was observed for Sm^{3+}, Nd^{3+}, Co^{2+}, Tm^{3+}, and Dy^{3+}, while the reverse was found for Cu^{2+}, Ni^{2+}, Gd^{3+}, and Ho^{3+} where $2\pi\nu T_{1m} > 1$.

Titration curves where the ESR signal amplitude and $P_{1/2}$ are plotted as functions of added metal concentration are shown in Fig. 1A. We waited about 15 minutes after each addition of metal before examining at 77°K. Of the ions studied, Pr^{3+} has the shortest relaxation time. At the highest concentration a reduction in ESR signal height of only a factor of 2 is observed. The ion Dy^{3+} is a very effective relaxation agent, changing $P_{1/2}$ by three orders of magnitude.

The S-shaped titration curves of Fig. 1A indicate a saturation of all metal binding sites at a metal concentration of $10^{-2}M$. This corresponds to 6×10^{20} metal binding sites per gram of dried material. The *p*H varies from 5.5 to 3.8 as metal is added and hydrogen ions are released.

Titration curves for type A and type B melanin are shown in Fig. 1B. Type B melanin was prepared from type A by digestion in 6*M* HCl in sealed tubes at 95°C for 120 hours, which removes protein (*2*). Figure 1B indicates that metal binding for type B melanin is less specific than for type A.

Figure 2 shows the result of metal-ion competition experiments. The sample was incubated for 2 to 3 hours in a $10^{-1}M$ solution of the first ion shown in brackets, which is diamagnetic. The free-radical ESR intensity was then plotted as a function of concentration of the second ion (usually Dy^{3+}). The incubation time in Dy^{3+} was 15 minutes for each experimental point. Data in Fig. 2 were obtained at room temperature (22°C).

The [Na^+, Dy^{3+}] curve is on Fig. 2 for reference. The chemical properties of La^{3+} and Dy^{3+} are similar, and the [La^{3+}, Dy^{3+}] curve indicates that the probability of Dy^{3+} replacing La^{3+} is determined primarily by the statistical abundance of the ions.

Incubation of melanin in zinc has been shown to increase the free-radical signal height (*11*). Thus the [Zn^{2+}, Dy^{3+}] titration curve starts from a higher level. We regard the well-defined steps in this curve as one of the most interesting results of this work. They apparently indicate the presence of several distinct types of binding sites. A similar step is observed in the [Ca^{2+}, Dy^{3+}] curve.

Since melanin binds metal very tightly, we compared the binding strength with that of a strong metal chelator, ethylenediaminetetraacetic acid (EDTA). We mixed $5 \times 10^{-3}M$ Gd^{3+} with a tenfold excess of EDTA and added it to melanin, observing the free-radical signal as a function of time after mixing. The results are shown in Fig. 3. Data are also plotted for a parallel experiment in which $5 \times 10^{-3}M$ Gd^{3+} was added to melanin and a tenfold excess of EDTA subsequently added. Equilibrium is reached in about 10^3 minutes at an intermediate reduction of the ESR free-radical signal corresponding to the level obtained when $1.5 \times 10^{-4}M$ Gd^{3+} is added to melanin in the absence of EDTA. Melanin thus has some sites that bind metals more tightly than EDTA and some that bind them less tightly. Bruenger *et al.* (*3*) found that EDTA competed more successfully with metal ions at *p*H 7.6 than our results indicate at lower *p*H.

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12. We thank Professor S. Lukiewicz of the Jagellonian University in Poland for providing the melanin samples. Supported in part by a grant to H.M.S. from the John Hartford Foundation.
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Oncogenic Transformation of Human Embryo Lung Cells by Human Cytomegalovirus

Abstract. *Persistent infection of human embryo lung fibroblasts with a genital isolate of cytomegalovirus resulted in oncogenic transformation of these cells. Immunofluorescence techniques detected virus-specific antigens, while microcytotoxicity tests established that the transformed cells share a membrane antigen with hamster cells transformed by inactivated cytomegalovirus. The transformed human cells induced progressively growing tumors in weanling athymic nude mice.*

Human cytomegalovirus (CMV) can induce oncogenic transformation of hamster embryo fibroblasts (*1*), and can stimulate synthesis of host cell DNA and RNA (*2*). These properties are commonly associated with known oncogenic herpesviruses (*3*). We reported (*4*) that cells from human prostate tissue which apparently had been infected in vivo with CMV grew in vitro to passage levels well above those routinely attained by normal cells. After a number of passages virus was no longer rescuable, although CMV-specific antigens and nucleic acid continued to be detected in the cells. It is uncertain whether these cells, which exhibited loss of contact inhibition, were transformed by CMV or whether they were chronically infected and then released virus at levels below detection. Therefore, further studies of the transforming capacity of the isolated virus (designated Mj) were undertaken. Since this strain replicated quite slowly in human embryo lung (HEL) cells, the studies were performed without inactivating the virus.

We report evidence that infection of HEL cells with the Mj strain of CMV can lead to long-term persistent infection, and that occasional cell transformants can arise in the cultures. The transformed cells contain CMV-specific membrane and intracellular antigens, and share common antigens with CMV-transformed hamster cells. Furthermore, the cells induce nondifferentiated tumors when injected into weanling athymic nude mice.

Monolayers of HEL cells were initially grown in Dulbecco's medium supplemented with 10 percent fetal calf serum and 0.075 percent sodium bicarbonate. For transformation experiments, Ham's medium with 20 percent fetal calf serum and 0.075 percent sodium bicarbonate was used. Standard procedures for the indirect immunofluorescence test were used for the detection of CMV-specific antigens in the transformed cells (*4*). Human antiserums to CMV were obtained from hospital patients. Fluorescein isothiocyanate–conjugated goat antiserum against human immunoglobulin G was purchased from Cappel Laborato-

ries, Downingtown, Pennsylvania. In tests for membrane antigens, unfixed cells grown on cover slips were incubated with immune serum, washed, and covered with the conjugated antiserum against human immunoglobulin G. For detection of intracellular antigens, the test for immunofluorescence against complement (ACIF) was used. Coverslip cultures were fixed with a mixture of equal parts of methanol and acetone for 2.5 minutes, then covered with heated antiserum against CMV and incubated at 37°C for 45 minutes. After repeated washings, 2 to 4 units of complement were added for 45 minutes at 37°C. Human serum negative for CMV antibody served as the complement source. The cells were washed again with phosphate-buffered solution and covered with fluorescein isothiocyanate–labeled goat antiserum against human complement for 45 minutes at 37°C. After final washings, the cells were mounted on slides in a mixture of one part phosphate buffer and nine parts glycerol. Appropriate positive and negative controls were included in each test.

In microcytotoxicity tests, normal hamster spleen cells and spleen cells from hamsters bearing CMV tumor isografts (line Cx-90-3B, T-2) or sensitized to CMV were tested against target cells in microtiter test plates as described (*4*). After overnight incubation, the cells were fixed and stained, and the number of attached target cells was counted visually. Spleen cell cytotoxicity was determined by calculating the percentage difference between the number of cells remaining in eight wells with normal spleen cells (N) versus those in eight wells with sensitized spleen cells (S) according to the equation

$$\text{Cytotoxicity}\,(\%) = 100\,(N - S)/N$$

Statistical analysis of the microcytotoxicity data was carried out by using Student's t-test. Probability values less than .05 were considered to be significant.

In the transformation experiments, HEL cells were inoculated with Mj strain of CMV at a multiplicity of infection estimated to be less than 0.001 plaque-forming unit (PFU) per cell. Two established CMV laboratory strains, C-87 and Ad-169, were also tested. The HEL cells were infected with these strains at multiplicities of 0.01 to 0.03 PFU per cell. Following infection, the cultures were passaged frequently, usually at 2-day intervals. Under these conditions, no long-term persistently infected HEL culture could be obtained with the laboratory strains of CMV. The virus-infected cells showed early accelerated growth, and their morphology changed to epithelioid. However, none of the cultures survived more than five in vitro passages. The cultures were kept under observation for 4 months with repeated changes of medium to see whether transformed foci might emerge. None did.

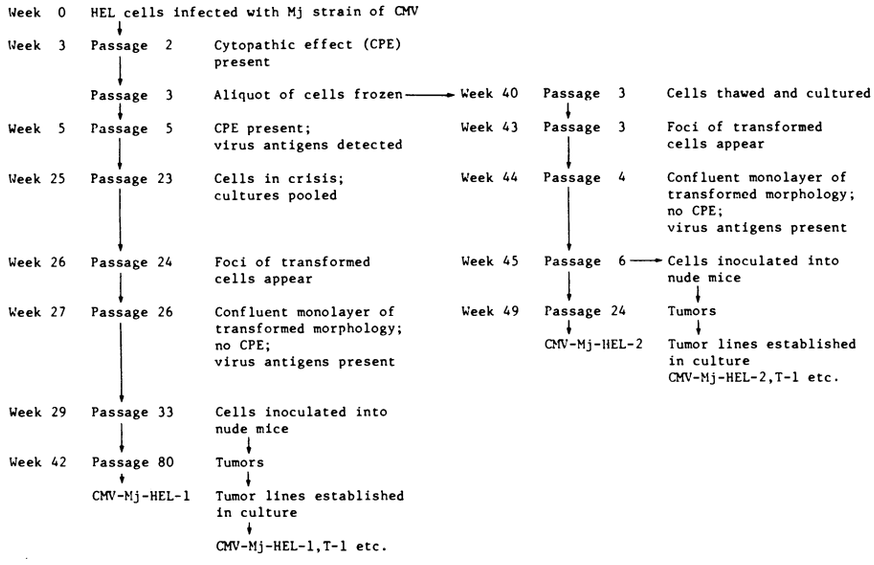

Fig. 1. In vitro history of human embryo lung cells transformed by human cytomegalovirus and development of tumor lines.

HEL cultures inoculated with Mj strain yielded different results. A persistent infection was established, with virus-induced cytopathology seldom spreading beyond 50 percent of the cell sheet. At passage 23, the cells entered crisis. In an attempt to save the cells, cultures from six 30-ml flasks were pooled into one flask. Shortly thereafter, foci of short fibroblastoid and epithelioid cells developed and within 1 week formed a confluent monolayer. Subsequently, the cells grew rapidly and could be subdivided every other day. The history of the cell line (designated CMV-Mj-HEL-1) is summarized in Fig. 1.

The morphologically transformed cell population was a mixture of short fibroblastoid and epithelioid cells (Fig. 2). No infectious virus could be recovered by in-

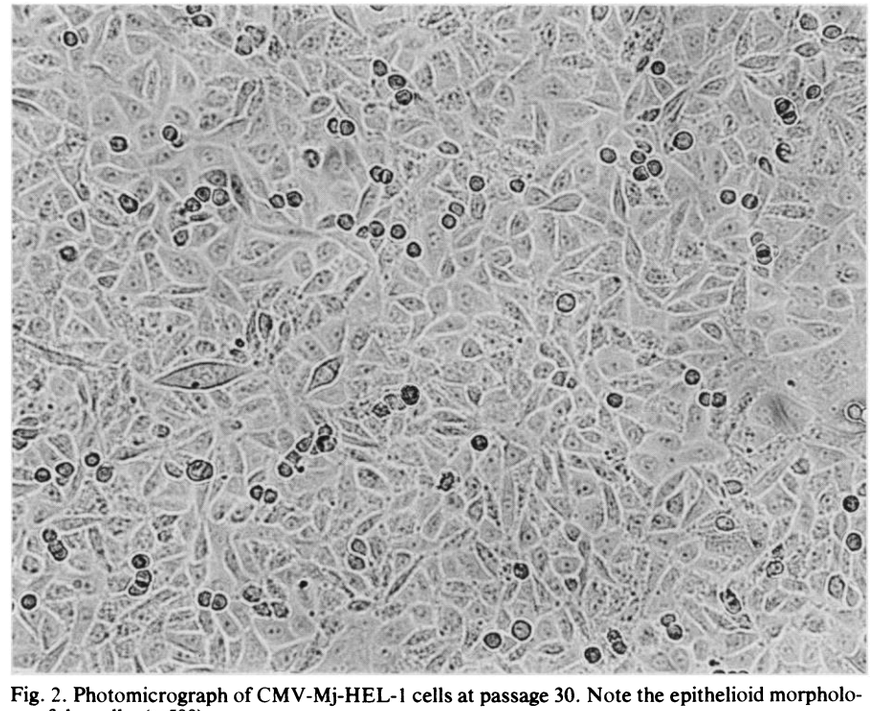

Fig. 2. Photomicrograph of CMV-Mj-HEL-1 cells at passage 30. Note the epithelioid morphology of the cells (×500).

Table 1. Cytotoxicity of spleen cells from hamsters sensitized to CMV or to isografts of CMV-transformed cells (line Cx-90-3B,T-2). The ratio of spleen cells to target cells was 500 : 1; HBC-1, human bladder carcinoma line 1.

Test	Sensitizing material	Target cells	Cytotoxicity (%)	P
1	CMV	CMV-Mj-HEL-1	19	< .05
		HEL	−2	
		HBC-1	0.2	
2*	CMV	Cx-90-3B,T-2	30	< .001
		CMV-Mj-HEL-1	23	
		CMV-Mj-HEL-2	25	< .01
		HEL	7	
3	Cx-90-3B,T-2	CMV-Mj-HEL-1	41	< .001
		CMV-Mj-HEL-1,T-1	31	< .001
		HEL	1	

*In test 2, sensitized and control spleen cells were passed through a nylon wool column before use.

oculation of cell extracts onto HEL cultures or by cocultivation with HEL cells. Irradiation with ultraviolet light (42 erg sec^{-1} mm^{-2}, 4 minutes at a distance of 200 mm) or treatment with iododeoxyuridine (100 μg/ml in medium for 3 days) also failed to induce infectious virus. CMV-specific membrane antigens were detected in these cells with five different human convalescent serums by indirect immunofluorescence (Fig. 3). Perinuclear and paranuclear dense fluorescent bodies were observed in most cells when the ACIF technique was used. A few cells also exhibited intranuclear staining. The antiserums did not react with normal HEL cells. CMV-immune serum was adsorbed with CMV-infected HEL cells (10^8 cells in 1 ml of immune serum for 1 hour at 37°C, and then overnight at 4°C). This adsorption removed the reactivity of the immune serum for the CMV-Mj-HEL-1 cell membrane. Adsorption with normal HEL cells did not affect reactivity. Hamster antiserum to herpes simplex virus type 2 or to PARA-7 tumor cells (hamster cells transformed by an adenovirus-SV40 hybrid) did not react with the CMV-Mj-HEL-1 cell surface.

Previous studies (*5*) had shown that CMV-transformed hamster cells were specifically killed by spleen cells from CMV-sensitized hamsters, or from hamsters bearing isografts of CMV-transformed cells. Therefore, tests were performed to determine whether such effector cells are also cytotoxic for the CMV-Mj-HEL-1 line. Table 1 shows results representative of the microcytotoxicity data. CMV-transformed HEL cells were consistently killed. The cytotoxicity was specific in that survival of normal HEL cells and human bladder carcinoma cells (designated HBC-1) was not significantly reduced. Thus, CMV-transformed human or hamster cells share a common virus-specific membrane antigen (or antigens).

Initial studies of the oncogenicity of the CMV-Mj-HEL line were performed with athymic nude mice (Life Sciences, St. Petersburg, Florida). Two to three weeks after the subcutaneous inoculation of 2×10^7 cells, progressively growing tumors appeared in six of nine animals. Initial histological studies of one tumor indicated that it was composed of small polygonal cells with large nuclei and scanty cytoplasm embedded in an abundant collagenous matrix. The cells were poorly differentiated but appeared to be of mesenchymal origin. Local invasion of adjacent structures was rare. Cells cultured in vitro from the tumor (designated CMV-Mj-HEL,T-1) were tested for CMV-related antigens. Intracellular and membrane antigens were found by indirect immunofluorescence. Also, spleen cells from tumor-bearing hamsters were cytotoxic for the human cells (Table 1, test 2). Thus, passage in vivo did not lead to loss of the CMV-specific antigens. None of 14 nude mice inoculated with 2×10^7 uninfected HEL cells developed tumors.

Three established human bladder carcinoma lines were being maintained in the laboratory when the HEL cells were transformed. The karyotype of one of the three lines (HBC-1) was identical to that of the CMV-Mj-HEL cells, raising the possibility that laboratory contamination had occurred. Therefore, HBC-1 cells were tested for CMV-specific antigens. No antigens were detected in the cells by immunofluorescence or by lymphocyte microcytotoxicity assay (Table 1) in repeated testing.

Normal cells and cells transformed by RNA virus generally respond to cytochalasin B treatment with one and occasionally two nuclear divisions, while cells transformed by DNA virus tend to become multinucleated (*6*). Treatment of CMV-Mj-HEL cells with cytochalasin B resulted in uncontrolled nuclear division, whereas nuclear division in HBC-1 cells remained controlled. Immunological studies and those with cytochalasin B provided no evidence that the HBC-1 cells carried adventitious CMV. It is possible, however, that the CMV carrier

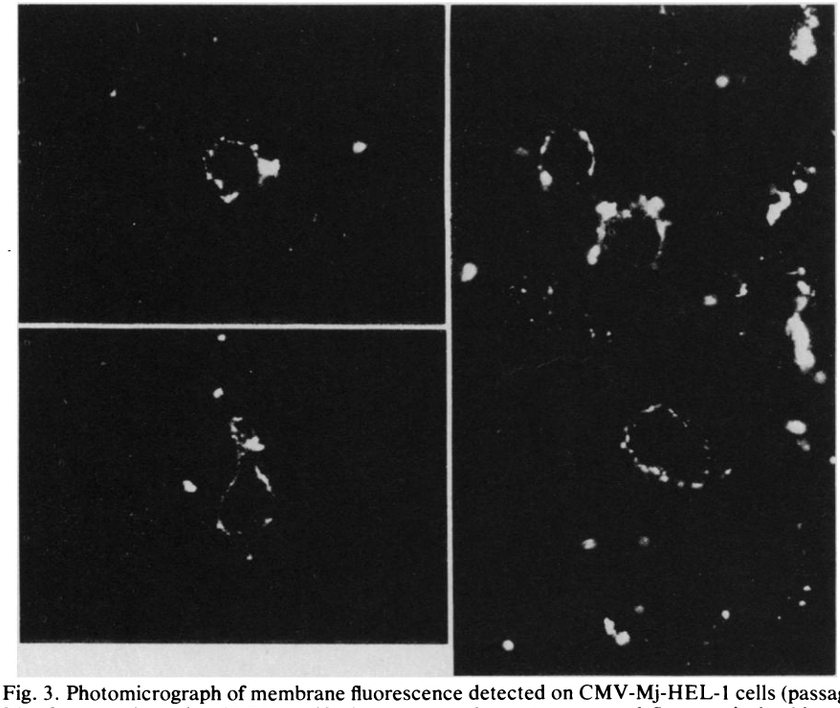

Fig. 3. Photomicrograph of membrane fluorescence detected on CMV-Mj-HEL-1 cells (passage 26) after reaction with CMV-specific human convalescent serum and fluorescein isothiocyanate–conjugated goat antibody against human immunoglobulin G (×1250).

HEL cells became contaminated with HBC-1 cells, and that the HBC-1 cells were subsequently transformed. Therefore, a subculture of CMV carrier HEL cells frozen before the introduction of the human bladder lines into the laboratory was thawed, cultured, and followed to see if a transformed line would again arise. Only a fraction of the frozen cells could be cultured. After 3 weeks, foci of transformed cells appeared (Fig. 1) and a stable cell line was developed. CMV-specific antigens could be demonstrated in this line (designated CMV-Mj-HEL-2) by immunofluorescence reagents. Furthermore, spleen cells sensitized to CMV were cytotoxic for the cells (Table 1). Thus far, no virus has been rescued from the cells. The cells induced progressively growing tumors in weanling athymic nude mice.

In summary, the present work indicates that human cells can be transformed by a recent isolate of CMV, and that such cells produce nondifferentiated tumors following transplantation to athymic nude mice. As such, the results constitute further evidence of the transforming capacity of CMV, and raise the possibility that the virus may have oncogenic potential in its natural host.

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Fossilized Eggs in a Pennsylvanian Blastoid

Abstract. *A single specimen of* Pentremites rusticus *(Echinodermata, Blastoidea) from the Early Pennsylvanian of Oklahoma has hundreds of apparent eggs in one of its abnormal anal hydrospire groups. This rare occurrence suggests that female blastoids in this sexually dimorphic species had modified their anal hydrospires for brooding eggs instead of for normal respiration.*

A single sectioned specimen (*1*) (out of a total of 658) of the fossil blastoid *Pentremites rusticus* Hambach (*2*) from the Early Pennsylvanian (Morrowan) of northeastern Oklahoma was found to contain several hundred small translucent spheres (Fig. 1, A to D) that are probably fossilized blastoid eggs. These eggs are confined to one group of the blastoid's anal (posterior) hydrospires, which are abnormally developed in *P. rusticus* [and in *P. angustus* Hambach (*2*), apparently a synonym (*3*)]. Hydrospires are the five pairs of thin-walled longitudinal calcite folds (Fig. 1B) hanging into the coelomic cavity from the ambulacral margins; most authors (*4*) have considered them respiratory structures. Our discovery is the first convincing example of echinoderm eggs known from the fossil record, and their presence in abnormal anal hydrospires suggests that these structures had been modified for reproduction.

The egglike spheres range from 0.12 to 0.20 mm in diameter and show a normal size distribution with mean and mode at 0.16 mm (Fig. 2). Their size is in the proper range for eggs in living echinoderms (*5*). They are uniformly smooth, yellow, translucent, spherical to slightly ellipsoidal objects showing no internal structure except for radiating contacts where calcite crystals have grown together (Fig. 1D). Electron microprobe analysis of these spheres indicates that their

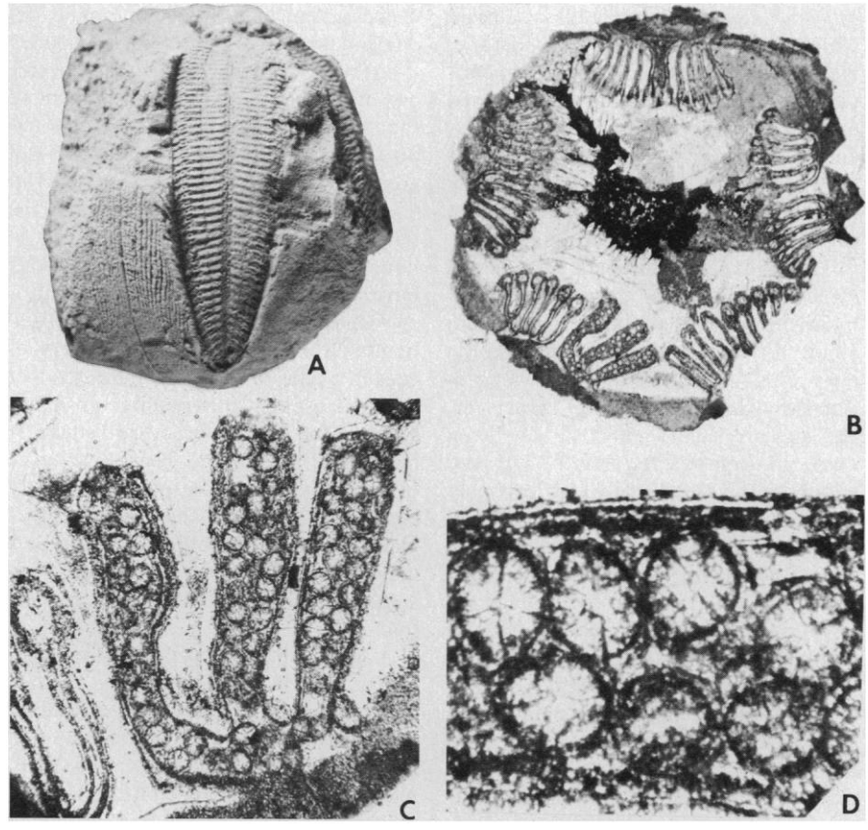

Fig. 1. *Pentremites rusticus* Hambach, plesiotype specimen OU 8372. (A) Reference photograph of complete blastoid (side view) before sectioning; note wide ambulacrum and slightly damaged calyx plates (× 3.7); (B) transverse thin section near middle of blastoid showing calyx plates and ambulacra on exterior surface (plates at lower right lost during grinding), thin calcite hydrospires hanging into coelomic cavity, and abnormal anal hydrospires (bottom) with left anal group containing numerous spherical eggs (×5); (C) enlargement of left anal hydrospire group showing eggs completely filling the three folds (× 23); (D) greatly enlarged eggs in one anal hydrospire fold showing degraded egg membrane and internal contacts where calcite crystals have grown together (× 92).