How an Eggshell Is Made

Eggshell is largely-crystalline calcium carbonate. The calcium comes partly from the hen's bones and when necessary the hen can mobilize 10 percent of her bone for this purpose in a day

by T. G. Taylor

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To a housewife an egg is an article of food, and its shell serves to protect it from physical damage and to prevent the entry of dirt and microorganisms. To the hen an egg is a potential chick, and the shell serves not only as a protective covering but also as a source of calcium for the embryo and as a membrane through which the embryo respires. The eggshell performs its various functions with high efficiency, which is remarkable considering the number of eggs (five to seven a week) that the hen turns out. What is even more remarkable is the process whereby the hen obtains the substantial supply of calcium needed for the formation of the eggshells. The element comes in large part from her bones. Indeed, in extreme cases the hen can mobilize for this purpose as much as 10 percent of her total bone substance in less than a day! The physiology of this unusual process rewards close examination.

In its immature state the egg is one of many oocytes, or unripened ova, in the ovary of the hen. Each oocyte is encased in a membrane one cell thick; the entire structure is termed a follicle. At any one time follicles of various sizes, containing yolks at different stages of development, can be found in the ovary. Normally follicles ripen singly at a rate of one a day in hens that are laying regularly. There are occasional pauses. On the other hand, two follicles sometimes ovulate at the same time, giving rise to a double-yolk egg.

Ovulation takes place within six or eight hours after the release of a high level of a hormone produced by the pituitary gland. The release of the hormone is related to the time of onset of darkness, and it normally occurs between midnight and about 8:00 a.m. It follows that the hen always ovulates in daylight. Moreover, since it takes about 24 hours after ovulation to complete the formation of the egg, the egg is also laid during the daylight hours.

Once the yolk is released from the ovary all the remaining stages of egg formation take place in the oviduct, which consists of several distinct regions: the infundibulum, the magnum, the isthmus, the shell gland (uterus) and the vagina. The oviduct, like the ovary, is on the left side of the hen's body; a vestigial ovary and a vestigial oviduct are sometimes found on the right side in a mature bird, but they normally degenerate completely during the development of the embryo. One can only speculate on the evolutionary reason for the disappearance of the right ovary and oviduct. A reasonable guess is that two ovaries were disadvantageous because of the problem of providing enough calcium for the shells of two eggs at once. Birds have enough of a job supplying calcium for one egg a day. Certain species of wild birds have retained two functional ovaries and oviducts. It is not known how ovulation is controlled in these species, but apparently wild birds do not lay two eggs in one day.

After the ovum is released from the follicle it is engulfed by the funnel-like infundibulum of the oviduct. It is here that the egg is fertilized in hens that have been mated. As the yolk passes along the oviduct, layers of albumen are laid down in the magnum. The proteins of the albumen, which constitute the egg white, are synthesized in the magnum from amino acids removed from the blood. The synthesis is continuous, and in the periods between the passage of yolks down the oviduct albumen is stored in the tissue of the magnum. The addition of the layers of albumen to the yolk takes about four hours.

The next stage in the formation of the egg is the laying down of two shell membranes, an inner one and an outer one, around the albumen. The membranes are formed in the thin, tubular isthmus. When the membranes are first laid down, they cover the albumen tightly, but they soon stretch. By the time the egg enters the shell gland they fit quite loosely.

The egg passes the next five hours in the process known as "plumping." This entails the entry of water and salts through the membranes until the egg is swollen. The plumping period appears to be an essential preliminary to the main process of shell calcification, which occupies the next 15 to 16 hours.

The shell is composed of calcite, which is one of the crystalline forms of calcium carbonate. A sparse matrix of protein runs through the crystals of the shell. The final stage in the formation of the egg is the deposition of a cuticle on the fully calcified shell; this is accomplished just before the egg is laid.

Let us now look at the structure of the eggshell in rather more detail. The shell is attached to the outer membrane by hemispherical structures known as mam millary knobs. Histochemical studies have shown that the cores of the knobs consist of a protein-mucopolysaccharide complex rich in acid groups, and that anchoring fibers run from the outer membrane into the knobs.

The cores of the mammillary knobs are laid down as the membrane-covered egg passes through the part of the oviduct called the isthmo-uterine junction; it is between the isthmus and the shell gland. It seems probable that the knobs are calcified soon after they are formed, before the egg enters the shell gland, and that they subsequently act as nuclei for the growth of the calcite crystals comprising the shell. Modern ideas on the mechanism of biological calcification—whether in bones, teeth, eggshells or any of the other places where calcium is deposited in animal bodies—emphasize the importance of crystal growth. Earlier theories seeking to explain the mechanism laid much stress on the role of precipitation of calcium salts from supersaturated solutions, but in the light of more recent evidence this concept no longer seems valid.

The mechanism whereby the mammillary knobs are calcified is not well understood. It is thought to involve the binding of calcium ions to the organic cores of the knobs by means of the sul-fonic acid groups on the acid-mucopoly-saccharide-protein material of which the cores are composed. It is suggested that the spatial arrangement of the bound calcium ions is the same as it is in the lattice of the calcite crystal, so that these oriented calcium ions act as seeds or nuclei for the growth of calcite crystals forming the shell. Some years ago my colleagues and I found that the isthmus contains extremely high concentrations of both calcium and citric acid, the former reaching a maximum of about 90 milligrams per 100 grams of fresh tissue and the latter about 360 milligrams. We concluded that the high level of calcium in this region may be of significance in the calcification of the mammillary knobs.

The main part of the shell was once known as the spongy layer but has more recently come to be called the palisade layer. It is composed of columns of tightly packed calcite crystals; the columns extend from the mammillary knobs to the cuticle. Occasional pores run up between the crystals from spaces formed where groups of knobs come together. The pores reach the surface in small depressions that are just visible to the unaided eye on the outside of the shell. It is through these pores that the embryo takes in oxygen and gives out carbon dioxide during the incubation of the egg.

The raw materials for the formation of the calcite crystals, namely the ions of calcium and carbonate, come from the blood plasma. The shell gland is provided with an extremely rich supply of blood. Careful measurements have shown that the level of plasma calcium falls as the blood passes through the gland when the calcification of a shell is in progress but does not fall when there is no egg in the gland.

Changes in the level of calcium in the blood of female birds during the breeding season have engaged the attention of many workers since 1926, when Oscar Riddle and Warren H. Rein-hart of the Carnegie Institution of Washington discovered that breeding hen doves and pigeons had blood calcium levels more than twice as high as those found in cocks or nonbreeding hens. Adult males, nonbreeding females and immature birds of both sexes have plasma calcium levels of about 10 milligrams per 100 milliliters, whereas the level in females during the reproductive period is usually between 20 and 30 milligrams per 100 milliliters. For many years it was assumed that the high level of plasma calcium found in laying females was related to the trait of producing eggs with calcified shells, but it is generally recognized now that it is related to the production of large, yolky eggs. The extra calcium in the blood of laying birds (as compared with nonlaying ones) is almost entirely bound to protein. In contrast, the level of ionic calcium, which is the form of calcium mainly used in the formation of the eggshell, is about the same in laying and nonlaying hens.

The particular protein concerned in the binding of the increased plasma calcium is the phosphorus-containing protein phosvitin. It is the characteristic protein of the egg yolk. Phosvitin has a great affinity for calcium: the greater the amount of this phosphoprotein in the blood, the higher the level of plasma calcium. Phosvitin is synthesized in the liver under the influence of estrogen and is carried in the blood (in combination with lipid material) to the follicles developing in the ovary. Similar proteins are found in the blood of all animals that lay yolky eggs, including fishes, amphibians and reptiles, and yet neither fishes nor amphibians lay eggs with calcified shells, and among the reptiles only the Chelonia (turtles and tortoises) and the Crocodilia do so.

In the passage of blood through the shell gland there is a fall in both the protein-bound calcium (also termed non-diffusible calcium because the molecules of the protein to which it is bound are too large to diffuse through a semipermeable membrane) and in the diffusible calcium, the latter being mainly in the form of calcium ions. The two forms of calcium appear to be in equilibrium with each other. It seems likely that calcium in the form of ions is taken up from the plasma by the shell gland and that the level of ionic calcium is partly restored by the dissociation of a portion of the protein-bound calcium.

So much for the calcium ions. The origin of the carbonate ions is much harder to explain. At the slightly alkaline level of normal blood (pH 7.4) their concentration is extremely low, and it is the bicarbonate ion that predominates.

Theories to explain the formation of carbonate ions center on the enzyme carbonic anhydrase, which is present in high concentration in the cells lining the shell gland. One theory assumes that two bicarbonate ions are in equilibrium with a molecule of carbonic acid and a carbonate ion, with the equilibrium strongly in favor of the bicarbonate ions. The hypothesis is that the carbonic acid is continuously being dehydrated to carbon dioxide gas under the influence of the carbonic anhydrase, and that carbonate ions continuously diffuse or are pumped across the cell membranes into the shell gland, where they join calcium ions to form the calcite lattice of the growing crystals in the eggshell. An alternative theory, proposed by Kenneth Simkiss of Queen Mary College in London, is that the carbonate arises directly in the shell gland by the hydration of metabolic carbon dioxide under the influence of carbonic anhydrase.

The main evidence in support of the intimate involvement of carbonic anhydrase in eggshell formation is that certain sulfonamide drugs, which are powerful inhibitors of the enzyme, inhibit the calcification of shells. By feeding laying hens graded amounts of sulfanilamide, for example, it is possible to bring about a progressive thinning of the shells. Eventually, at the highest levels of treatment, completely shell-less eggs are laid.

On the average the shell of a chicken's egg weighs about five grams. Some 40 percent of the weight, or two grams, is calcium. Most of the calcium is laid down in the final 16 hours of the calcification process, which means that it is deposited at a mean rate of 125 milligrams per hour.

The total amount of calcium circulating in the blood of an average hen at any one time is about 25 milligrams. Hence an amount of calcium equal to the weight of calcium present in the circulation is removed from the blood every 12 minutes during the main period of shell calcification. Where does this calcium come from? The immediate source is the blood, but the ultimate source is the food. It has been demonstrated, however, that during the period of shell formation the hen is unable to absorb calcium from the intestines rapidly enough to meet the full requirement of the shell gland, no matter how much calcium is supplied in the food. When the rate of absorption from the gut falls short of the rate at which calcium is removed from the blood by the shell gland, the deficit is made good by the liberation of calcium from the skeleton.

This process has been demonstrated convincingly by the use of a radioactive isotope of calcium, calcium 45. Cyril Tyier of the University of Reading fed the isotope to laying hens daily and employed autoradiography to detect the amount of radioactive calcium deposited in the eggshells. (Beta particles given off by the calcium 45 of dietary origin blackened the X-ray film that was in contact with sections of shell, and the distribution of the isotope was thus visualized.) After the hens had been fed the radioactive calcium for a week the skeleton became intensely labeled, so that it was no longer possible to distinguish food calcium from bone calcium deposited in the shell. Accordingly the labeled calcium was withdrawn from the food, so that any calcium

45 deposited in the shells from then on must have come from the skeleton. Radioactive calcium appeared in abundance in the shells.

The mobilization of skeletal calcium for the formation of eggshell increases as the dietary supply of calcium decreases. When food completely devoid of calcium is fed, all the shell calcium comes from the bones. If a hen is fed a low-calcium diet, she will mobilize something like two grams of skeletal calcium in 15 to 16 hours. That is 8 to 10 percent of the total amount of calcium in her bones. Clearly hens cannot continue depleting their skeleton at this rate for long. When the food is continuously low in calcium, the shells become progressively thinner.

The hen's ability to mobilize 10 percent of her total bone substance in less than a day is quite fantastic but not unique: all birds that have been studied are able to call on their skeletal reserves of calcium for eggshell formation, and the rate of withdrawal is impressively high. This ability is associated with a system of secondary bone in the marrow cavities of most of the animal's bones. The secondary bone, which is called medullary bone, appears to have developed in birds during the course of evolution in direct relation to the laying of eggs with thick, calcified shells.

Strange to say, considering the fact [k]- that people had been killing birds for food for thousands of years and examining bones scientifically for at least a century, this unusual bone was not reported until 1916, when J. S. Foote of Creighton Medical College observed it in leg bones of the yellowhammer and the white pelican. The phenomenon was then forgotten until Preston Kyes and Truman S. Potter of the University of Chicago discovered it in the pigeon in 1934.

Medullary bone is quite similar in structure to the cancellous, or spongy, bone commonly found in the epiphyses (the growing ends) of bones. It occurs in the form of trabeculae, or fine spicules, which grow out into the marrow cavity from the inner surface of the structural bone. In males and nonbreeding females the marrow cavities of most bones are filled with red marrow tissue, which is involved in the production of blood cells. The spicules of medullary bone ramify through the marrow without interfering with the blood supply.

Medullary bone is found only in female birds during the reproductive period, which in the domestic fowl lasts-many months. (In wild birds it lasts only a few weeks.) Medullary bone is never found in male birds under normal conditions, but it can be induced in males by injections of female sex hormones (estro-gens). In hen birds medullary bone is produced under the combined influence of both estrogens and male sex hormones (androgens). It is thought that the developing ovary produces both kinds of hormone.

The formation and breakdown of medullary bone have been studied more closely in the pigeon than in any other bird. Pigeons lay only two eggs in a clutch; the second egg is laid two days after the first one. A pigeon normally lays the first egg about seven days after mating. The medullary bone is formed during this prelaying period. By the time the first egg is due to be provided with its shell, the marrow cavities of many bones of the skeleton are almost filled with bone spicules, which have grown steadily since the follicles developing in the ovary first started to secrete sex hormones.

About four hours after the egg enters the shell gland marked changes begin in the medullary bone. Within a few hours its cellular population has been transferred from one dominated by osteoblasts, or bone-forming cells, to one dominated by osteoclasts, or bone-destroying cells. The phase of bone destruction continues throughout the period of shell calcification. The calcium released from the bone mineral is deposited on the shell as calcium carbonate, and the phosphate liberated simultaneously is excreted in the urine.

The breakdown of the medullary bone persists for a few hours after the egg is laid. Then, quite suddenly, another phase of intense bone formation begins. This phase lasts until the calcification of the shell of the second egg starts; at that time another phase of bone destruction begins. No more bone is formed in this cycle. Resorption of the medullary bone continues after the second egg of the clutch is laid until, a week or so later, all traces of the special bone structure have disappeared and the marrow cavity regains its original appearance.

What mechanism might account for '[v] the rapid change from bone formation to bone destruction and vice versa? One suggestion is that variations in the level of estrogen control the cyclic changes in the medullary bone. There can be little doubt that the high level of estrogen plus androgen in the blood plasma is primarily responsible for the induction of medullary bone during the prelaying period; the drop in the level of estrogen or androgen or both after the second egg of the clutch is laid might well give rise to the bone destruction. It is difficult to see, however, how the fine degree of control necessary to induce bone destruction when calcification of the first eggshell is due to start, and to reverse the process soon after it is completed, can be exercised by changes in the secretion of sex hormones, presumably from the single follicle present in the ovary and possibly from the recently ruptured follicle.

The control mechanism that my colleagues and I consider more likely is one mediated by the parathyroid gland. The role of this gland is to regulate the level of calcium ions in the blood. A drop in the level of plasma calcium causes the release of parathyroid hormone from the gland, and the hormone brings about a resorption of bone tissue through the agency of the bone cells (osteoclasts and enlarged osteocytes). Both organic matrix and bone mineral are removed together, and the calcium and phosphate are released into the blood. The level of plasma calcium is thus restored; the phosphate is excreted.

Bone resorption under the influence of parathyroid hormone is largely due to an increase in the number and activity of osteoclasts. The histological picture observed in the medullary bone of pigeons at the height of eggshell calcification bears a strong resemblance to the resorption of bone in rats and dogs following the administration of parathyroid hormone. Leonard F. Belanger of the University of Ottawa and I have recently shown that the histological changes in the medullary bone of hens treated with parathyroid hormone were very similar to those occurring naturally during eggshell formation.

It has been shown that the level of diffusible calcium in the blood drops during eggshell calcification in the hen; the stimulus for the release of parathyroid hormone is therefore present. The hypothesis that the parathyroid hormone is responsible for the induction of bone resorption associated with shell formation is also consistent with the time lag between the end of the calcification of the eggshell of the first egg in the pigeon's clutch and the resumption of medullary bone formation.

When hens are fed a diet deficient in calcium, they normally stop laying in 10 to 14 days, having laid some six to eight eggs. During this period they may deplete their skeleton of calcium to the extent of almost 40 percent. It is interesting to inquire why they should stop laying instead of continuing to lay but producing eggs without shells. Failure to lay is a result of failure to ovulate; once ovulation takes place and the ovum enters the oviduct, an egg will be laid, with or without a hard shell.

The question therefore becomes: Why do hens cease to ovulate when calcium is withheld from their diet? The most probable answer seemed to us to be that the release of gonadotrophic hormones from the anterior pituitary gland is reduced under these conditions. To test this hypothesis we placed six pullets, which had been laying for about a month, on a diet containing only .2 percent calcium—less than a tenth of the amount normally supplied in laying rations.

After five days on the deficient diet, when each hen had laid three or four eggs, we administered daily injections of an extract of avian pituitary glands to three of the experimental birds. During the next five days each of these hens laid an egg a day, whereas two of the untreated hens laid one egg each during the five days and the third untreated hen laid three eggs. We concluded that the failure to produce eggs on a diet deficient in calcium is indeed due to a reduction in the secretion of pituitary gonadotrophic hormones.

The mechanism of pituitary inhibition under these conditions has not been established. It is possible that the severe depression of the level of plasma calcium inhibits the part of the brain known as the hypethalamus, which is known to be sensitive to a number of chemical influences. The secretion of gonadotro-phins in mammals is brought about by hormone-like factors released by the hypothalamus, but it is not known if the same mechanism operates in birds.

Plainly the laying of eggs with highly calcified shells has profound repercussions on the physiology of the bird. The success of birds in the struggle for existence indicates that they have been able to meet the challenge imposed on them by the evolution of shell making. Many facets of the intricate relations between eggshell formation, the skeletal mobilization of calcium, the ovary and the parathyroid and anterior pituitary glands await elucidation, but the general picture is now clear.