The Activity of Amoebocytes and of Alkaline Phosphatases during the Regeneration of the Shell in the Snail, Helix aspersa

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SUMMARY

Experiments show that in Helix aspersa amoebocytes repair a damaged shell after transporting calcium carbonate and proteins from other parts of the shell, or from the digestive gland. The mantle is firmly applied to the damaged area and over it exudes fluid rich in amoebocytes. These secrete, and rapidly calcify, a protein membrane. Similar additional calcified membranes give firm protection after 24 hours.

The shell serves as a reservoir of calcium and protein, invaluable to a terrestrial gastropod. Amoebocytes can carry calcium back to the body. Extensive shell damage is repairable provided enough reserves remain: a snail cannot rebuild an entirely new shell. Thickening occurs whenever calcium and protein are available. Shell growth, however, initiated by the secretion of the periostracum, exclusively formed by the mantle skirt, happens only in conjunction with body growth.

Provided feeding activities are normal, alkaline phosphatases are abundant during shell repair, especially around digestive gland lime cells. Shell repair and digestion are closely associated. Amoebocytes, ineffective in an active, starved snail, can repeatedly repair damaged shell when a snail feeds, if only on filter paper: for once stimulated ample calcium and proteins are available for them in the shell.

INTRODUCTION

Calcium is undoubtedly one of the important substances affecting the life of any mollusc, and particularly those that depend on a protective shell for their survival from desiccation, if exposed to air, or from flooding, if exposed to fresh water. The wide distribution of so common a terrestrial gastropod as Helix aspersa Müller may well depend on the success with which this snail has been able to overcome problems of calcium metabolism. Whereas
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A marine gastropod may need to absorb only a small proportion of the abundant calcium present both in its food and in the surrounding sea-water to produce an adequate shell, Helix aspersa must possess some extremely efficient means of absorbing a very high percentage of the small quantities of calcium present in its food. That Helix aspersa does possess this power is shown by feeding snails from hatching on lettuce, which contains 0.025–0.05 per cent. calcium: when 208 days old a snail kept on this diet had laid down in its shell 130 mg. of calcium, having eaten during this time little more than the 260 gm. of lettuce that would represent 100 per cent. efficiency in calcium absorption.

In the hot summer of 1947 it was noticeable that those snails which had thicker shells survived in exposed positions much better than the thinner-shelled animals. But the protective advantage given by the production and maintenance of a thick shell is not the only benefit that efficiency in the use of calcium confers. Experiments showed that the shell is used as a calcium reserve, readily accessible for a variety of purposes: for example, if the snail’s shell is damaged it can quickly be repaired from these stores.

Earlier work on calcium metabolism established that in many gastropods the digestive gland is the principal organ for the storing of calcium, but exactly how this material is transported to the shell has been left largely unexplained. Some have assumed that it is carried in solution in the blood (Prenant, 1928; Manigault, 1933), but Sioli (1935) proved this to be incorrect for Helix pomatia at least, since at a time when he demonstrated its withdrawal from the digestive gland for the repair of the shell he found no increase in the amount in solution in the blood. As well as in this unsettled question of the transportation of calcium, previous workers differed in their interpretation of the processes of shell formation and repair. Dakin (1912), Crofts (1929), and others held that the pallial epithelium secreted the shell, although none studied in detail the physiological processes involved. On the other hand, de Waele (1930) considered that the extrapallial fluid, which lies between the pallial epithelium and the shell, contained a ‘protein-calcium carbonate complex’ from which the calcium carbonate would be precipitated at a definite carbon dioxide tension. Such a ‘protein-calcium carbonate complex’ is, however, unknown chemically.

Methods

It was found that if a piece of shell of Helix aspersa be removed, a new piece would be regenerated over this area in a few hours. This fact was made use of in a series of experiments designed to study both the transport of calcium from the digestive gland to the shell and the formation of the regenerated piece of shell. Pieces of regenerated material and of the digestive gland were fixed either in 10 per cent. formalin neutralized with borax, or in ice-cold 95 per cent. alcohol. They were stained for calcium with gallamine blue (Stock, 1949), and, when required, counterstained with purpurin. For more accurate histological details comparable samples were fixed with Susa, or
Flemming without acetic, and stained with Heidenhain's iron haematoxylin. Snails used in the experiments on regeneration had a piece of shell removed that was approximately 1 sq. cm. in area. Unless this piece was removed sufficiently high up the visceral hump the snail would retract and push its head and foot through the hole, in some cases using this new opening for weeks, even laying down new shell to cover its exposed mantle. If the removal was from the mouth of the shell itself the snail usually only withdrew farther into the shell, but any exposure of the mantle that could not be overcome by further contraction into the shell was rapidly followed by the laying down of a new piece of shell. Failure to do this (if, for instance, too much of the snail's shell was removed) was fatal, although before death the snail would attempt to cover the whole of the exposed area.

**EXPERIMENTAL WORK ON CALCIUM METABOLISM**

**The Storage of Calcium in the Digestive Gland**

That certain cells within the digestive gland of many gastropods store calcium has been known since the work of Barfurth (1880) and Biedermann and Moritz (1899). In *Helix aspersa* sections of the tubules of the digestive gland show that these 'lime' cells are separated one from the other by three or four columnar digestive cells. The lime cells that store calcium have large basal nuclei and are some 50 μ in height. Those that occur in the sections along the sides of the tubules are columnar and are some 30 μ in width, whilst those lying at the corners are pyramidal and measure some 50 μ across their base (fig. 1). The calcium is stored within minute protein spheres which lie near the nucleus and, if abundant, fill the whole of the basal region of the cell. It was at first assumed that the stores would consist of crystals of calcium phosphate as Krijgsman (1928) had so described them in *Helix pomatia*. Tests for phosphate within these spheres both in *Helix aspersa* and *Helix pomatia* were, however, always negative, whereas tests with acids plainly showed the presence of carbonates. Similarly, in both species, the calcium appeared in an amorphous state and never as crystals. When a snail is fed on a diet rich in calcium and proteins, or is allowed to accumulate good reserves during natural feeding (by preventing the formation of epiphragms), these cells become packed with protein spheres that are filled with an amorphous calcium carbonate. When the number of spheres becomes very great the cells bulge into neighbouring ones. If the snail be fed on a diet that is rich in proteins but contains no calcium these cells will store 'empty' protein spheres in as large numbers as are present when the diet is rich in calcium. When, on the other hand, the snail is fed on a diet with calcium, but with no protein, its digestive gland cells contain no stores of calcium, probably because there is nothing to prevent the carbonate from being dissolved in body fluids. Tested sections of digestive gland showed the protein envelope to be definitely protective since fairly strong acids were needed to liberate the calcium carbonate from these spheres.
All the cells of the digestive gland are in contact at their base with haemo-coelic spaces, within which are found two types of amoebocytes that play an important part in the transport of calcium from the digestive gland to the shell. The more numerous of these amoebocytes (type A) are 10 ¿-20 ¿ in diameter (according to the extension of the cells), and are found not only in blood spaces of the digestive gland but also around those lime cells that are storing calcium: they have only a small amount of cytoplasm and this seldom contains sufficient calcium to stain markedly with gallamine blue. Larger amoebocytes (type B) extending to 50 ¿ in length, with nuclei 10 ¿ long, also occur in these blood spaces and store calcium both in their nuclei and in their cytoplasm.

The Formation of New Shell over a Damaged Area

If a piece of shell be removed over the digestive gland there will be at first some loss of extrapallial fluid that has been lying between the mantle and the shell. Further loss of this fluid is prevented by parts of the mantle being firmly applied to the cut edges of the shell. It may then be seen that a rich
FIG. 2. Type A (small) and Type B (large) amoebocytes absorbing calcium from digestive gland cells.

A. Calcium spherule.
B. Large amoebocytes (type B) with calcium deposits both in the nucleus and in the cytoplasm (blackened areas staining heavily with gallamine blue).
C. Haemocoelic space.
D. Small amoebocytes (type A).
E. Boundary of the cells of the adjacent digestive gland lobule.
F. Area from which all the calcium spherules have been absorbed.
G. Areas in which the calcium has been absorbed but the collapsed protein spheres still remain.
H. Lumen of a digestive gland lobule.
supply of blood is sent to the area, since the pallial vessels appear distended ...d a slight prick to one of them causes a considerable spurt of blood, demonstrating the pressure that exists at this time within them. Some of the blood as a consequence passes through the walls and so keeps the whole of the exposed mantle well moistened. Microscopic examination of the extravasated blood shows it to be richer in amoebocytes, types A and B, than the blood from other sources (for example, extrapallial fluid from undamaged regions, or ventricular samples). A delicate organic skin quickly begins to form, at first in isolated patches; but after 2 or 3 hours it extends over the whole area, is partly calcified and can be removed for microscopic study. After 24 hours the pieces become too thick for this purpose. Regeneration of the shell consists of the laying down of a succession of calcified organic membranes, each welded to the other, and the whole building up an ever thicker and stronger structure. Amoebocytes packed with reserves of protein and calcium play an important part both in the formation of the organic membranes and in their calcification. When an organic membrane is being formed (fig. 3), groups of amoebocytes (type B) secrete fine protein networks as a mosaic of small polygonal pieces that become built into a continuous membrane.

Calcification of the membrane is carried out by amoebocytes (types A and B), depositing calcium carbonate on to the newly formed membrane where it crystallizes and becomes surrounded by a protein envelope, the whole
structure being known as a calcospherite. The crystals are radially striated, chemical and optical tests showing them to be calcite. The size of the crystal depends on the amount of calcium carbonate available for its formation. The larger amoebocytes (type B) tend to orientate themselves and secrete collectively, the resultant crystalline plate being extensive. The smaller amoebocytes (type A), however, secrete individually, each crystal, though small, being larger than the amoebocytes itself (fig. 4).

The margins of the membrane are calcified first, but pathways are left uncovered until the rest of the membrane is calcified and amoebocytes are able to move freely to the centre. As soon as this has occurred further amoebocytes will begin to form the next membrane and this will be calcified in the same way. Normally amoebocytes are not trapped between successive membranes, but move away from the area after secreting their calcium and protein; and when the regenerated piece is removed for microscopic study, only those amoebocytes that are actively engaged in regeneration will be found adhering to the side that is in contact with the extrapallial fluid. The one exception to this was found in a snail that was regenerating at a time when it was being fed on a diet very rich in calcium and proteins. Successive layers were here laid down so rapidly that it appeared as though some of the amoebocytes (type B) had not had time to move away before they were covered by the secretions of others. The amoebocytes were far more abundant in those snails that regenerated several new pieces of shell in a few weeks than in snails with undamaged shells.

If the regenerated shell be decalcified, the last-formed membrane breaks up into many tiny pieces, the secretions of each group of amoebocytes as yet remaining separate. The membranes formed earlier, however, remain welded together, not only each as an entire sheet, but as one single mass; this is because the protein envelopes, whilst forming around the crystals, adhere firmly to the membrane on which the crystals are being laid down. During decalcification strong acids puncture these membranes but the whole protein framework remains otherwise intact.

Removal of Calcium and Protein from the Digestive Gland and from the Shell

Examination of sections of the digestive gland of snails regenerating pieces of shell showed that both the reserves of calcium carbonate and their enveloping protein spheres are removed by amoebocytes. The smaller amoebocytes (type A) absorb calcium only into their nuclei (having very little cytoplasm), and are to be found in large numbers in and around the lime cells and within the neighbouring blood spaces. The larger amoebocytes (type B) often lie several cells deep in the areas of calcium absorption and between such areas and the damaged shell they line the walls of the blood spaces and appear to move along them to the regenerating region (fig. 5).

When these larger amoebocytes are absorbing calcium from the lime cells they appear enlarged and vacuolated, and their nuclei and certain parts of their cytoplasm then stain very heavily with gallamine blue. They ingest the
calcium and protein independently from the lime cells, the protein and some of the calcium remaining in vacuoles in the cytoplasm while some of the calcium becomes absorbed into the nucleus. These larger amoebocytes are found laden with calcium not only in the areas of the digestive gland where absorption is taking place, but also in large numbers lining the walls of blood spaces leading towards the mantle, in extrapallial fluid and in the regenerating shell.
itself. That *Helix aspersa* can remove calcium from the shell may be demonstrated by placing fragments of the shell on the area where regeneration is taking place. It is then found that these fragments are attacked by numbers of amoebocytes, which absorb the calcium and protein required for the formation of the new shell. All around the fragments, stretching to areas where regeneration is taking place, will be seen long lines of amoebocytes, their nuclei packed with calcium, whilst the fragments will be etched away in the areas where these amoebocytes have been working. Fairly soon, however, the fragments will be welded on to the regenerated shell and will be covered over by new calcified layers. Examination of the inner surface of the shell of regenerating snails showed several areas, usually a short distance from the damaged region, that were quite unlike anything ever seen in the undamaged shell. Owing to pigmentation it was not always possible to demonstrate conclusively that such areas have been etched away: some could have been areas where materials had previously been unevenly deposited, although this would have been an unusual occurrence, since the inner surface of the snail's shell is normally very smooth.

**Phosphatase Activity during Shell Regeneration**

The rapidity with which regeneration of damaged shell took place suggested that there might be an enzymatic stimulation of amoebocytic activity. Since
alkaline phosphatases play an important part in the process of calcification in mammals these enzymes might similarly be important in molluscs. Manigault (1939) has in fact demonstrated biochemically that during regeneration of the shell in *Helix pomatia* there is an increase in the amount of alkaline phosphatase in the mantle, in extracts of the digestive gland, and in the blood. Treating the subject biochemically he has not shown the precise location of the enzymes, and cytological tests were therefore necessary on the appropriate tissues in *Helix aspersa*. The Gomori technique was used for this, the materials having been fixed in ice-cold 90 per cent. alcohol; controls, staining calcium only,
were always made to prevent confusion between the distribution of calcium and of the enzyme. As soon as regeneration began there was a marked increase in the amount of alkaline phosphatases that were present in the digestive gland. Here the areas richest in the enzyme were those parts of the haemocoelic spaces where the small amoebocytes were massed together for the absorption of materials for the repair of the shell. The nuclei of the cells of the small amoebocytes stained very densely and there was much extracellular enzyme, making some of the areas appear so black that the individual amoebocytes within them were difficult to distinguish. The small amoebocytes lying within the cells of the digestive gland and ready to absorb calcium, similarly, gave strongly positive reactions for alkaline phosphatases (figs. 6 and 7). The larger amoebocytes, which previous experiments had shown to
carry much of the calcium in their nuclei and cytoplasm, did not give positive results for alkaline phosphatases. The pallial epithelial cells also did not appear to contain this enzyme, but the pallial blood-vessels and their longitudinal and circular muscles gave strongly positive results. During feeding, both normal and regenerating snails showed alkaline phosphatases present in the crop, in the salivary gland, and in certain oesophageal and intestinal regions. The kidney, particularly of regenerating snails, often contained large numbers of small amoebocytes similar to those found in the digestive gland and, like the latter, containing alkaline phosphatases. Tests on the material of the regenerated shell showed alkaline phosphatases within the nuclei of small amoebocytes.

**DISCUSSION**

The removal of pieces of 1 sq. cm. from the margin of the shell did not provide a means of studying either the process of repair or of normal formation of the shell, the snail merely adapting the shape of the pallial edge to the new shell edge. Regeneration occurred only when so much of the anterior region of the shell was removed that part of the visceral hump remained unprotected when the snail was fully retracted. Exposure of any part of the visceral pallium stimulated regeneration, the mantle being applied quickly to the cut edges of the shell and serving as a platform over which new shell was laid down, the only delay occurring when the snail could push its body through the hole, which interfered mechanically with the formation of the new shell. When sufficient raw materials were available, extensive areas could be repaired, the whole of the exposed surface being protected in a few hours. A snail was not able to regenerate an entire shell: although some attempt was always made, the digestive gland reserves were either inadequate or could not be mobilized with sufficient speed to prevent desiccation. Even when the snail was kept in a humid atmosphere failure to repair the injury within a day was usually fatal. Whereas shell repair occurred when the snail’s life was endangered by the exposure of delicate membranes, normal growth was correlated with growth of the snail as a whole and a mere removal of a piece of shell neither endangered the animal’s life nor would it stimulate enlargement of the shell.

Regeneration experiments demonstrated that in *Helix aspersa* amoebocytes transport the raw materials for the formation of the shell either from the digestive gland or from other areas of the shell. The numbers of amoebocytes present in any area that is directly concerned with shell regeneration are far in excess of those in the same areas under other conditions. The origin of these amoebocytes is of considerable interest. The epithelium of both mantle and intestine are likely places for their formation, for regions have been seen in them where cells appear to be actively budding, although more definite evidence is needed that these cells are in fact amoebocytes. It is possible, on the other hand, that the amoebocytes are blood-cells that divide in the plasma, but no evidence of this has been found. The part the mantle plays in the regeneration
of the shell also needs further investigation. In sections of *Helix aspersa*, the epithelial cells of the mantle were never found to contain reserves of calcium, and it cannot therefore be considered that they play any part in the calcification of the organic membrane, nor is there any evidence to support a suggestion that they help in its secretion. Normally there is a layer of extrapallial fluid between the mantle cells and the regenerating shell, and consequently their secretions, if any, would pass first into this fluid, whereas the thin organic skin formed during the repair of the shell appears to be produced entirely by the secretions of the amoebocytes. The membranes are not comparable in either thickness or texture with the periostracum, although they appear to be identical with the matrix laid down during the normal process of thickening the shell. The periostracum can be secreted only at the mantle edge and its formation appears to be the essential initial step in the formation of new as distinct from regenerated areas of the shell. When a gastropod stops growing, therefore, as *Nucella* does at sexual maturity (Moore, 1936), further growth of the shell is limited to processes comparable to thickening which are within the power of the whole mantle, such as the production of internal ridges or teeth. Although normal growth of the shell has not been studied in detail, it has often been observed, in the course of this work, that before a new piece of periostracum is formed, the edge of the pallium becomes highly distended with blood and extends beyond the rim of the shell, thus forming a platform over which new material can be added to extend the aperture of the shell.

In *Helix aspersa* the substances that are used for the formation of the shell are first stored in the digestive gland. The calcium is stored as a carbonate and is protected from solution in body fluids both there and in the shell by being enclosed in a sphere of protein. The snail, if fed on a diet rich in protein, but free from calcium, will store ‘empty’ protein spheres and if called upon to regenerate under such circumstances will lay down from these stores repaired shell that is almost entirely proteinaceous. Whenever calcium or protein or both were abundant in the diet, excess was stored in the digestive gland and in the shell. Snails fed with excess calcium carbonate had the inner surface of the whole shell covered with a white layer of the carbonate that is easily distinguishable from the pigmented surface normally found during feeding on plants. If the snail was regenerating its shell at a time when excess calcium and protein were being fed to it, it would still store part of this excess over the whole of the shell as well as using some for repairs. Regeneration and normal thickening appear to be the same process: this can occur in any part of the shell and consists of the production of calcified organic membranes built one on to the other.

In all the experiments on shell repair a close correlation was found between digestion and regeneration. A snail kept active, but without food, rapidly lost the power of repairing its shell, yet if the digestive processes remained active, even if the snail was feeding on cellulose, the amoebocytes could continue to function, laying down, if necessary, one renewed piece of shell after another.
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The activation both of amoebocytes and of alkaline phosphatases in the digestive gland and in the region of the shell where damage is being repaired, appears to be correlated with digestion. The amoebocytic cells responsible for the mobilization of calcium from the digestive gland or from the shell may also be concerned with the absorption of calcium from the gut. Yonge (1926, 1928, 1935) has shown that in many molluscs amoebocytic cells play an important part in food absorption. Such cells are as numerous as are the small amoebocytes in Helix and, like them, they are able to pass freely through the tissues. Yonge (1926) described the phagocytes of Ostrea edulis as being 'everywhere numerous in the blood vessels, connective tissue and epithelia, and free in the gut and mantle'. A marine lamellibranch has abundant calcium available in the sea-water and in its food, and this may explain why it does not store it in the digestive gland. Collip (1921) has discussed how intertidal lamellibranchs would need to withdraw calcium from their shell to prevent increased acidity in the tissues during the anaerobic state existing when the animal is not covered by water. Sioli (1935) likewise suspected, but was unable to prove, that Helix pomatia withdrew calcium from its shell for regeneration and other purposes. He found in one of his experiments on regeneration that the digestive gland and the kidney were supplying only 20 per cent. of the calcium present in the newly formed shell, and that even if the snail had withdrawn the whole of the calcium from its soft tissues, this would still be insufficient to account for the total amount of calcium present in the regenerated piece. Tests with Helix pomatia similar to those described for Helix aspersa showed that both these snails have amoebocytes that can absorb calcium from shell fragments placed over the damaged area. The shell of these snails serves far more than as a mere protective covering, for throughout the animal's life it remains an important reservoir for substances that are continually needed, notably calcium and protein.

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REFERENCES

— 1921. Ibid., 49, 297.
Dakin, W. J., 1912. Ibid., No. 20, London (Williams & Norgate).
Wagge—Regeneration of the Shell in Helix aspersa

—— 1925. Ibid., 59, 403.
—— 1926. Ibid., 14, 295.