difference was small or non-existent in homotypic and non-lung heterotypic aggregation experiments (Fig. 1*b–e* and our unpublished results). Microscopic examination of wells containing 4+ aggregates of lung and F13 cells revealed practically no free cells, indicating complete aggregation (Fig. 2*a*). Heterotypic aggregates formed with lung cells and B16 lines or with other organ cell suspensions were heterogeneous in cell composition (Fig. 2*b*). The existence of mixed aggregates of tumour and organ cells was confirmed by double labelling techniques (work unpublished). Soon after initiating the assay (10 min) there was little evidence of nonspecific cell aggregation (Fig. 1*e–f*).

The preferential adhesion to lung cells of the increasingly metastatic B16 variant lines seems to be paralleled by tumour cell behaviour *in vivo*. Injection of $^{125}$I-labelled cells of the B16 variant into the tail vein of syngeneic mice results in 100% lung arrest of the B16-F11 and -F6 lines and approximately 80% lung arrest of the F1 line within 2 min. By 4 h 90, 80 and 55% and after 3 d 47, 35 and 2% of lines F11, F6 and F1, respectively remain in the lungs[14]. These results parallel closely the number of resulting lung tumour colonies visible within 3–4 weeks in the animals. Subcutaneous implantation of B16 metastatic variant cells in syngeneic hosts followed by removal of the primary subcutaneous tumours 1 to 2 weeks later results in spontaneous lung metastases within 6–8 weeks, the number formed being proportional to the number of *in vivo* selections F10>F5>F1 (ref. 14). These results indicate that initial cell–cell recognition during tumour cell arrest in specific organs may be one of the most important steps in the spread of blood-borne tumours.

Recent observations suggest that the success of tumour implantation and subsequent metastatic growth are determined by unique cell surface properties. Treatment of tumour cell surfaces with trypsin, neuraminidase[15–17], heparin[18,19] or dextran[20] before intravenous introduction modifies arrest and tumour distribution. Surface interaction of blood-borne tumour cells with lymphocytes[14] and platelets[21] can also affect the fate of circulating tumour emboli. Using B16 metastatic variants, Bosmann *et al.*[22] reported differences in surface proteases, glycosidases, glycosyltransferases and electrophoretic mobilities between low and high metastatic lines. These studies suggest the importance of cell surface properties in determining the *in vivo* fate of tumour cells.

Our results argue against an entirely nonspecific trapping mechanism for circulatory tumour cell arrest[1,2] and suggest that cellular recognition is important in determining the organ specificity of metastatic tumour spread. Since highly metastatic B16 tumour cells cause virtually complete aggregation of suspended lung cells, it is possible that recognition occurs through surface structures common to most, if not all, lung cells. Several types of cells are obtained by the dispersion techniques used here (for example, lung tissue yields endothelial, type I and II epithelial cells, fibroblasts and others) (Fig. 2), but contaminating blood cells such as erythrocytes, platelets, glass-adherent phagocytic cells and so on can be removed by density gradient separation, particle phagocytosis and other techniques. The role of blood cell interactions in the arrest of tumour emboli[14,21] was not examined here, but we are investigating the adhesion of tumour cells to platelets, lymphocytes and macrophages that affect implantation. The ultimate biochemical determination of the surface structures involved in the adhesion of tumour cells to organ cells is feasible using closely related B16 tumour lines with different recognition and metastatic properties.

These studies were supported by a contract from the Tumour Immunology Program of the US National Cancer Institute and grants from the US Public Health Service, Human Cell Biology Program of the US National Science Foundation and the Jane Coffin Childs Memorial Fund for Medical Research. J. L. W. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research at the Salk Institute.

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Received February 18; revised April 2, 1975.

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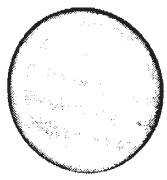
# Embryonic use of egg shell calcium in a gastropod

THE function of the calcified outer eggshell found in many gastropod eggs which are deposited on land has been the subject of considerable speculation. Various theories of its relationship to desiccation, mechanical support, protection against predators, physiological buffering and possible calcium source have been discussed[1–4]. This report presents the first quantitative analysis proving that a gastropod embryo uses the egg shell calcium in forming its embryonic shell, the protoconch.

The egg of the land pulmonate *Anguispira alternata* (Stylommatophora: Endodontidae) was examined during its development. This snail deposits eggs which have a heavily calcified, solid $CaCO_3$ shell made of calcite[5]. Each snail deposits 20–50 eggs, 2.5 mm in diameter, at 20 min intervals during a laying period. Hatching time is approximately 50–60 d at 20 °C; in land pulmonates there is usually a very large amount of variance in the rate of development and consequently in the time of hatching.

The egg shell and egg contents (albumen fluid) were analysed separately for calcium. After 20, 30, and 43 d incubation at 20 °C the eggs were opened and the embryos and egg shells analysed separately for total calcium content (Tables 1 and 2). In the freshly deposited egg, the albumen and single-celled

**Fig. 1** Soft X-ray radiograph of a live, newly deposited *Anguispira alternata* egg. Bar represents 2 mm. For the generation of soft X rays, a GE Special Model F-5 machine with a beryllium tube and tungsten target was used. Operating conditions were 25 kV at 5 mA with Du Pont Ortho-Cronar Litho Film.



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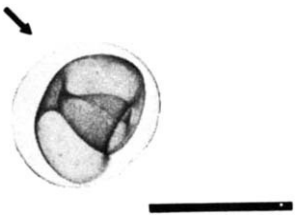

**Fig. 2** Egg incubated at 20 °C for 43 d. The egg shell is now thinner and the embryonic shell, the protoconch, has formed at its expense. Arrow indicates where the snail will hatch. Live egg, exposure and printing as for Fig. 1. Bar represents 2 mm.

zygote together contain less than 1% of the total egg calcium; all the rest is in the egg shell. Between 20 and 30 d incubation, the embryo begins to absorb significant amounts of calcium from the egg shell. By 43 d of development, the embryos have absorbed up to 60% of the egg shell calcium. The range of results in Table 2 is a reflection of the staggering of developmental rates in land snails.

**Table 1** Mean calcium distribution in the freshly deposited egg

| Sample | Sample size | mg Ca per egg |
|---|---|---|
| Intact egg (shell + albumen) | 5 | 0.608 |
| Intact egg | 5 | 0.490 |
| Albumen | 10 | 0.003 |
| Albumen | 5 | 0.002 |

The method of calcium analysis, neutron activation analysis, involves the conversion of a portion of $^{48}Ca$, a stable isotope, to $^{49}Ca$, which is unstable with a half life of 8.72 min, and measuring the characteristic $\gamma$ radiation emitted. The source of neutrons was the Ford Reactor in the Michigan Memorial Phoenix Project, at the University of Michigan, the pneumatic tube system providing a flux of about $5 \times 10^{12}$ n cm$^{-2}$ s$^{-1}$. All the samples, in the milligramme range, were placed in polyethylene vials (cleaned with nitric acid) and freeze dried. They were then irradiated for 1 min, allowed to cool for 3 min, the entire vial was then placed near a 30 cm$^3$ germanium–lithium detector, FWHM 2.3 keV, connected to a 4,096 channel multichannel analyser calibrated to approximately 1 keV per channel with a CoCs source. Each sample was counted for 400 s. The calcium was measured using the 3,083 keV line of $^{49}Ca$ with reagent grade $CaCO_3$ powder as the standard. Calculations of the statistical counting errors and variability of controls and standards indicate a possible error of ± 10%. Whether these minute (2 mm) and very thin (30 μm) egg shells were crushed or not, made no difference in the results at this level of analysis.

Figures 1 and 2 show X-ray radiographs of a freshly deposited egg and an egg incubated for 43 d respectively. Both eggs were placed on the same piece of film, exposed and then printed together. Note that whereas the egg shell is made of calcite, the protoconch is made of another polymorph of $CaCO_3$, aragonite. The arrow in Fig. 2 indicates the area in which the embryo has most greatly weakened the egg shell, and at which it will break out. The mechanism of egg shell dissolution and reabsorption is not clear. When the embryo hatches, it consumes any egg shell remnants which are left.

There is evidence that this process of egg shell dissolution occurs in many other families of snails[6]. Calcified eggs have also been reported from land archaeogastropods[7] and from some of those mesogastropods which deposit their eggs on land[8]. It is suggested that land snails evolved the calcified egg shell early in their ancestry. For these terrestrial animals, calcium is often the limiting factor[9]. It would seem to be of great advantage to have the body shell already calcified by the time of hatching, for protection against small predators.

Embryonic absorption of egg shell calcium has been documented in some insects[10], some reptiles and birds[11]. A study of the calcium mobilisation in gastropod embryos with these other groups would be most rewarding from a comparative point of view.

**Table 2** Calcium distribution in eggs incubated at 20 °C ± 1

| No. of days incubated | mg Ca in egg shell | mg Ca in embryo | %Total Ca in embryo |
|---|---|---|---|
| 20 | — | 0.003* | — |
| 30 | 0.601 | 0.126 | 17 |
| 30 | 0.510 | 0.190 | 27 |
| 30 | 0.646 | 0.126 | 16 |
| 43 | 0.285 | 0.366 | 56 |
| 43 | 0.326 | 0.218 | 40 |
| 43 | 0.601 | 0.126 | 17 |
| 43 | 0.338 | 0.246 | 42 |
| 43 | 0.523 | 0.146 | 22 |
| 43 | 0.211 | 0.346 | 62 |
| 43 | 0.646 | 0.126 | 16 |
| 43 | 0.253 | 0.302 | 54 |
| 43 | 0.510 | 0.190 | 27 |
| 43 | 0.522* | 0.299* | 36* |

* Ten specimens combined in a single analysis.

I thank Norman E. Kemp and James N. Cather for help, and Jack Jones for assistance with the activation analysis.

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Received January 28; accepted April 3, 1975.

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# Depressant action of TRH, LH-RH and somatostatin on activity of central neurones

THREE structurally identified hypothalamic-adenohypophyseal regulatory peptides—thyrotropin-releasing hormone (TRH)[1,2], luteinising hormone releasing hormone (LH–RH)[3,4] and growth hormone-release inhibiting hormone (somatostatin or SRIF)[5], have now been localised both in extrahypothalamic regions of the central nervous system (CNS)[6–9] and in certain extraneural regions, such as the pineal gland[10]. There is increasing evidence that these peptides may affect behaviour by direct action on the brain. Systemic administration of TRH to rats induces behavioural effects independent of the pituitary–thyroid axis[11]; LH–RH can induce mating behaviour in hypophysectomised female rats[12], and somatostatin potentiates the behavioural effects of d,l-dopa[13]. These findings together with evidence of high affinity binding sites for TRH in extrahypothalamic tissues[14], identification of LH–RH in synaptosome fractions of brain[15] and localisation of LH–RH[16,17] and somatostatin[18,19] in nerve terminals suggest a role for these peptides in neuronal function. Applying TRH, LH–RH and somatostatin directly to central neurones microiontophoretically, we have found that these peptides have a potent depressant action on the activity of neurones at several levels (cerebral and cerebellar cortex, brain stem and hypothalamus) of the neural axis.

Experiments were performed on male Sprague Dawley rats anaesthetised with urethane (1.25 mg kg$^{-1}$ intraperitoneally in 25% w/v solution) or pentobarbital (50 mg kg$^{-1}$ intraperitoneally with supplemental intravenous doses). In experiments in which extracellular records were obtained from neurones in the cerebral or cerebellar cortices or the cuneate

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