that do not alter the cells' genome, in a manner perhaps similar to that of certain plant tumors (*2*). Unfortunately, chromosomal analysis of the embryos that result from transfer of diploid tumor cells does not permit one to distinguish between development guided by the transplanted nucleus and parthenogenetic development resulting from inadvertently retained maternal genetic material. Diploid gynogenesis can result from either fusion of the nucleus of a mature maternal gamete with a nucleus retained from the second polar body or fusion of daughter nuclei after one mitotic division of the maternal gamete nucleus.

We believed that use of a nuclear marker would provide evidence that the tumor nucleus was participating in development (*3*). Nuclear markers for *R. pipiens* thus far developed appear unsuited for experiments on the transfer of tumor nuclei because expression of the marker gene is delayed until metamorphosis (*4*). Transplantation of the nucleus of a triploid tumor cell into an enucleated egg makes it possible to distinguish between development initiated by the transplanted nucleus and that guided by an inadvertently retained maternal nucleus. Thus, triploid tadpoles resulting from the transplantation of triploid tumor nuclei can reasonably be interpreted as resulting from development initiated by the tumor nucleus and not from gynogenetic development. Triploid tumors are readily available because of a procedure for tumor induction developed by Tweedell (*5*) and the ease of producing triploid embryos by hydrostatic pressure (*6*).

Commercially obtained northern leopard frogs were induced to ovulate by intracoelomic injection of pituitary glands (*7*). Freshly extruded eggs were fertilized with a sperm suspension. Triploidy was produced by hydrostatic pressure sufficient to repress formation of the second polar body (*6*). Control diploid embryos were obtained from the same clutch of eggs but were not subjected to pressure.

Diploid and triploid tadpoles, Shumway stage 25 (*8*), were injected in the pleuroperitoneum with a virus-containing mitochondrial fraction prepared from a frog renal adenocarcinoma (*5*). Injected tadpoles were reared in individual 1-liter aquariums and were fed a diet of boiled lettuce. The chromosome number of each injected tadpole, as well as that of tadpoles developing from nuclear transplantation, was ascertained by aceto-orcein squashed preparations of the tail tip (*9*).

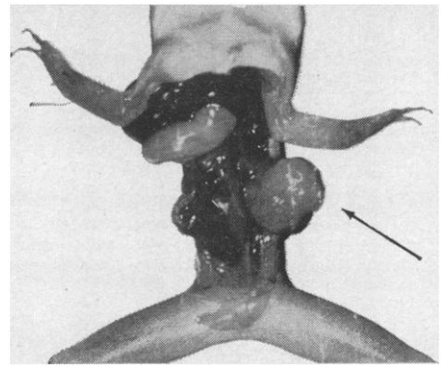

Fig. 1. Left renal tumor (arrow) of recently metamorphosed triploid frog.

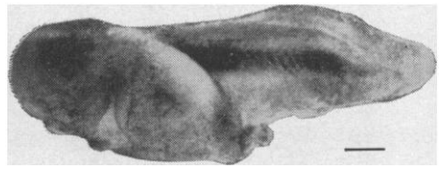

Fig. 2. Tumor nuclear transplant tadpole with well-formed head, body, and tail. The scale is 1 mm.

Forty larvae injected with the tumor fraction were reared to metamorphic or postmetamorphic stages. Twelve of 17 diploid and 10 of 23 triploid frogs developed massive renal adenocarcinomas (Fig. 1).

The procedure for nuclear transplantation was modified from that of King (*10*). Donor tumors were dissociated in calcium- and magnesium-free Niu-Twitty solution containing $5 \times 10^{-4} M$ ethylenediaminetetraacetic acid. Several completely dissociated tumor cells were drawn into a glass micropipette and injected into a previously enucleated egg. The diameter of the micropipette slightly exceeded that of the tumor cells so that little or no distortion in the shape of the cell occurred during implantation.

In 143 transfers of triploid tumor nuclei, 33 partial and complete blastulas developed. Eight of the blastulas gastrulated, and seven of these developed to swimming embryos. All seven embryos were probably triploid. The triploid chromosome number ($3n = 39$) was appraised by chromosome count in five embryos. No ring chromosomes or chromosome bridges were observed; more subtle chromosomal abnormalities may exist but were not detected. Triploidy was assessed in the other two embryos by a comparison of the mean nuclear diameter of the transplant embryo with that of known diploid and triploid control embryos.

From 181 nuclear transfers of diploid renal tumors, 14 embryos developed to swimming stages. All of the embryos were diploid. These tadpoles did not differ in their development from tadpoles of triploid transplants.

Observation of living triploid tadpoles revealed functional tissues of many types. Ciliated epithelium propelled the tadpoles in the culture dishes. The tadpoles swam when stimulated. Functional receptors, nerve tissue, and striated muscle are necessary for swimming. Cardiac muscle pumped blood cells through external gills. "Suckers" secreted abundant mucus. A pronephric ridge, eye anlage, nasal pit, and an open mouth were clearly discernible, as was the differentiation of head, body, and tail (Fig. 2). The tail fin regenerated after being clipped for chromosome study. The multipotency of the tumor genome was thus obvious before histological examination of the transplant tadpole.

Sections of embryos developed from transplanted triploid tumor nuclei were stained with Feulgen and fast green and revealed, in addition to those already listed, the following tissues: brain, spinal cord, optic cup with lens, auditory vesicle, somites, pronephric tubules, pharynx, midgut, and notochord.

Triploidy is not a flawless nuclear marker. It may occur infrequently among parthenogenetic tadpoles. Parmenter reported embryos of various ploidy and one triploid tadpole from among 26 parthenogenetic tadpoles (*11*). If we assume one triploid in 26 developing embryos (3.8 percent) is representative of the frequency of parthenogenetic tadpoles, then the probability of parthenogenesis causing development of the seven tadpoles is $P = .038^7$ (1.1×10^{-10}). We reject parthenogenesis as a plausible interpretation of our data.

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Immune Adherence by the Fourth Component of Complement

Abstract. *An immune complex becomes reactive in immune adherence after the specific binding of the fourth component of complement (C4). Immune adherence produced by the fourth component of complement is indistinguishable from classical immune adherence in terms of all parameters tested, except that it is entirely independent of the participation of the second and third components of complement.*

Immune adherence (IA) is the term used by Nelson (*1*) to describe the complement-dependent adherence of immune complexes to certain nonsensitized indicator particles, such as human erythrocytes. It has been widely used as a sensitive in vitro test for the detection of antigen, antibody, or bound complement components (*2*); it has also received some attention as a method for demonstrating isoantibody or preformed immune complexes in tissue sections (*3*), on isolated lymphocytes (*4; 5*, p. 223), or on tumor cells (*6*). Nishioka and Linscott first showed that the generation of an IA reactive complex required the specific binding of only the first four components—C1, C2, C3, and C4—of the complement system (*7*). Among these components, C1 and C2 are required only to mediate the specific binding to the immune complex of C4 and C3, respectively. While IA reactivity was readily evident after the fixation of small amounts of C3, it could not be demonstrated before the binding of this component. Furthermore, several investigators have shown that there is direct dependence of IA on the number of bound C3 molecules per cell (*5*, p. 245; *8*). There is thus little doubt that bound C3 is capable of mediating the IA reaction.

Recent studies indicate that specifically bound C4 can produce IA which is indistinguishable from classical IA, although it is entirely independent of C3. The reactivity of C4 in IA first became evident when it was observed that concentrated preparations of highly purified C4 were consistently reactive in IA.

For the study of C4-dependent IA (*9*), sensitized sheep erythrocytes (EA) bearing C1 (EAC1) were added to dilutions of purified C4 (*10*) to allow formation of EAC1,4. For the generation of C3-dependent IA, the intermediate complex EAC1,4 was added to dilutions of a mixture of purified C2 (*11*) and C3 (*12*) where C2 was present in excess. Human type O erythrocytes were subsequently added as indicator particles to dilutions of washed, water-hemolyzed EAC1,4 and EAC1,-4,2,3 (*13*). The agglutination titer was appraised by inspection of the sedimented erythrocytes. Coombs-type agglutination tests with antiserums directed against C3 or C4 and various complement intermediates were also performed (*14*).

Approximately 30 preparations of highly purified C4 were tested for their ability, in conjunction with EAC1, to produce IA. These preparations were IA reactive; furthermore, the resulting IA titer was directly dependent upon the C4 concentration in the reaction mixture. No reactivity was evident upon omission of C4, nor was IA produced with C4 preparations which had become inactivated with respect to hemolytic activity of C4. The reaction was also abolished upon heat denaturation (60°C, 30 minutes) or hydrazine (0.015*M*, 37°C, 45 minutes) treatment. A requirement for active C1 for the generation of IA reactivity was indicated by the observations that completely negative reactions resulted on omission of C1, after C1 removal from EAC1 by treatment with 0.02*M* EDTA, or after abrogation of C1 reactivity on EAC1 by treatment with $10^{-2}M$ diisopropylfluorophosphate. These studies indicate that IA reactivity results only after interaction of active C1 with active C4.

The data presented in Fig. 1 show the IA reactivity of ^{125}I-labeled, specifically bound C4 on EAC1,4 (*15*). This reactivity was first evident at 2500 to 3000 bound C4 molecules per cell and maximum reactivity was reached with about 9000 C4 molecules per cell. Between these values the IA titer was a function of the number of C4 molecules per cell. For comparison, Fig. 1 also shows the relation between the number of molecules of specifically bound C3 on EAC1,4,2,3 and the IA titer. For the preparation of EAC1,-4,2,3 only IA negative EAC1,4 complexes were used; that is, cells which contained less than 2500 C4 molecules per cell. Very few molecules of specifically bound C3, approximately 60 per cell, were required to produce a positive IA reaction. Between 60 and about 3000 C3 molecules per cell, where maximum reactivity was reached, the IA titer was dependent on the number of bound C3 molecules per cell. Both IA-positive EACl,4 and EACl,-4,2,3 were undiminished in reactivity after treatment with either EDTA or diisopropylfluorophosphate.

The extreme sensitivity of IA in detecting cell-bound C3 indicated that contamination of purified C4 with traces of C2 and C3 and resulting generation of a few C1,4,2,3 sites must be ruled out as the explanation for the IA reactivity of EAC1,4. One reaction of comparable sensitivity to IA in the detection of cell-bound C3 is the agglutination of cells bearing C3 by potent antiserums to this component. Therefore, numerous preparations of IA-positive EAC1,4 were examined for reactivity with antiserum to C3. Agglutination was not observed. The results of some of these experiments are shown in Fig. 2 which demonstrates the maximum IA reactivity obtained with four different EAC1,4 preparations as well as the completely negative patterns observed when the same cells were tested with antiserum to C3. By contrast, in parallel experiments,